Oxidation of Corticosteroids to Steroidal-21-oic Acids by Human Liver Enzyme†

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ABSTRACT: An enzyme that oxidizes corticosteroids to acidic metabolites has been purified from postmortem human liver. The most rapidly oxidized substrate was 11-deoxycorticosterone (DOC). Other corticosteroids were oxidized at rates that were 10% or less of DOC. The products of DOC oxidation were 3,20-dioxopregn-4-en-21-oic acid and 20-hydroxy-3-oxopregn-4-en-21-oic acid. The 20-keto acid was the predominant metabolite in all enzyme preparations. Keto acid and hydroxy acid were not interconverted. Enzyme activity was assayed by measuring the transfer of tritium from [21-^{3}H]DOC to water. The enzyme is yellow, and has spectral maxima at 278 and 405 nm. Inhibition by *o*-phenanthroline suggests that it may be a metalloenzyme. Molecular weight was estimated at 74 000 ± 8 000; a pH maximum occurred at pH 8–8.5. This enzyme may participate in the in vivo conversion of corticosteroids to the acidic metabolites that we have described previously (H. L. Bradlow et al. (1973), *J. Clin. Endocrinol. Metab. 37,* 811).

The pathways of corticosteroid metabolism which lead to the neutral urinary metabolites in man have been intensively studied. The metabolites that have been identified account for close to 100% of the steroids recovered from the neutral fraction of urine (Fukushima et al., 1960; Gray and Shaw, 1965a,b). However, these neutral metabolites represent 70% or less of the total excreted steroid (Peterson, 1971; Brooks, 1964; Taylor, 1969; Zumoff et al., 1967; Peterson et al., 1955; Peterson, 1971; Migeon et al., 1956). The remaining polar urinary metabolites have been generally neglected. We have recently demonstrated that an important part of the highly polar unidentified steroids in urine are acids and contain the 21-oic acid moiety. So far, four steroid acids which contain the 20-hydroxy-21-oic acid side chain derived from cortisol have been unequivocally identified. Their quantitative significance is emphasized by the fact that they represent 5–30% of the total metabolites of cortisol (Bradlow et al., 1973). Preliminary data suggest that 20-oxo-21-oic acids are also metabolites of corticosteroids (Bradlow et al., 1973).

These results prompted us to attempt to isolate enzymes from human tissues which catalyze the oxidation of the ketol side chains of corticosteroids to acidic metabolites. In this paper, we report the partial purification of an enzyme from human liver which mediates the oxidative metabolism of corticosteroids to steroidal carboxylic acids.

Materials and Methods

Human liver was obtained postmortem from several subjects. Detailed studies were undertaken with enzyme from a female, age 49, who died of unknown causes; a male, age 69, with a diagnosis of tongue cancer; and a female, age 40, who died of a primary brain tumor. The properties of the enzymes in each case were similar. Livers from three other sources of uncertain history were used in more restricted studies. For the detailed studies in this report liver from the 49 year old female was used.

[21-^{3}H]Corticosteroids were synthesized as described by Willingham and Monder (1973). Their identities and homogeneity were established by thin-layer chromatography and by cocrystallization to constant specific activity with authentic unlabeled steroids. [1,2-^{3}H]-11-Deoxycorticosterone[1] (specific activity 45.9 Ci/mmol) and [4-^{14}C]-11-deoxycorticosterone (specific activity 54.3 Ci/mol) were purchased from New England Nuclear Corp. Unlabeled steroids were bought from Steraloids Corp., Pawling, N.Y. Sephadex gels were obtained from Pharmacia Co. Silica gel G precoated thin-layer plates (0.25 mm thick plastic sheet, Machery-Nagel Co., Germany) were used for most of the analytical chromatography. For preparative purposes, Quantagram PLQF (Quantum Ind., N.J.) plates were used. All chemicals and solvents were reagent grade.

Enzyme Assay. Oxidation of the 21-tritio side chain results in loss of label from the steroid as tritiated water (Willingham and Monder, 1974). Enzyme activity was therefore determined by measuring the tritium incorporated into water. A volume of 0.02–0.05 ml of purified enzyme containing 100 μg of protein was mixed with 0.025 M Tris-HCl (pH 8.0) followed by 1.3 nmol of [21-^{3}H]-11-deoxycorticosterone (specific activity 37.0 μCi/μmol) dissolved in 10 μl of ethanol. Total volume was 2.0 ml. After 2 h of incubation at 37°, the reaction was stopped by immersing the mixture in a freezing bath at −70°. Enzyme activity was determined after lyophilization of the incubation mixture by measuring the amount of tritium appearing in the water of the conden-

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[1] Abbreviations used are: DOC, 11-deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 21-dehydro-11-deoxycorticosterone, 3,20-dioxopregn-4-en-21-al; cortisol, 11β,17,21-trihydroxypregn-4-ene-3,20-dione; epicortisol, 11α,17,21-trihydroxypregn-4-ene-3,20-dione; tetrahydrocortisol, 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one; 2α-methylcortisol, 11β,17,21-trihydroxy-2α-methylpregn-4-ene-3,20-dione; 11-deoxycortisol, 17,21-dihydroxypregn-4-ene-3,20-dione; 2α-methylcortisone, 17,21-dihydroxy-2α-methylpregn-4-ene-3,11,20-trione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; prednisolone, 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; 6α-fluoroprednisolone, 6α-fluoro-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; 6α-methylprednisolone, 11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

sate.

