the manganese or the exchange rate is slow for this inhibitory binding orientation.

From our results we conclude that the active-site region of thermolysin contains two binding sites, or at least two nonoverlapping possible binding orientations, for these small inhibitor molecules. The site seen by NMR observation of the perturbation of the inhibitor resonances is a tight but apparently nonproductive binding. The other site, or orientation, while much weaker is productive in the sense that binding at this site, or in this mode, partially displaces water from the bound metal ion and apparently is bound in an orientation which prevents catalytically effective binding of substrate. Preliminary x-ray crystallgraphic results (Kester and Matthews, unpublished results) are consistent with this interpretation. The inhibitor appears to bind at two positions. One of these suggests that the inhibitor acts as a metal-ion ligand at the active site while the inhibitor occupies the hydrophobic binding pocket in its other orientation.

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Studies on 21-^{3}H-Labeled Corticosteroids: Evidence for Isomerization of the Ketol Side Chain of 11-Deoxycorticosterone by a Hamster Liver Enzyme†

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ABSTRACT: We have previously observed that liver and other tissues catalyze the transfer of tritium from 11-deoxy-[21-^{3}H]corticosterone to water (Willingham, A. K., and Monder, C. (1974), *Endocr. Res. Commun. 1,* 145–153). We now have found that most of the tritium is lost in a reaction that involves exchange of the label with water protons. This exchange is enzyme-catalyzed, yields unlabeled 11-deoxycorticosterone (DOC), and is reversible. The enzyme was purified about 150-fold. It required no added cofactors. The enzyme was yellow, with spectral maxima at 272 and 403 nm, was inhibited by *o*-phenanthroline and EDTA, and was reactivated by Co^{2+}, Fe^{2+}, or Mo^{5+}. Co^{2+} and Fe^{2+} themselves enhanced the activity of the purified enzyme. The apparent molecular weight was 390 000. [21*S*-21-^{3}H]DOC was detritiated faster than the 21*R* epimer. Although 1 equiv of deuterium was quickly incorporated into DOC when the enzymic reaction occurred in D_2O, prolonged incubation resulted in the exchange of both 21-methylene hydrogens. Loss of tritium was not due to a reversible oxidation of the 21-alcohol. A newly synthesized steroid, 20-hydroxy-3-oxo-pregn-4-en-21-al (isoDOC) was converted to DOC by the enzyme. In order to explain these results, we propose that the enzyme is an isomerase that reversibly interconverts both DOC and isoDOC through a common enediol intermediate.

Corticosteroids undergo a wide range of catabolic transformations in man and other animals. Study of the excreted end products of these reactions are valuable in reconstructing metabolic pathways, and in the diagnosis of disease. In most laboratories, it has been the practice to study the "neutral" steroid metabolites of urine, that is, the fraction of the total steroids remaining in the organic extract of urine after the solvent phase has been washed with alkali. It has been pointed out by several investigators (Peterson, 1971; Brooks, 1964; Taylor, 1969; Fukushima et al., 1960) that the neutral and conjugated urinary steroids account for only a part of the total metabolites excreted, and a number of workers have suggested that, among other possibilities, steroid acids may be formed and passed into the urine in addition to the other well-known metabolites (Brooks, 1964; Taylor, 1969; Southcott et al., 1956; Lowy et al., 1969; Gray and Shaw, 1965). It indeed appears that important steroid acids are discarded in the alkali

† From the Research Institute of the Hospital for Joint Diseases, New York, New York 10035. *Received December 13, 1976.* This investigation was supported by grants from the National Institute of Arthritis, Metabolism and Digestive Diseases (AM09006), the National Cancer Institute (CA 14194), and United States Public Health Service General Research Support Grant RR 5589. H. J. Lee and S.-W. Oh were trainees of the National Cancer Institute (CA5215). C. Monder was a Career Development Awardee of the United States Public Health Service (AM06841).

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wash. We have recently identified in human urine a class of acidic metabolites of cortisol which have in common the 20-ol-21-oic acid side chain (Bradlow et al., 1973). These steroids represent up to 30% of the steroid metabolites in urine. The mechanism by which the organism is able to convert corticosteroids to steroidal acids is therefore of obvious interest. Progress toward this goal was greatly eased by our synthesis of 21-^{3}H-labeled corticosteroids (Orr and Monder, 1975; Willingham and Monder, 1973) which enabled us to follow the metabolism of the side chain directly. In the course of attempting to isolate an enzyme from hamster liver which catalyzes the oxidation of corticosteroids to 20-hydroxy acids, we obtained a preparation which stimulated the loss of tritium from 21-^{3}H-labeled corticosteroids.

In this paper we describe the partial purification and properties of this enzyme and present evidence that it is an isomerase.

Materials and Methods

Materials. Male Golden hamsters weighing about 100 g were purchased from Chick Line Co., Newfield, N.J., and maintained on a standard chow diet. Unlabeled steroids were bought from Steraloids, Inc., Pawling, N.Y., and 4-^{14}C-labeled corticosteroids from New England Nuclear Corp., Boston, Mass. 2α-Methylcortisone, 11α,17,21-trihydroxyprogesterone, 6α-fluoroprednisolone, 2α-methylhydrocortisone, 6α-methylprednisolone, and 6α-fluoroprednisolone were a gift of Dr. Nina Hollander. Steroids were purified when necessary by thin-layer chromatography on silica gel plates with appropriate developing solvents. Deuterium oxide, isotopic purity 99.7%, was from New England Nuclear Corp. All other chemicals and solvents were reagent grade.