Molecular size of enzyme was estimated by gel filtration on columns of Sephadex G-200 (1.5 cm × 78 cm). Elution was carried out with 0.01 M sodium phosphate buffer (pH 7.0). Calibration curves were made with proteins of known molecular size, including human γ-globulins (mol wt 160 000), bovine serum albumin (mol wt 67 000), ovalbumin (mol wt 45 000), and myoglobin (mol wt 17 800). V_0 was determined with blue dextran 2000. Optical spectrum of purified enzyme was measured in 0.01 M sodium phosphate buffer (pH 6.0) using a Cary 15 spectrophotometer.

Radioactivity was determined in a Packard 3380 scintillation spectrometer, using external standardization. Aquafluor (New England Nuclear Corp.) was used as scintillation fluid. Radioactivity profiles on thin-layer chromatograms of ^{14}C compounds were traced with a Packard Model 7201 radiochromatogram scanner.

Chemical Synthesis of 20-Hydroxy-3-oxopregn-4-en-21-oic Acid. The 21-methyl ester of 20-hydroxy-3-oxopregn-4-en-21-oic acid was prepared by rearrangement of 3,20-dioxopregn-4-en-21-al catalyzed by cupric ion (Lewbart and Mattox, 1963). The steroid methyl ester was purified on silica gel plates, using chloroform–methanol (98:2, v/v). The area containing the steroid ester was scraped off the plate and the steroid was eluted with acetone. To 20 mg of steroid methyl ester dissolved in 0.5 ml of methanol was added 1 ml of water and 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 thin-layer chromatography, using the upper phase of toluene–acetic acid–water (50:50:10, v/v). Melting point of purified free acid was 158–160° with decomposition; λ_{max} (ethanol) 238 nm (ε 13 300). 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 melting point of methyl ester was 204–207° with decomposition; mass spectrum (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 $C_8H_{12}O$.

Enzymatic Synthesis of 20β-Hydroxy-3-oxopregn-4-en-21-oic Acid from 3,20-Dioxopregn-4-en-21-oic Acid. 3,20-Dioxopregn-4-en-21-oic acid (25 mg) dissolved in 5 ml of methanol was incubated with 75 mg of NADH and 0.4 mg of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Boehringer-Mannheim Inc.) in 150 ml of 0.1 M Mes buffer (pH 5.1) for 24 h at room temperature. The incubation mixture was then adjusted to pH 1 with 3 N HCl and extracted with ethyl acetate. The extract was transferred to a preparative silica gel G plate and developed with chloroform–methanol–formic acid (84:16:1, v/v). The band corresponding to authentic 20-hydroxy-3-oxopregn-4-en-21-oic acid was scraped off, transferred to a Soxhlet apparatus, and extracted for 8 h with acetone. The acetone was blown off under a stream of warm nitrogen. Average yield was 14.2 mg. Anal. Calcd for $C_{21}H_{30}O_4 \cdot H_2O$: C, 69.2; H, 8.8. Found: C, 69.5; H, 8.5. Ir (KBr) 2.95, 3.4, ~4 (shoulder), 5.8, 6.1, 7.2, 9.2 μ. Ir spectrum corresponded to that of chemically synthesized acid.

The product was converted to its 21-methyl ester with excess diazomethane in ether and checked for purity in benzene–ethanol (84:16 v/v) and chloroform–methanol (98:2 v/v). A single ultraviolet-opaque spot was seen in each solvent system at R_f 0.47 and 0.26, respectively. Anal. Calcd for $C_{22}H_{32}O_4 \cdot 0.5H_2O$: C, 71.5; H, 9.0. Found: C, 71.5; H, 9.0. Ir (KBr) 2.95, 3.4, 6.0, 6.3, 7.1, 9.5, 9.8 μ. Ir spectrum corresponded to synthetic ester.

Table I: Purification of Enzyme from Human Liver.[a]

Fraction	Total Protein (mg)	Detritiation Activity (× 10^6 dpm)	Specific Activity (dpm/mg of Protein)	Fold Purification
Homogenate	8 380	5.57	663	1
12 000*g* sup	3 340	4.25	1 273	1.9
50–70% $(NH_4)_2SO_4$ precipitate	660	1.76	2 672	4.0
Sephadex G-100 eluate	189	1.07	5 670	8.6
CM-Sephadex C-50 eluate	25	1.05	42 100	63.4

[a] Activity represents ^3H incorporated into ^3H_2O in 2 h at 37° in a system containing 0.025 M Tris-Cl (pH 8.0), enzyme, and 8 × 10^4 dpm of [21-^{3}H]-11-deoxycorticosterone (37.0 μCi/μmol) in a final volume of 2.0 ml.

Reduction of 20-Hydroxy-21-oic Acid to 20,21-Diol. The steroid in dry tetrahydrofuran was refluxed with a sevenfold weight excess of lithium aluminum hydride for 75 min. Residual reducing agent was destroyed with ethyl acetate. Solids were coagulated with a few drops of saturated aqueous sodium sulfate followed by 50 mg of anhydrous magnesium sulfate. The reduced steroid in dry chloroform was stirred overnight with manganese dioxide, filtered, and concentrated. The resulting products were found to migrate with authentic 20,21-diols on thin-layer plates containing pH 9.2 sodium borate impregnated silica gel. With methylene chloride–acetone (65:35, v/v) mobilities were 0.11 (20α) and 0.20 (20β). With chloroform–methanol (90:10, v/v) mobilities were 0.26 (20α) and 0.37 (20β).

3,20-Dioxopregn-4-en-21-oic Acid. This keto acid was synthesized from 3,20-dioxopregn-4-en-21-al by the method described by Monder (1971).

4-^{14}C-Labeled Steroid Acids. [4-^{14}C]-20β-Hydroxy-3-oxopregn-4-en-21-oic acid and [4-^{14}C]-3,20-dioxopregn-4-en-21-oic acid were synthesized from [4-^{14}C]-11-deoxycorticosterone (specific activity 54.3 Ci/mol), by applying the preparative methods described above on a micro scale.

Results

Purification of Enzyme. Approximately 30 g of human liver was homogenized for 2 min with 100 ml of 0.25 M sucrose in 0.01 M sodium phosphate buffer (pH 7.0) in a Waring Blendor. All steps in the purification procedure were performed at 3°. The homogenate was centrifuged at 12 000 *g* for 30 min. About 70% of the activity was retained in the supernatant fraction. The supernatant solution was subjected to ammonium sulfate fractionation. The precipitate collected between 50 and 70% saturation was taken up in 10 ml of 0.01 M phosphate buffer (pH 7.0) and applied to a Sephadex G-100 column (2.5 × 76 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). Elution was performed with the same buffer; 5-ml fractions were collected. Enzyme activity was eluted in a single peak in fractions 38–44. The pooled fractions were concentrated by

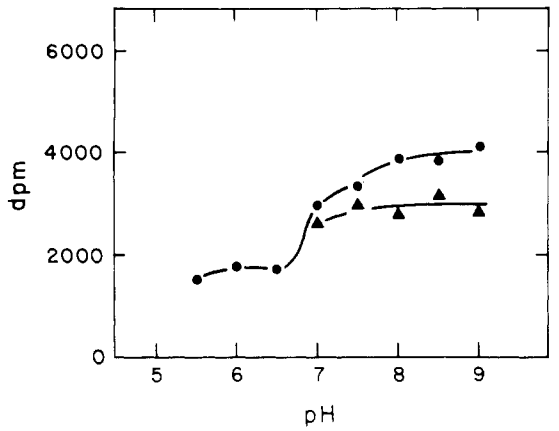


FIGURE 1: pH dependence of 21-detritiation activity of 21-oic acid synthetase. (●) 0.01 M sodium phosphate; (▲) 0.025 M Tris-Cl.

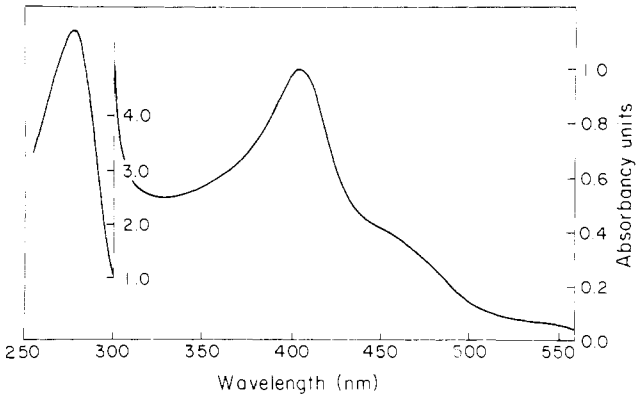


FIGURE 2: Optical spectrum of 21-oic acid synthetase. A solution of 3 mg of protein/ml of 0.01 M sodium phosphate (pH 7.0) was scanned in a Cary Model 15 spectrophotometer. Light path was 10 mm.

ultrafiltration (Diaflo membrane XM-50, Amicon Corp.), and rechromatographed on a CM-Sephadex C-50 column (2.0 × 40 cm). Elution was achieved with a linear gradient consisting of 300 ml of 0.01 M sodium phosphate buffer (pH 6.0) which was maintained at constant volume by the gradual addition of 300 ml of 0.1 M sodium phosphate buffer (pH 8.0). Enzyme was eluted in the breakthrough volume as a symmetrical peak. The purification procedure yielded a 63-fold increase in detritiation activity and 18% recovery of the total activity of the homogenate. A summary of the purification is presented in Table I.

Characteristics of Enzyme. The detritiation of [21-^{3}H]DOC[1] was linear with enzyme concentration up to 0.1 mg of protein/ml of incubation mixture, and linear with incubation time up to 2 h. The enzyme at 4° lost 50% of its activity within 2 weeks and lost most of its activity after 1 month.

The dependence of enzyme activity on pH is illustrated in Figure 1. Activity increased up to pH 8, and plateaued beyond this value. Detritiation activity was somewhat higher with 0.01 M sodium phosphate buffer than with 0.025 M Tris-HCl buffer. The purified enzyme was pale yellow and had absorption maxima at 278 and 405 nm in 0.01 M phosphate buffer (pH 6.0) (Figure 2). Enzyme solution emitted green fluorescence when viewed with a long-wave ultraviolet light. Molecular size of enzyme as determined by Sephadex G-200 gel filtration was calculated to be 74 000 ± 8 000 daltons using human γ-globulin, bovine serum albumin, ovalbumin, and myoglobin as reference standards.