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

Determination of Enzyme Catalyzed Detritiation Activity. The amount of tritiated water formed as a result of incubation of 21-^{3}H-labeled corticosteroids with hamster enzyme preparation was determined by measuring radioactivity in the condensate which was obtained by lyophilization of the total incubation mixture. Reaction mixtures contained 0.025 M Tris-HCl buffer (pH 8.0), 1×10^5 dpm of 21-^{3}H-labeled corticosteroid in 10 μL of 90% ethanol, and 0.1 to 0.3 mg of enzyme in a total volume of 2.0 mL except where otherwise noted. In the standard assay, 11-deoxy[21-^{3}H]corticosterone (48.5 Ci/mol) was used. Reactions were carried out at 37 °C and stopped by freezing the mixture at −70 °C. Controls contained all the assay components except enzyme.

Chromatography. Sephadex G-100 columns, 2.5 × 70 cm, for preparative chromatography and Sephadex G-200 columns, 1.5 × 78 cm, for analytical separation, were equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. Samples were applied, and fractions of 5 mL were collected at a rate of 30 mL per h for the preparative and 18 mL per h for analytical columns. The analytical columns were calibrated with proteins of known molecular weight by the method of Siegel and Monty (1966). Void volume was measured with Blue Dextran (Pharmacia Fine Chemicals). One milligram of enzyme in buffer was applied in a total sample volume of 0.5 mL and the elution of enzyme was monitored by ultraviolet absorption at 280 nm and by the detritiation assay. Total volume of the analytical column was decreased by less than 3% for eight consecutive chromatographic runs. A DEAE[1]-Sephadex A-50 column, 1.5 × 40 cm, was equilibrated with 0.025 M Tris-HCl, pH 8.0; elution of applied protein was effected by the application of a Tris-HCl buffer gradient resulting from the introduction of 150 mL of 0.25 M Tris-HCl, pH 6.0, into 300 mL of 0.025 M Tris-HCl, pH 8.0. Fractions of 5 mL were collected at a rate of 10 mL per h. A CM-Sephadex C-50 column, 2.5 × 40 cm, was equilibrated with 0.01 M sodium phosphate buffer, pH 6.0. Sample was applied and eluted with a gradient formed by mixing 300 mL of 0.01 M sodium phosphate, pH 6.0, and 300 mL of 0.1 M sodium phosphate buffer, pH 8.0. Fractions of 5 mL were collected at the rate of 40 mL per h.

Steroid substrates and metabolites were purified by preparative chromatography on 0.5-mm thick plates of silica gel (GF_{254}, E. Merck, Darmstadt). The following solvent systems were used as indicated in the text. (A) chloroform–methanol, 90:10 v/v; (B) chloroform–methanol, 98:2 v/v; (C) upper phase of toluene–acetic acid–water, 50:50:10 by volume; (D) *n*-hexane–acetic acid–water, 35:5:10 by volume.

Preparation of 21-^{3}H-Labeled Corticosteroids. A number of 21-^{3}H-labeled corticosteroids were synthesized as described by Willingham and Monder (1973) using sodium borotritiide. The two enantiomers of 11-deoxy[21-^{3}H]corticosterone and [21-^{3}H]cortisol were synthesized by reduction of 21-dehydrocorticosteroids with 21-hydroxysteroid dehydrogenase and [4*S*-4-^{3}H]NADH to give the 21-^{3}H-labeled 21*S* isomer and that of 21-^{3}H-labeled 21-dehydrocorticosteroid with the enzyme and NADH to give 21-^{3}H-labeled 21*R* isomers (Willingham and Monder, 1974).

The labeled corticosteroids were purified on a prepacked column of silica gel (silica gel 60, column size A, E. Merck, Darmstadt) with chloroform–methanol (9:2 v/v) as developing solvent. In each case 21-tritiated corticosteroids cocrystallized with authentic steroid from ethanol–hexane or acetone–hexane with no change in specific activity. Steroid remaining in the supernatant liquors retained the same specific activities as the crystals. The labeled steroids corresponded in mobility to authentic steroids on thin-layer plates with ethyl acetate, chloroform–methanol (98:2 v/v), and benzene–ethanol (84:16 v/v). Specific activities of the tritiated steroids ranged from 36 to 50 Ci per mol. The 21-dehydrocorticosteroids required for these preparations were synthesized by oxidation of corresponding corticosteroids with cupric acetate (Monder and Furfine, 1969). In experiments using 11-deoxy[4-^{14}C, 21-^{3}H]corticosterone, 4-^{14}C- and 21-^{3}H-labeled steroids were mixed to achieve the indicated ratios.

Synthesis of 20β-Hydroxy-3-Keto-pregn-4-en-21-al. This steroid to which we have given the trivial name "isoDOC", was synthesized as described by Oh and Monder (1976). Anal. Calcd for $C_{21}H_{30}O_3 \cdot 0.5H_2O$: C, 74.4; H, 9.20. Found: C, 75.0; H, 8.95. The following were also determined: mp 140 °C dec; λ_{max} methanol 241 nm (16 400); NMR (ppm) 18 CH_3, 0.88; 19-CH_3, 1.19; 4-H, 5.69; 20-CHOH, 4.06 (multiplet; doublet in D_2O, J = 10 Hz); 21-CHO, 9.65.

Incorporation of Deuterium into DOC. Into each of 25 tubes were placed 3.85 mL of 0.025 M Tris-HCl buffer (pH 8.0) in ^2H_2O (final concentration, 96.0% D_2O), 0.55 mg of hamster liver enzyme, and 500 μg of DOC in 0.05 mL of methanol. Final volume was 4.0 ml. Incubation was performed for designated intervals at 37 °C. The mixture was saturated with sodium chloride and extracted with ethyl acetate. Steroid was purified on a silica gel column with chloroform–methanol (99.5:0.5) as the developing solvent. The steroid was washed

[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; SD, standard deviation.

TABLE I: Purification of Enzyme from Hamster Liver.

Fraction	Volume (ml)	Total protein (mg)	Total detritiation act. (dpm × 10^6)	Spec. act.[a] (dpm/mg of protein)	Fold purification
Homogenate	127	17100	28.1	164	1
Centrifugation, 12 000*g*, supernatant	105	8250	27.7	336	1.8
50–70% $(NH_4)_2SO_4$ precipitate	7.6	634	9.0	1420	8.7
Sephadex G-100 eluate	65	312	7.6	2437	14.8
CM-Sephadex C-50 eluate	5.0	17.5	4.5	25700	156

[a] Total ^3H incorporated into ^3HOH in 2 h at 37 °C in a system containing 0.025 M Tris (pH 8), enzyme, and 1.06 × 10^5 dpm of [21-^3H]-11-deoxycorticosterone (45.8 μCi/μmol) in a final volume of 2.0 mL.

TABLE II: Substrate Specificity of 21-^3H-Tritiated Steroids.[a]

21-Tritiated Steroid	^3H recovered as water % of [21-^3H]DOC
11-Deoxycorticosterone	100
Cortisol	1.8 ± 0.05
11-Deoxycortisol	11.5 ± 0.3
Corticosterone	11.6 ± 0.3
Prednisolone	0.91 ± 0.02
2α-Methylcortisol	2.25 ± 0.06
6α-Fluoroprednisolone	0.91 ± 0.02
6α-Methylprednisolone	1.17 ± 0.03
11α,17α,21-Trihydroxyprogesterone	4.45 ± 0.11
2α-Methylcortisone	14.6 ± 0.4
20-Keto-5α-pregnan-21-al	0
3,20-Diketo-4-pregnen-21-al	0
Tetrahydrocortisol	0.4

[a] Appearance of tritium in water was determined after incubating 1.3 × 10^{-9} mol (ca. 1 × 10^5 dpm) of 21-^3H-labeled steroid (dissolved in 10 μL of 90% ethanol), 0.2 mg of enzyme, and of 0.025 M Tris-HCl buffer (pH 8.0) in a volume of 2.1 mL. Reaction was carried out at 37 °C for 2 h. In this time, 39.5% of ^3H in DOC was transferred to water. Values are for three determinations ± SD.

with H_2O to remove exchangeable deuterium and recrystallized from dichloromethane and hexane.

Mass Spectrometry. Mass spectra were obtained on a Du Pont Model 21-492B mass spectrometer with a 21-094 computerized data system. Mass spectra were recorded at an electron ionizing energy of 70 eV. Source temperature was 210 °C. The range of probe temperature was from room temperature to about 300 °C.

Results

Purification of Enzyme. Six hamsters were decapitated and the livers were immediately chilled to 3 °C. All subsequent steps were performed at 3 °C. The combined tissues were homogenized for 2 min with 5 volumes of 0.025 M sucrose in 0.01 M sodium phosphate buffer, pH 7, in a Waring blender. The homogenate was centrifuged at 12 000*g* for 30 min to remove nuclei, mitochondria, and debris. Most of the enzyme activity remained in the supernatant fraction.

Solid ammonium sulfate (31.5 g/100 mL of supernatant solution) was added to the stirred supernatant solution. After 30 min, the resulting precipitate was collected by centrifugation at 12 000*g* for 30 min. To each 100 mL of the supernatant phase, 13.7*g* of ammonium sulfate was added. After 30 min of stirring, the precipitate was collected by centrifugation. The fractionation step increased the specific activity of enzyme about fivefold.

The precipitate from the ammonium sulfate fractionation was dissolved in 5 mL of 0.01 M sodium phosphate buffer, pH 7, and filtered through a Sephadex G-100 column. Fractions of 5.0 mL were collected. Enzyme, as measured by detritiation activity, was eluted in a single peak accompanied by a red contaminant (fractions 30 to 40). Active fractions were combined and concentrated to 5 mL with an Amicon Filter XM-50, under nitrogen pressure.