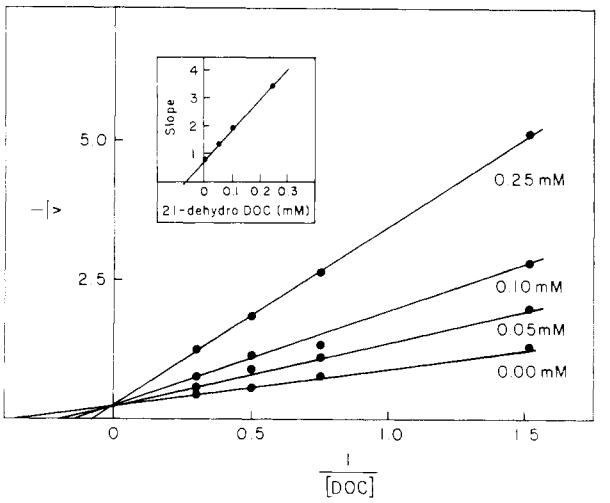


FIGURE 3: Effect of 21-dehydro-DOC (3,20,21-trioxopregn-4-ene) on detritiation of [21-^{3}H]DOC by 21-oic acid synthetase. Ordinate: reciprocal of rate of recovery of 21-^{3}H as water in 2 h at 37° (DPM × 10^{-4}). Abscissa: reciprocal of μM DOC concentration. Values for 21-dehydro-DOC as constant variable are indicated in the figure at the corresponding primary plot lines. Inset shows secondary plot.

Table II: Substrate Specificity of Enzyme.[a]

[21-^{3}H]Corticosteroids	Enzyme Activity (% of [21-^{3}H]DOC)
11-Deoxycorticosterone	100
Corticosterone	9.3 ± 1.3[b]
Cortisol	6.5 ± 0.6
11-Deoxycortisol	11.2 ± 1.5
Epicortisol	3.4 ± 1.3
Tetrahydrocortisol	0.9 ± 0.4
2α-Methylcortisol	3.4 ± 0.2
2α-Methylcortisone	5.7 ± 0.7
Prednisolone	7.8 ± 1.2
6α-Methylprednisolone	4.4 ± 0.2
6α-Fluoroprednisolone	0.4 ± 0.3

[a] 1.3 nmol of [21-^{3}H]corticosteroids (specific activity 14–49 μCi/μmol) was incubated under standard assay conditions. [b] Mean ± SEM (n = 3).

Kinetics. Double reciprocal plots of the initial velocity of detritiation activity against [21-^{3}H]DOC concentration gave an apparent K_m of 2.8 × 10^{-6} M. Double reciprocal plots of velocity vs. [21-^{3}H]DOC with different concentrations of 21-dehydro-11-deoxycorticosterone as constant variable were also obtained. The results indicated that 21-dehydro-11-deoxycorticosterone is a competitive inhibitor (Figure 3). The inhibition constant with respect to 21-dehydro-11-deoxycorticosterone was estimated as 6.8 × 10^{-5} M.

Substrate Specificity. Table II compares the detritiation rates of [21-^{3}H]corticosteroids including the tritiated derivatives of cortisol, epicortisol, tetrahydrocortisol, 2α-methylcortisol, 11-deoxycortisol, 2α-methylcortisone, corticosterone, prednisolone, 6α-fluoroprednisolone, and 6α-methylprednisolone with that of [21-^{3}H]-11-deoxycorticosterone. [21-^{3}H]-11-Deoxycorticosterone, the best substrate, was detritiated at a rate about ten times greater than [21-^{3}H]-11-deoxycortisol or [21-^{3}H]corticosterone and 15 times faster than [21-^{3}H]cortisol. Tetrahydrocortisol and 6α-fluoroprednisolone were not detritiated at measurable rates.

Effect of Cofactors, Inhibitors, and Metal Ions. None of the cofactors added to the incubation mixture including NAD^+, $NADP^+$, FAD, glutathione, and thiamine pyro-

Table III: Effect of Inhibitors on Enzyme Activity.[a]

Inhibitors	Enzyme Activity (% of Control)
None	100
p-Chloromercuribenzoic acid	101.3 ± 5.4
N-Ethylmaleimide	161.1 ± 6.1
Iodoacetic acid	106.9 ± 8.5
EDTA	101.7 ± 2.4
o-Phenanthroline	51.7 ± 5.2
o-Phenanthroline + Co^{2+}	109.8 ± 3.3
o-Phenanthroline + Fe^{2+}	92.4 ± 3.8

[a] Standard assay conditions were used as described under Materials and Methods. Each indicated component was present in the final reaction mixture at a concentration of 1×10^{-4} M.

Table IV: Effect of Metal Ions on Detritiation Activity.