The concentrated enzyme solution was introduced into a CM-Sephadex C-50 column (2.5 × 40 cm). A gradient of increasing ionic strength and pH was established by the introduction of 300 mL of 0.1 M sodium phosphate, pH 8.0, into a reservoir of 0.01 M sodium phosphate, pH 6.0. Enzyme was eluted in a sharp, symmetrical yellow peak between 50 and 65 mL of effluent. The active enzyme fractions were pooled together and concentrated on an Amicon filter as described above. Overall procedure yielded 120- to 156-fold increase in specific detritiation activity and 16% recovery of total activity of homogenate. A concentrated solution of the enzyme was pale yellow. The enzyme solution showed absorption maxima at 272 and 403 nm. A summary of the purification procedure is presented in Table I.

When DEAE-cellulose was placed in contact with the enzyme, either in a batch procedure or on a column, the enzyme became inactive within 15 min. Incubation of enzyme with 0.1 nM triethylamine, the main functional group of the resin, did not result in loss of activity. We are unable to explain the detrimental effect of DEAE-cellulose on the enzyme. Activity was lost entirely after boiling the enzyme solution for 3 min. The enzyme lost little activity after 18 h of incubation at 37 °C. Accompanying the "detritiation" activity in the freshly isolated preparation was enzyme activity which catalyzed the net oxidation of 11-deoxycorticosterone to 21-oic acids (Martin and Monder, 1976). This latter activity contributed about 10% to the net transfer of tritium to water. The enzyme responsible for acid formation was unstable and was inactivated after several days at 4 °C. Consequently, if the "detritiation" enzyme was held in the refrigerator for a week, the side chain oxidation no longer made a net contribution to the loss of tritium from the [21-^3H]-11-deoxycorticosterone. All experiments described were performed with enzyme freed of oxidase activity in this way. All attempts to separate the oxidizing enzymes from the "detritiase" were unsuccessful.

pH Dependency. The effect of pH on enzyme activity with [21-^3H]DOC as substrate in 0.01 M Tris-HCl and 0.01 M sodium phosphate buffer was studied. Rate of detritiation by the enzyme gradually increased with pH and reached a plateau at pH 8 which persisted to pH 9.0. Though the rate was about 7% higher in Tris-HCl buffer than in sodium phosphate buffer, the pattern of the pH–activity curve did not appear to depend on the nature of the buffer.

TABLE III: Effect of Organic Inhibitors and Metal Ions on Detritiation Activity of Hamster Enzyme.[a]

Addition	Detritiation rate (% of control)	Addition	Detritiation rate (% of control)
p-Chloromercuribenzoate	74	Cobaltous chloride	144
N-Ethylmaleimide	100	Ferrous sulfate	136
Iodoacetate	99	Molybdenum pentachloride	116
EDTA	78	Mercuric chloride	78
o-Phenanthroline	6	Silver nitrate	84
o-Phenanthroline + $CoCl_2$	41	Cupric acetate	84
o-Phenanthroline + $FeSO_4$	37		

[a] Values are expressed as percent of control containing none of the components listed. Each indicated component was present in the final reaction mixture at a concentration of 2×10^{-4} M. Manganese chloride, ferric chloride, ferric ammonium sulfate, zinc chloride, lead acetate, and lithium chloride were without effect.

TABLE IV: Loss of Tritium from [21-^{3}H]DOC when Incubated with Various Proteins.[a]

Protein source	Additions	Detritiation % of hamster enzyme
None	None	0
Hamster enzyme	None	100
Hamster enzyme	+ Fe^{2+}	137
Bovine serum albumin	None	0.2
Bovine serum albumin	+ Fe^{2+}	0.9
Bovine serum albumin	+ Co^{2+}	0.5
Lactate dehydrogenase	None	1.3
Lactate dehydrogenase	+ Fe^{2+}	1.5
Lactate dehydrogenase	+ Co^{2+}	1.3

[a] Incubations were performed as described in Table II. The vessels contained 2×10^{-4} M metal ion, 0.075 mg of protein, and 6×10^4 dpm of [21-^{3}H]DOC.

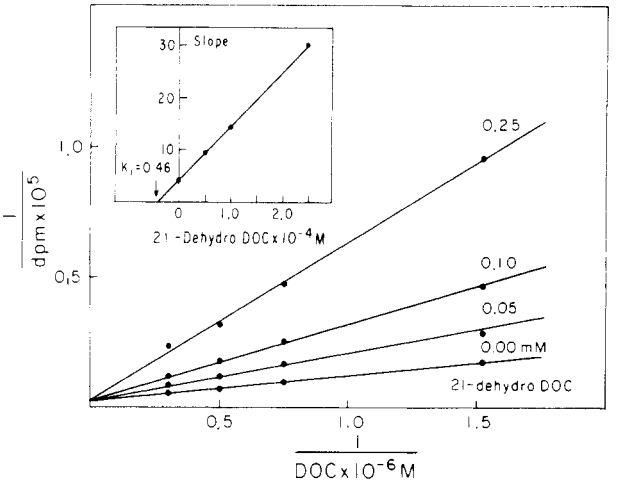


FIGURE 1: Effect of 3,20-dioxo-pregn-4-en-21-al (21-dehydroDOC) on detritiation of 11-deoxy[21-^{3}H]corticosterone. Insert shows secondary graph of the primary double reciprocal plots. Steroids were incubated with enzyme in 2 mL of 0.025 M Tris (pH 8.0) for 2 h at 37 °C. Velocity of reaction was taken as the rate of transfer of tritium to water.