Metal Ions[a]	Enzyme Activity[b] (%)
None	100.0
Cobaltous chloride	112.0 ± 8.6
Ferrous sulfate	97.6 ± 3.6
Ferric chloride	99.9 ± 1.8
Ferric ammonium sulfate	101.5 ± 2.8
Molybdenum pentachloride	86.6 ± 5.0
Magnesium chloride	100.9 ± 5.7
Manganous chloride	105.4 ± 5.6
Mercuric chloride	76.1 ± 10.0
Zinc chloride	78.9 ± 1.1
Cupric acetate	55.9 ± 2.0
Lead acetate	99.9 ± 2.4
Lithium chloride	100.0 ± 3.3
Silver nitrate	28.2 ± 3.7

[a] Final concentration of metal ions was 1×10^{-4} M. [b] Values are mean ± SEM.

phosphate affected the rate of detritiation. Organic sulfhydryl inhibitors, *p*-chloromercuribenzoate, *N*-ethylmaleimide, and iodoacetate, did not show any inhibitory effect. Unexpectedly, *N*-ethylmaleimide increased the enzyme catalyzed detritiation activity. We cannot yet provide an explanation for this unusual effect. 1×10^{-4} M *o*-phenanthroline inhibited activity by 50%. Inhibition by *o*-phenanthroline was reversed by 10^{-4} M Co^{2+} or 10^{-4} M Fe^{2+} (Table III). Various metal ions were examined for their direct effect on detritiation activity. Results are shown in Table IV. Co^{2+} showed a slight activating effect at 1×10^{-4} M, but Fe^{2+} did not, in spite of its ability to reverse *o*-phenanthroline inhibition. Moderate to severe inhibition by Zn^{2+}, Hg^{2+}, Cu^{2+}, and Ag^{+} was observed. Since organic sulfhydryl inhibitors failed to affect enzyme activity, inhibition by these metal ions could be due to protein denaturation. However, the inhibitory effect of Cu^{2+} might also be explained by the fact that cupric ions catalyze the chemical conversion of DOC to 21-dehydro-11-deoxycorticosterone which, as indicated above, is a strong competitive inhibitor of the enzyme.

Isolation and Identification of the Reaction Product. Purified enzyme containing 3.2 mg of protein was incubated with 2.0 μCi of [4-^{14}C]DOC (specific activity 54.3 μCi/μmol) in 50 μl of ethanol, 0.14 μCi of [21-^{3}H]DOC in 40 μl of ethanol, and 0.025 M Tris-Cl (pH 8.0) for 4 h at 37°. Total volume was 12 ml. After removing the aqueous phase from the incubation mixture by lyophilization, the dried residue was redissolved in a small amount of distilled water and transferred to an extraction funnel. Glassware was rinsed with a small amount of acetone, which combined with the water phase. This solution was adjusted to pH 1 with 3 N HCl, and the steroids were extracted with ethyl acetate exhaustively. The combined organic phase was dried under a stream of nitrogen, redissolved in acetone, and streaked on a 20 × 20 cm silica gel G plate. Development of the chromatogram was performed with ethyl acetate. Two major radioactive peaks were observed. The unconverted substrate, DOC (R_f 0.37), was the predominant radioactive peak; the other was a polar fraction which remained at the origin, and which represented 24% of the total. Polar metabolites were extracted with methanol and further chromatographed with the upper phase of toluene–acetic acid–water (5:5:1 v/v); or with benzene–ethanol–formic acid (84:16:1 v/v). A single radioactive peak cochromatographed with authentic 20-hydroxy-3-oxopregn-4-en-21-oic acid in both systems, with R_f 0.38 and 0.39, respectively. The profile was not symmetrical. Therefore, the radioactive acid was extracted and converted to the 21 methyl ester with diazomethane. The methyl derivatives moved as two peaks in benzene–ethanol (84:16), R_f 0.47 and 0.54, and in chloroform–methanol (98:2 v/v), R_f 0.20 and 0.38. The more polar component corresponded to authentic 20-hydroxypregn-4-en-21-oic acid 21-methyl ester.

To confirm the identity of the 20-hydroxy steroid acid, the radioactive product was cocrystallized with authentic 20β-hydroxy-3-oxopregn-4-en-21-oic acid 21-methyl ester from methylene chloride–ligroine. Initial specific activity was 2085 ± 45 dpm of ^{14}C per mg of product. Subsequent crystallization yielded specific activity of 3100 ± 90 dpm/mg with no change for two further recrystallizations. It was concluded that about ⅔ of the product corresponded to the 20β-hydroxy acid ester.

The oxidation of DOC to the 20-hydroxy acid proceeded with retention of tritium. The ^{3}H/^{14}C ratio of the original substrate was 5.77. The residual substrate recovered after incubation had a ^{3}H/^{14}C ratio of 5.96, a value not significantly different from that of the substrate. The ^{3}H/^{14}C ratio of the acid was 3.04, indicating that about half of the original tritium content was retained in the hydroxy acid metabolite. The tritium in the original [21-^{3}H]DOC was completely accounted for as water, acid metabolites, and unconverted residual DOC.

Stereochemistry of the Side Chain. The configuration of the side chain of the [1,2-^{3}H]hydroxy acid synthesized by the liver enzyme from [1,2-^{3}H]DOC was determined by converting it to [1,2-^{3}H]-20,21-dihydroxypregn-4-en-3-one by treatment with lithium aluminum hydride and manganese dioxide and comparing the mobility of the reduced product with authentic epimeric diols. The 20β-hydroxy standard was prepared by reducing stereochemically homogeneous 20β-hydroxy-3-oxopregn-4-en-21-oic acid with lithium aluminum hydride. The 20α-diol was obtained by reduction of the 20-hydroxy acid resulting from cupric acetate catalyzed rearrangement of 3,20-dioxopregn-4-en-21-al (Lewbart and Mattox, 1963). The epimeric diols were separated on silica impregnated with 0.1 M sodium borate. From 7000 cpm of acid, 290 cpm of β form and 115 cpm of α form were obtained. The ratio of isomers was $\beta/\alpha = 2.5$.

3,20-Dioxopregn-4-en-21-oic Acid. The component whose methyl ester had a greater mobility on thin-layer plates than that of the 20-hydroxy acid-21 ester was invariably present in greater quantities than the 20-hydroxy acid. Its mobility corresponded with that of the 20-oxo acid ester

in benzene–ethanol (84:16), R_f 0.54, chloroform–methanol (98:2), R_f 0.38, and methylene chloride–acetone (3:1), R_f 0.49.

To establish the identity of the metabolite, the free tritiated acid fraction was prepared from [1,2-^{3}H]DOC with liver enzyme as described earlier. After fractionation by thin-layer chromatography, the total acidic metabolites were incubated with 20β-hydroxysteroid dehydrogenase and NADH to convert keto acid to hydroxy acid. The mixture contained 0.15 μmol of authentic 20-keto acid, 4.8 $\times$ 10^5 dpm of the tritiated acid metabolite fraction, 0.22 μmol of NADH, and 160 μg of 20β-hydroxysteroid dehydrogenase in 0.1 M sodium phosphate (pH 6.5). Total volume was 2.0 ml. Control contained buffer in place of enzyme. After 21.5 h of incubation at room temperature the acid fraction was isolated, esterified with diazomethane, and separated on thin-layer plates with chloroform–methanol (98:2). The ratio of 20-oxo acid/20-hydroxy acid, initially 4.9, was found to be 0.24 after treatment with the 20β-hydroxysteroid dehydrogenase, clearly showing that the 20-keto group had been reduced by the enzyme.

In a separate experiment, the 21-methyl ester of the suspected [1,2-^{3}H]-20-oxo acid was cocrystallized with authentic 3,20-dioxopregn-4-en-21-oate 21-methyl ester to constant specific activity from diethyl ether–hexane. The data support the conclusion that 3,20-dioxopregn-4-en-21-oic acid was a product of the action of the liver enzyme on 11-deoxycorticosterone.

Absence of Interconversion of 20-Oxo and 20-Hydroxy Acids. The simultaneous presence of both 20-hydroxy and 20-oxo acids may be the consequence of their interconversion, or alternatively, may result from a bifurcation in the metabolism of an intermediate precursor common to both acids. Three lines of evidence show that each of the products was not converted to the other. (a) [4-^{14}C]-3,20-Dioxopregn-4-en-21-oic acid (2 $\times$ 10^4 dpm, 16.5 nmol) incubated for 2 h at 37° in air with the complete system (0.7 mg of enzyme, 0.4 μmol of cobaltous chloride, and 0.1 M sodium phosphate (pH 8.0), volume, 2.0 ml) was recovered unchanged; (b) 20 β-hydroxy-3-oxopregn-4-en-21-oic acid (2 $\times$ 10^4 dpm; 16.5 nmol) was recovered unaltered after incubation under the same conditions; (c) addition of 2.5 $\times$ 10^{-5} M NADH or NADPH to incubation mixtures containing [4-^{14}C]-11-deoxycorticosterone did not change the yield of acid or the ratio of 20-oxo to 20-hydroxy acid (oxo/hydroxy = 4.2).

Ratio of 20-Oxo Acids and Other Steroid Acids Made by Human Liver Enzyme Preparation. Table V summarizes data accumulated during this investigation in which the extent of formation of 20-oxo acid relative to other acid metabolites by purified enzyme from a sampling of postmortem livers are compared. Because of the very limited number of specimens, and the variability in the condition of the livers as they were received, only very limited conclusions can be drawn from the table. No attempt was made to prove the identity of the radioactive material in the 20-hydroxy acid methyl ester region. In most cases, an acid ester that moved slightly slower than the 20β-hydroxy acid ester standard was present. This did not correspond in mobility to 3-oxoandrost-4-en-20-oic methyl ester. Calculations were made on the assumption that this radioactivity was entirely 20-hydroxy acid ester. The evidence indicates that all livers contained enzyme which oxidized DOC to both 20-oxo and 20-hydroxy acids. All enzymes from whatever source made more 20-oxo than 20-hydroxy acid.

Table V: Ratio of 20-Oxo to 20-Hydroxy Acid Formed from 11-DOC.