Kinetics. Detritiation activity was linear with time for 2 h. The rate fell off during prolonged incubation. With [21-^{3}H]DOC as substrate, the initial reaction velocity was proportional to the amount of enzyme added. Double reciprocal plots of the initial rate of detritiation by enzyme against [21-^{3}H]DOC concentration were linear. No inhibition was observed with high concentration of DOC as substrate. An apparent K_m of 3.8×10^{-6} M was obtained.

In Table II is presented the relative velocities at which a number of 21-tritiated corticosteroids were detritiated. No correlation with respect to structure of the steroid and rate of tritium loss could be established. It was not possible to correlate the velocity with published values of steroid potency (Ringler, 1964) with respect to glycogen deposition, anti-inflammatory activity, or urinary sodium. The loss of tritium from tetrahydrocortisol is of doubtful significance.

Inhibitors. Table III summarizes the effects of a number of organic inhibitors and metal ions on activity. Of the reagents that react with sulfhydryl groups, *p*-chloromercuribenzoate alone was a weak inhibitor of the enzyme. In contrast, iodoacetate and *N*-ethylmaleimide were ineffective. The most significant inhibition of enzyme activity, 94%, was exhibited by *o*-phenanthroline. EDTA also showed an inhibitory effect, though this was less than that of *o*-phenanthroline. Inhibition by *o*-phenanthroline was partially overcome by addition of Co^{2+} or Fe^{2+} ions. Activation of enzyme to which no inhibitor was added was obtained directly with the transition metals Co^{2+}, Fe^{2+}, and Mo^{5+}. Most of the metals tested including Pb^{2+}, Li^+, Zn^{2+}, and Fe^{3+} did not influence enzyme activity. Some of the heavy metals, such as Hg^{2+}, Ag^+, and Cu^{2+}, showed weak inhibition at 2 mM concentration. Inhibition of activity of Hg^{2+} and Ag^+ may be due to interaction with SH groups of the enzyme, and inhibition of detritiation activity by Cu^{2+} might be due to formation of the steroidal-20-oxo-21-al which was found to be a strong competitive inhibitor (Figure 1).

Effects of Coenzymes. The effects of a number of coenzymes on detritiation activity with [21-^{3}H]-11-deoxycorticosterone were determined. Neither NAD, NADP, FAD, thiamine pyrophosphate, nor glutathione, alone or in combination, influenced enzyme activity.

Estimation of Molecular Weight. Molecular weight determination was presented in terms of K_{av}, as proposed by Laurent and Killander (1964). Molecular weight of the enzyme was estimated by plotting K_{av} vs. log molecular weight of standard proteins. A value of 389 000 ± 12 000 was obtained for three independent determinations.

Exchange of Tritium of [21-^{3}H]DOC with Water. Hamster liver enzyme catalyzed a transfer of tritium from [21-^{3}H]DOC into water. Figure 2 shows that 50% of the tritium exchanged in 2 h; 95% was exchanged in 24 h. No tritium was lost from [4-^{14}C, 21-^{3}H]DOC in the absence of enzyme. Table IV shows that, when bovine serum albumin replaced the detritiating enzyme at an equivalent concentration, tritium loss was not initiated. Rabbit muscle lactate dehydrogenase stimulated detritiation at a rate that was less than 1% of the hamster enzyme. Ferrous sulfate or cobaltous chloride, which stimulated hamster liver "detritiase", did not stimulate activity with the

TABLE V: Tritium Incorporation into DOC from ^3H_2O.

		Experiment 1[a]			Experiment 2[b]		
Substrate	Enzyme	Found (A)[c]	Expected (B)[c]	(A)/(B)	Found (A)[c]	Expected (B)[c]	(A)/(B)
DOC	+	5740	10000	0.57	1390	2200	0.63
DOC	−	92	0		24	0	

[a] Experiment 1. One-tenth milligram of DOC in 0.05 mL of methanol was incubated for 20 h in tritiated water (94 μCi/mL) buffered with 0.025 M Tris (pH 8.0) with 0.55 mg of enzyme. Final volume was 2.0 mL. Values given in dpm/mg of steroid. [b] Experiment 2. One-tenth milligram of DOC in 0.05 mL of methanol was incubated for 20 h in tritiated water (20 μCi/mL) buffered with 0.025 M Tris (pH 8.0) with 2.2 mg of enzyme. Final volume was 10 mL. Values given in dpm/mg of steroid. [c] Columns A present actual incorporation of tritium into steroid. Columns B indicate the maximum specific activity of the steroid based on the incorporation of 1 mol of tritium per mol of steroid.