Patient	Sex	Age	Diagnosis	Oxo/Hydroxy
1	F	49	Unknown	2.62 ± 0.32 (3)
2	M	69	Ca of tongue	1.91 ± 0.28 (2)
3	F	73	Pneumonia	2.22 ± 0.66 (2)
4	M		Unknown	8.59 ± 1.05 (2)
5	F	40	Brain tumor	5.00 (1)
6	Unknown		Unknown	6.08 ± 1.29 (3)

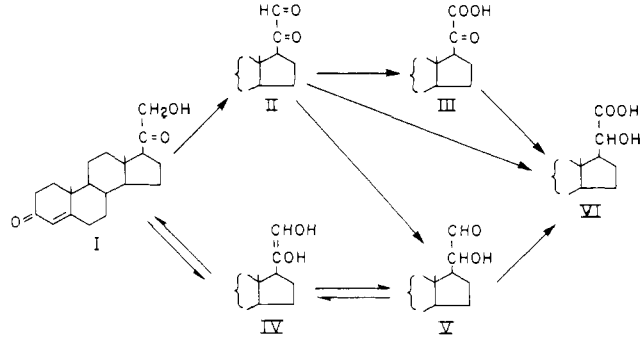
Discussion

The possibility that corticosteroids are oxidized in humans to carboxylic acids to any important extent has never been seriously considered. A number of authors have hinted at the possibility that such transformations may occur (Gray and Shaw 1965a,b; Southcott et al., 1956; Bailey et al., 1966; Pal, 1967) but these observations have never been followed up. Specific acid metabolites of corticosteroids have been isolated from human urine only in recent years by Gerhards et al. (1971) and by us (Bradlow et al., 1973).

The oxidative metabolism of steroids and sterols to carboxylic acid derivatives is by no means a newly described phenomenon. The bile acids, oxidation products of sterol metabolism, have been studied in great detail (Nair and Kritchevsky, 1971,1973). The carboxylic acid intermediates formed during cholesterol biosynthesis (Hornby and Boyd, 1971; Rahimtula and Gaylor, 1972; Bloxham and Akhtar, 1971) are examples of the obligatory participation of carboxylates in the metabolic transformation of steroids.

We have shown that carboxylic acids are quantitatively important products of corticosteroid metabolism in man (Bradlow et al., 1973) in vivo. Four acid metabolites of cortisol have been identified in urine. From an examination of the structure of the ketol (I) and β-hydroxy acid (VI) moieties excreted (Scheme I) it is obvious that several steps are required to effect the metabolic transformations of the side chain alone. Possible pathways are summarized in Scheme I.

Scheme I



Although our investigations with intact subjects have provided considerable information about the paths of metabolism leading to the steroid acids, there are limitations to the amount and kind of interpretable data that can be obtained in this way. A direct study of the enzymes responsible for the synthesis of the steroidal 21-oic acids is therefore necessary to provide important complementary data. Our ability to isolate and characterize an enzyme from human liver which catalyzes the oxidative conversion of the corticosteroid side chain to 21-oic acids has been greatly facilitated by the availability of 21-tritiated corticosteroids (Wil-

lingham and Monder, 1973). Advantage is taken of the fact that the enzyme-mediated oxidation of the ketol side chain results in the obligatory transfer of tritium from carbon 21 of the steroid to water (Willingham and Monder, 1974). Therefore, the increase in tritium-labeled water with time provides a handy measure of enzyme activity.

Our most difficult problem has been our inability to obtain postmortem livers less than 12–14 h after death. The purified enzyme is sixfold less active than one which we have isolated from hamster liver (Lee and Monder, 1973). We do not know what changes in the activity or properties of the enzyme may have occurred between the time the patient died and our receipt of the specimen. It may be that human liver contains less activity or the enzyme deteriorates rapidly after death. In spite of these reservations, the human and hamster liver enzymes have many characteristics in common including spectral properties, kinetic behavior, and substrate specificity.

The most rapidly oxidized substrate was 11-deoxycorticosterone and for this reason it was used in most of these studies. The comparatively rapid oxidation of 11-deoxycorticosterone has also been demonstrated in intact subjects (C. Monder and H. L. Bradlow, to be published). The underlying basis for the substrate specificity of this enzyme is not known. Perhaps the relative efficiencies of the various substrates depend on their polar properties. We have found positive correlation between mobility on paper in the formamide–chloroform system of Zaffaroni (Engel, 1963), mobility on thin-layer plates in chloroform–methanol (95:5), and detritiation (unpublished observations).

The fact that the enzyme is inhibited by *o*-phenanthroline and the activity is restored by cobaltous or ferrous ions, indicates that transition metals are associated with the active site of the enzyme. The enzyme is yellow with a spectral maximum at 405 nm. It is possible, therefore, that the color of the enzyme is due to organically bound transition metal. Whether the chromophore is essential for stabilizing the enzyme or plays a catalytic role can only be established by further investigation.

It is not possible to provide the enzyme with an adequately descriptive name. Two classes of product, the 20-hydroxy and 20-keto-21-oic acids, are generated. We suggest, therefore, that the name "steroid-21-oic acid synthetase" be used tentatively for purposes of identification. The enzyme may be either a multienzyme complex despite its relatively small size of 74 000 daltons, or a group of very similar enzymes which cannot be resolved by the techniques we used. The hydroxy acids formed from 11-deoxycorticosterone were a mixture of 20α and 20β epimers. These results are in qualitative accord with our finding that both enantiomers of the 20-hydroxycortoic acids are excreted into urine after infusion of labeled cortisol (Bradlow et al., 1973). The existence of enzymes of restricted stereochemical specificity provides the simplest explanation of our results.

Further evidence for the complexity of the enzyme system is provided by a consideration of the intermediate steps required to convert a ketol side chain to a hydroxy acid side chain. The possible metabolic paths are summarized in Scheme I. Sequences I → II → III → VI and I → II → VI are unlikely. The 21-dehydro intermediate, II, is a strong competitive inhibitor of the oxidation and formed acid products to a much smaller extent than DOC when incubated with the enzyme. In addition, keto acid was not converted to hydroxy acid. The ineffectiveness of glutathione supports our in vivo (Monder et al, 1975) and in vitro evidence (Monder and Wang, 1973a) that the 20-hydroxy-21-oic acids are not formed from 21-dehydrocorticosteroids via a glyoxylase reaction (II → VI). Unless it can be shown that synthesis of VI occurs by way of a nonexchangeable pool of II, we may eliminate all pathways that utilize 21-dehydrocorticosteroids as intermediate. The remaining possibility in the scheme utilizes on isomerization step (Rose, 1976) through an enediol (I → IV → V) followed by an oxidation at carbon 21 with an aldehyde dehydrogenase (V → VI) perhaps via keto aldehyde dehydrogenase (Monder and Wang, 1973a,b). The retention of tritium in the side chain of the hydroxy acid is consistent with this pathway.

The enzyme activity which we have segregated from human liver mediates several new and unique transformations of corticosteroids. We have demonstrated that it oxidizes corticosteroids to 20-hydroxy and 20-keto acids, and have thus provided a basis for further biochemical studies of what we believe to be important new pathways of corticosteroid metabolism.

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Protocatechuate 3,4-Dioxygenase from *Acinetobacter calcoaceticus*†

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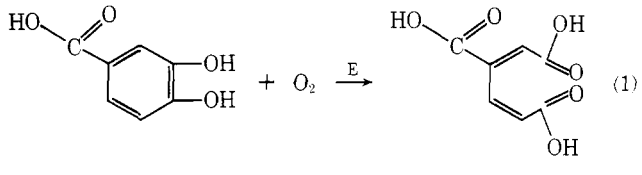
ABSTRACT: Protocatechuate 3,4-dioxygenase (PCD) from *p*-hydroxybenzoate-induced cells of *Acinetobacter calcoaceticus* was purified by heat and protamine sulfate treatment, ammonium sulfate fractionation, DEAE-cellulose, and Sephadex G-200 column chromatography. The enzyme appears to be homogeneous by ultracentrifugation and acrylamide gel electrophoresis. This is the first report of PCD purified from *Acinetobacter.* For comparison, crystalline *Pseudomonas* PCD was also obtained. The enzymes from *Acinetobacter* and *Pseudomonas* are quite similar in their molecular weight, molecular size, and iron content. The specific enzyme activity of PCD from *Acinetobacter* is about one-third of that from *Pseudomonas,* despite their similar iron content. Visible and circular dichroism spectra indicate some conformational differences between these two enzymes. Protocatechualdehyde, a competitive deadend inhibitor, binds *Pseudomonas* PCD more effectively than *Acinetobacter* PCD. *p*-Hydroxymercuribenzoate, specific for free -SH groups, inhibits only *Acinetobacter* PCD and shows no effect on *Pseudomonas* PCD. Amino acid analyses reveal very low proline and methionine content with higher lysine, glutamic acid, and isoleucine compositions for *Acinetobacter* PCD. Other properties, including active center conformation, were studied and discussed.

The oxidative attack on simple aromatic compounds by microorganisms leads to formation of catechol or protocatechuic acid as the last intermediate product with an aromatic structure. Protocatechuate 3,4-dioxygenase (PCD)[1] (protocatechuate:oxygen 3,4-oxidoreductase, EC 1.13.11.3), a nonheme trivalent iron-containing enzyme, catalyzes the conversion of protocatechuate to β-carboxy-*cis,cis*-muconic acid (eq 1).



(1)

The enzyme activity has been reported from several sources (Stanier and Ingraham, 1954; Gross et al., 1956; Cain and Cartwright, 1960; Ornston, 1966). The protein isolated from *Pseudomonas aeruginosa* was first crystallized by Fujisawa and Hayaishi (1968). No other laboratories have reported the crystallization of PCD (personal communication with Drs. O. Hayaishi and H. Fujisawa). The valence state of the iron and other properties of the *Pseudomonas* PCD were also reported (Fujisawa et al., 1972a,b; Fujiwara and Nozaki, 1973).

In our continuing effort to understand the nature of oxygenases and the conformation of their active centers, we have been investigating the PCD induced in *Pseudomonas aeruginosa* (Zaborsky et al., 1975; Hou, 1975). We have demonstrated that the primary binding site for organic substrate is located in the amino acid residues of the enzyme protein and is distinct from the iron-containing catalytic site. Recently, we have isolated PCD from a completely different genus, the bacterium *Acinetobacter calcoaceticus.* We hope that a detailed comparison of these two enzymes might provide information on the conformation of the active sites and the essential characteristics required for the catalytic activity. We now report on the purification and physical-chemical properties of *Acinetobacter* PCD and compare them with the crystalline PCD from *Pseudomonas.*

Materials and Methods

Assay of PCD. The enzyme activity was assayed both spectrophotometrically and by measuring oxygen consumption. In the spectrophotometric assay, the decrease in absorbance at 290 nm (MacDonald et al., 1954) was measured with a Beckman automatic recording spectrophotometer Model DBG at 24 °C. The assay system contained, in a final volume of 3.0 ml, 1.2 μmol of protocatechuic acid, 150

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[1] Abbreviations used are: PCD, protocatechuate 3,4-dioxygenase; PC, pyrocatechase.