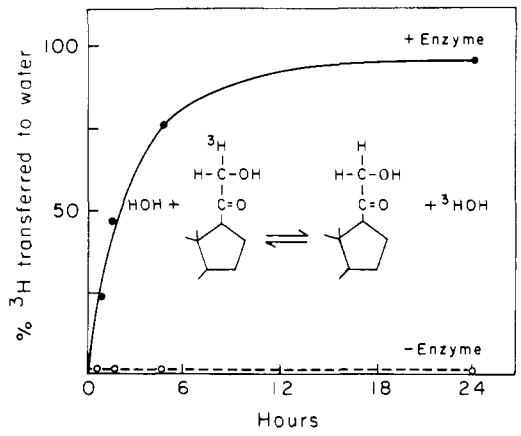


FIGURE 2: Enzyme-catalyzed exchange of steroid tritium with water. Reactants and products are shown in insert. [21-^{3}H]DOC, 9×10^5 dpm, in 0.05 mL of ethanol and 2.2 mg of enzyme were incubated in 10 mL of 0.025 M Tris (pH 8.0) at 37 °C. Final DOC concentration was 1.05×10^{-6} M.

other proteins. The detritiation of [21-^{3}H]DOC by hamster liver enzyme was therefore not due to a nonspecific protein or to a metal-catalyzed reaction.

Identification of the Ditritiated Product. The chromatographic mobility of the steroid after incubation for 24 h with hamster liver enzyme was identical with that of 11-deoxycorticosterone on thin-layer plates in three solvent systems: chloroform–methanol (98:2); dichloromethane (8:2); benzene–ethanol (84:6). In each system the ratio of the remaining ^{3}H to ^{14}C remained constant. This ratio did not change when the steroid was converted to the 21-acetate and rechromatographed. Specific activity and isotope ratio remained constant after cocrystallization of the steroid product with authentic DOC. The detritiated steroid was therefore unmetabolized 11-deoxycorticosterone. Recovery of [4-^{14}C]DOC was 95, 95, 99, and 86% of the initial value after 1, 2, 5, and 24 h of incubation, respectively.

Incorporation of Tritium from Tritiated Water into Deoxycorticosterone. The reversibility of the tritium exchange reaction was tested by incubating unlabeled DOC with enzyme in tritiated water. Table V shows that the exchange reaction was reversible. In 20 h, about 60% of the theoretical amount of tritium was incorporated in the steroid assuming that only one site exists on DOC for ^{3}H exchange. The rate of incorporation of tritium into the steroid appears to be considerably slower than its loss since equilibrium was attained more rapidly in the latter case.

Requirement of C-20 Carbonyl Group for Tritium Exchange. The carbonyl group at carbon 20 is essential for ^{3}H $\rightleftarrows$ ^{1}H exchange to occur. When position 20 was converted to an alcohol, exchange was abolished. [21-^{3}H]-20β,21-Dihydroxypregn-4-en-3-one was not detritiated by hamster enzyme.

Evaluation of 21-Dehydrocorticosterone as an Intermediate. A reversible oxidation of DOC to 21-dehydroDOC provides a possible explanation for the exchange reaction. The known 21-hydroxysteroid dehydrogenases (Monder and White, 1965) utilize NAD or NADP as a cosubstrate. We found that 21-hydroxysteroid dehydrogenase did not catalyze the net loss of tritium from the [4-^{14}C, 21-^{3}H]-labeled DOC at pH 8.5 to 9.5 over a 90-min incubation period with NAD^+. Therefore, this enzyme does not participate in the exchange reaction described. When 21-dehydroDOC was incubated with the detritiation enzyme with or without NADH, no DOC was formed. The introduction of 21-dehydroDOC into a system containing [21-^{3}H]DOC and "detritiase" inhibited the detritiation competitively, as seen in Figure 1. The K_I of detritiation with respect to 21-dehydroDOC was 4.6×10^{-5} M. [21-^{3}H]-21-DehydroDOC lost no tritium with the detritiase.

Stereochemistry of Detritiation. Chemical reduction of 21-dehydroDOC with sodium borotritiide yielded an approximately equimolar mixture of the epimeric 21*S* and 21*R* forms of the tritiated steroid alcohol (Orr and Monder, 1975). We expected that the enzyme catalyzed detritiation would occur preferentially with only one of the isomers. At first we decided to study the reaction with 21-deuterated steroid by monitoring the change in splitting pattern of the nuclear magnetic resonance spectrum during dedeuteration using techniques we had developed previously for 21-deuteriocortisol (Orr and Monder, 1975). This proved not to be feasible for 21-deuterioDOC because the pattern of the geminal 21-hydrogens of DOC was not sufficiently resolved on the Varian HR-220 spectrometer to provide unequivocal data. We decided therefore to study the removal of tritium from the epimeric derivatives [21*S*-21-^{3}H]DOC and [21*R*-21-^{3}H]DOC. The system contained 2.5×10^{-7} M substrate (45 Ci/mol), 0.14 mg of enzyme protein, and 0.025 M Tris (pH 8), in 2 mL final volume. After 2 h at 37 °C, [21*S*-21-^{3}H]DOC and [21*R*-21-^{3}H]DOC had lost 20.3 and 8.7% of their tritium, respectively. These results confirm the results of Willingham and Monder (1974), who used a crude hamster liver homogenate. The "detritiase" preferentially removed the 21*S* tritium, but did not show absolute stereospecific selectivity.

Incorporation of Deuterium into DOC. In order to ascertain that label was incorporated into the side chain of the steroid, DOC was incubated with the enzyme in deuterated water. The distribution of molecules incorporating deuterium were (percent of total mass ions): D_0 (67.7), D_1 (22.4), D_2 (10), D_3 (0) after 2 h of incubation; D_0 (11.0), D_1 (27.4), D_2 (56.8), D_3 (4.9) after 20 h of incubation. Even at 2 h of incubation, a significant fraction of the steroid molecules contained two deuteria. The proportion of molecules incorporating one deu-

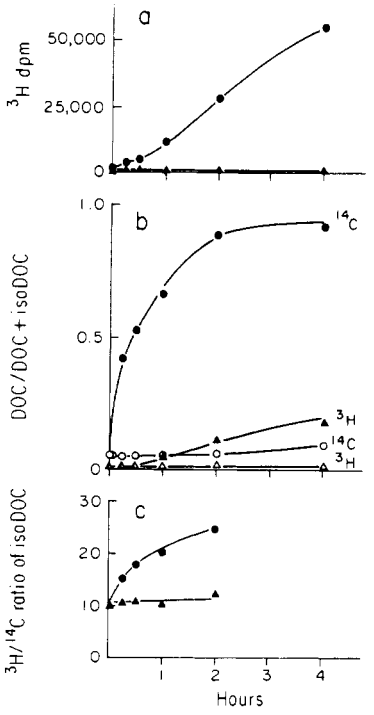


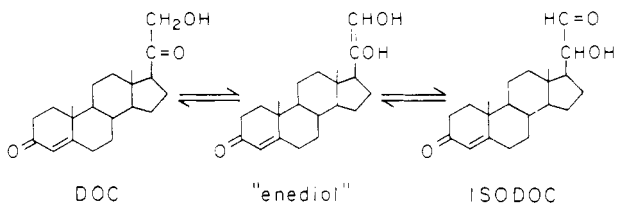
FIGURE 3: Conversion of isoDOC to DOC by hamster liver enzyme. [4-^{14}C]isoDOC (2.9×10^4 dpm) and [20α-^{3}H]isoDOC (2.74×10^5 dpm) in 0.05 mL of ethanol were incubated with 0.14 mg of enzyme in 0.025 M Tris, pH 7.0. Volume was 2.0 mL. Final DOC concentration was 2.4×10^{-6} M. Buffer replaced enzyme in controls. Tritiated water was removed from the incubation mixture by lyophilization. The residues were extracted with ethyl acetate and chromatographed to separate DOC and IsoDOC. The components were then transferred to scintillation vials and counted. (a) Appearance of tritium in water (●—●) with enzyme; (▲—▲) no enzyme; (b) Retention of ^{14}C and ^{3}H in DOC. (●—●) ^{14}C with enzyme; (○—○) ^{14}C, no enzyme; (▲—▲) ^{3}H with enzyme; (△—△) ^{3}H, no enzyme. (c) Change in ^{3}H/^{14}C ratio of remaining isoDOC (●—●) with enzyme; (▲—▲) no enzyme.

terium remained constant between 2 and 20 h, while the proportion containing two deuteria increased to two-thirds of the total in this time. A small fraction of the steroid molecules contained three deuteria. Examination of the fragmentation pattern showed that the ion m/e = 292 was homogeneous and contained only the natural abundance of deuterium. The label was therefore incorporated only into the side chain.

20-Hydroxy-3-oxo-pregn-4-en-21-al (IsoDOC) as a Substrate. Of several mechanistic possibilities, loss of tritium from [21-^{3}H]DOC through enolization appeared to be the most likely. The equilibrium relationship is shown in Scheme I.

The scheme predicts that 20β-hydroxy-3-oxo-pregn-4-en-21-al (isoDOC) is converted to DOC by the enzyme. A mixture of [4-^{14}C]isoDOC (Lippman and Monder, 1976) and

SCHEME I: Proposed Interconversion of DOC and isoDOC.[a]



[a] DOC and isoDOC exist in a reversible equilibrium with each other through an enediol intermediate. The scheme provides a working model to explain how conversion of isoDOC to DOC and the exchange of protons between DOC and water is catalyzed by the enzyme.

[20α-^{3}H]isoDOC made by the microscale modification of method of Oh and Monder (1976) using sodium borotritiide was incubated with enzyme. At intervals, aliquots of the incubation mixture were lyophilized, extracted with ethyl acetate and chromatographed in the developing system to separate the steroids. Figure 3 shows the distribution of ^{14}C and ^{3}H in DOC and isoDOC and water over a 4-h incubation period. Exchange of tritium with water, measured as increase in tritiated water, proceeded slowly during the first 30 min of incubation and then more rapidly during the ensuing 3.5 h. Loss of tritium from isoDOC in the absence of enzyme was negligible (Figure 3a). The formation of DOC was rapid. After 4 h of incubation, 95% of the isoDOC was converted to DOC (Figure 3b). The [20α-^{3}H]isoDOC rearranged to form DOC much more slowly than unlabeled isoDOC, as shown by the rapid enrichment of isoDOC with ^{3}H. The ^{3}H/^{14}C ratio of the remaining isoDOC increased from 9.6 to 24.7 in 2 h (Figure 3c).

Discussion

The ability of hamster liver and other tissues to remove tritium from [21-^{3}H]-labeled corticosteroids has been noted previously by Willingham and Monder (1974). In this paper we have described the partial purification of an enzyme that catalyzes this reaction. Our original rationale was that loss of tritium from 21-^{3}H-labeled corticosteroids is a measure of the extent of oxidation of the steroid side chain to carboxylic acids. We found, however, that transfer of tritium to water was far greater than could be accounted for by the amount of steroid acid formed. The reason for this, as demonstrated in this paper, was that the excess tritium was lost from the 21-^{3}H-labeled steroid with no accompanying metabolic change. We do not know the nature of the chromophore of this yellow enzyme, or the relation of the stimulation by metal ions to the activity of the native enzyme. Organic sulfhydryl blockers and the metal ions Hg^{2+} or Ag^+ did not inhibit activity and it was concluded that free sulfhydryl groups do not participate in the reaction.

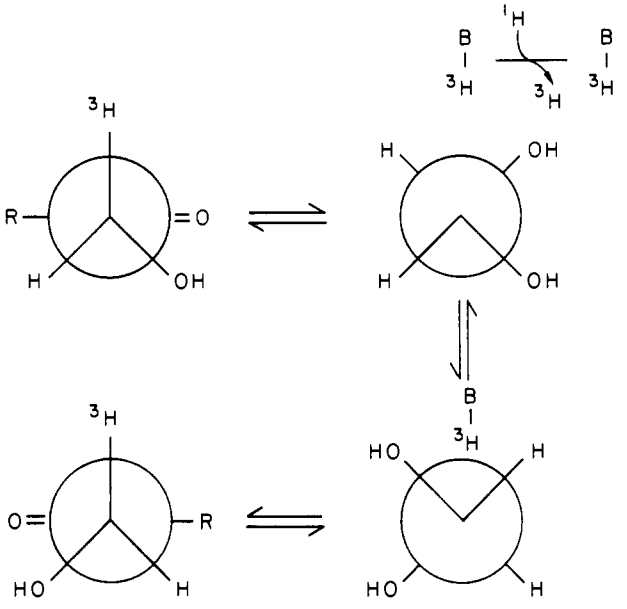
The enzyme probably catalyzes the reversible formation of an enediol. Eger et al. (1972) have suggested that, in the relatively nonpolar environment of the steroid binding site of a protein, the formation of corticosteroid 17β-enediols may be facilitated by the presence of metal ions such as cobaltous ion. The ability of ferrous and cobaltous ions to enhance detritiation by our enzyme is consistent with this proposal. Alternative pathways involving reversible oxidation and reduction at position 21 could not account for the tritium exchange with water. No reduction of 21-dehydroDOC to DOC occurred and 21-dehydroDOC inhibited the reaction. The enzyme did not catalyze the oxidation of DOC to 21-dehydroDOC. An alternative possible intermediate, 20,21-dihydroxy-pregn-4-en-3-one, was not a substrate, indicating the obligatory requirement of a 20-carbonyl function. We considered the possibility that an enol could be generated from an aldol or ketol, and accordingly showed the rapid interconversion of DOC and isoDOC by the enzyme. The equilibrium was far in the direction of the ketol.

The isomerization of [20-^{3}H]isoDOC to DOC occurs in part with the loss of tritium to water and in part with the retention of tritium as [21-^{3}H]DOC. In two experiments, the proportion of isoDOC relative to DOC at equilibrium was 0.013 ± 0.006. The ^{3}H/^{14}C ratio of isoDOC increased from 9.6 to 24.7 in 2 h of incubation, while the ^{3}H/^{14}C ratio of DOC formed in this time was 0.35. Evidently, during the conversion of isoDOC, the tritium at position 20 was preferentially exchanged with water, and transferred to position 21 of DOC to a much smaller extent.

The labeling of steroid in tritiated or deuterated water catalyzed by enzyme occurred with the incorporation of tracer mostly, but not exclusively, in one stereochemical orientation on carbon 21. Removal of label from the enantiomeric tritiated 21-carbons was also not absolutely stereospecific. These results are inconsistent with an enediol intermediate which is firmly and stereospecifically bound to the isomerase (Rieder and Rose, 1959).

An explanation for the stereochemical results could be made if a limited dissociation of the enediol from the enzyme stabilized by a divalent metal ion is assumed followed by rotation of the side chain around the 17–20 bond. The postulated epimerization could be enzyme mediated, although we have as yet no evidence for an epimerase acting on this substrate. The participation of a divalent metal ion in the reaction is consistent with a *cis*-enediol mechanism. Although tritium exchange with the bound *cis*-enediol is favored, rotation of the side chain in the transition state permits the incorporation of tritium at either the *si* or *re* faces, as shown in Scheme II.

SCHEME II: Hypothetical Scheme to Show Isomerization and Epimerization of Steroid Side Chain.



We conclude that the loss of tritium from [21-^{3}H]DOC results from the exchange with water of a proton at C-21 mediated through an enediol transition state. The intramolecular transfer of the proton between C-20 and C-21 can be rationalized within this mechanism. The detritiation enzyme is therefore an isomerase. Further study of its properties should reveal the extent of its similarity to other known isomerases (Rose, 1975), and its role in corticosteroid metabolism.

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