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OXIDATION OF CORTICOSTEROIDS TO STEROIDAL 20-HYDROXY-21-OIC ACIDS BY MOUSE LIVER

A. R. PURKAYSTHA, K. O. MARTIN, A. GOLDBERG and C. MONDER*
The Population Council, Center for Bio-medical Research, 1230 York Avenue, New York, NY 10021, U.S.A.

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SUMMARY

We have studied the enzyme catalyzed oxidation of 11-deoxycorticosterone to 20-hydroxy-3-oxo-4-pregnen-21-oic acid (pregnolic acid) in mouse liver. Enzyme activity, though variable, was higher than that of other species. Pregnolic acid was identified as the free acid, as the methyl ester by thin layer chromatography, and as the *p*-bromophenacyl ester by high performance liquid chromatography. With [4-^{14}C, 21-^{3}H]-DOC as substrate, exchange of tritium with water (interpreted as due to the reversible isomerization of the ketol to aldol form of the side chain) and the overall conversion of the ketol side chain to hydroxy acid was catalyzed by the post-microsomal supernatant fraction. Although we could not physically separate tritium exchange and acid production, pregnolic acid formation could be decreased or eliminated while tritium exchange was retained, consistent with our previous conclusion that isomerization to aldol was a precondition for acid formation. In preparations that made no acid, [4-^{14}C]-DOC was recovered, depleted of tritium. The rate of exchange of [21S, 21-^{3}H]-DOC with water was faster than [21R, 21-^{3}H]-DOC. The stereochemistry of pregnolic acid at C-20 was 85–90% R (i.e. 20α-hydroxy-21-oic acid). The K_m for isomerase with [21RS-21^{3}H]-DOC was 4.3×10^{-5} M; K_m for pregnolic acid formation was 8.0×10^{-5} M. Corticosterone was oxidized to acid metabolites at 20% the rate of DOC.

INTRODUCTION

Humans and lower animals oxidize corticosteroids to acidic end-products in quantitatively significant amounts [1–3]. It is not known if these oxidative pathways represent catabolic alternatives to the well-known reductive pathways or if they have other physiological significance. We originally undertook an examination of the details of the oxidation sequence utilizing hamster liver preparations [4]. With this model we established that corticosteroids are oxidized by enzymes located in the liver cytosol to 20-hydroxy-21-oic acids. The oxidation step followed rearrangement of the ketol side chain to the aldol configuration [5]. We have recently begun to investigate another animal, the mouse, which has the advantage of having a higher level of liver enzyme activity than the hamster. This paper describes our initial studies with the mouse liver system.

MATERIALS AND METHODS

Materials

Diphospho-[4-^{3}H]-pyridine adenine dinucleotide ammonium salt, [4(*n*)-^{3}H]-NAD, 50 mCi/mmol, was bought from Amersham Corp., IL. Unlabeled NADH, disodium salt, was from Sigma Chemical Co. 11-deoxy-[4-^{14}C]-corticosterone (54 mCi/mmol) and Aquafluor were bought from New England Nuclear Corp. Sephacryl S-200 and Sephadex G-25 (coarse) were bought from Pharmacia Corp. DE-52 (DEAE-cellulose) was purchased from Whatman Corp. Diazomethane for esterification of steroid acids was generated from Diazald (*N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide) according to the directions of the manufacturer, Aldrich Chemical Co., Inc.

Animals

Male Swiss mice weighing 22–23 g were from the Charles River Laboratories. They were kept in our animal quarters in plastic cages containing six mice each. Day:night cycle was 10 h:14 h. Temperature was maintained at 26°C. Purina Chow and water were given *ad lib*. The mice were used when they weighed 27–29 g. DBA/2J and C57BL/6J mice from Jackson Labs were used in some experiments.

Preparation of (4S)-[4-^{3}H]-NADH and [21-^{3}H]-DOC

(4S)-[4-^{3}H]-NADH was synthesized by incubating 0.05 ml of ethanol, 50 μg of horse liver alcohol dehydrogenase (Boehringer-Mannheim, SA 27U/mg), [4-^{3}H]-NAD (50 μCi) and 0.85 ml of 0.01 M sodium pyrophosphate, pH 9.5, in 1.0 ml final vol., for 30 min. To this mixture was added 330 μg of 21-dehydro DOC prepared as described by Oh and Monder[6], 0.1 ml of 21-hydroxysteroid dehydrogenase (SA 0.3 μmol/min/mg protein) purified by the method of Monder and White[7], 500 μg of pyrazole and 1.7 ml of 0.1 M sodium phosphate, pH 6.3, in a final vol. of 3.0 ml. When the absorbance at 340 nm no longer de-

* To whom correspondence should be sent.

creased, the incubation mixture was saturated with sodium chloride and extracted with ethyl acetate. The organic phase was washed with saturated aqueous sodium chloride, and water removed with sodium sulfate. The radioactive steroid was purified by chromatography. Yield of (21S)-[21-^{3}H]-DOC was 5.12 μCi (10.9 μCi/μmol).

(21R)-[21-^{3}H]-DOC was synthesized in the same way with [21-^{3}H]-21-dehydroDOC and unlabeled NADH. Specific activity of the product was 8 μCi/μmol. (21 R, S)-[21-^{3}H]-DOC (SA 37 mCi/mmol) was made by reduction of 21-dehydroDOC with sodium borotritiide (100 mCi/mmol) (New England Nuclear) [8]. 20α and 20β isomers of 20 hydroxy-4-pregnen-21-oic acid (pregnolic acid) were made by base rearrangement of 21-dehydroDOC [9].

Preparation of liver cytosol

Livers were homogenized in 4 vol. of 0.25 M sucrose in 0.01 M sodium phosphate, pH 7.0, with a Teflon–glass axial homogenizer. Homogenates were centrifuged at 100 *g* for 15 min, then the supernatant fluids were centrifuged at 105,000 *g* for 60 min. The resulting post-microsomal supernatant fraction was used for studies directly or subjected to further fractionations.

Column chromatography

A column of Sephacryl S-200, 2.5 × 76.2 cm was equilibrated with 0.01 M sodium phosphate, pH 7.5, containing 0.5 M sodium chloride. During chromatography, the flow rate was 37 ml per h; 4.8 ml aliquots were collected.

A DE-52 column, 0.9 cm × 13 cm, V_t = 8.3 ml, was washed overnight with 0.05 M tris, pH 8.0. An aliquot of 1.0 ml of enzyme was transferred to the column and washed in with 40 ml of the same buffer. Elution of enzyme was initiated with a gradient of sodium chloride (0–1 M) in 0.05 M Tris, pH 8.0. Fractions of 2.0 ml were collected.

Sephadex G-25 (coarse) columns, 0.9 cm × 7.9 cm, or 0.9 cm × 23.5 cm, were prepared. Total bed volume of the former was 5.0 ml, and of the latter was 15 ml. The columns were equilibrated with 0.01 M sodium phosphate, pH 7.0. One ml aliquot of cytosol was placed on the column and eluted with the same solvent. Fractions of 1.5 ml were collected.

High performance liquid chromatography of *p*-bromphenacyl derivatives of steroid acids was done on reverse phase columns (octadecylsilyl or phenyl) using alcohol–water mixtures as described by Farhi and Monder[10].

Measurement of isomerase activity and steroid acid synthesis

The exchange of tritium at C-21 with water is a measure of isomerase activity. To perform the assay, 20 μl of cytosol, 10 μl of [4-^{14}C, 21-^{3}H]-DOC and 0.97 ml of 0.025 M Tris, pH 8.0, were incubated at 37°C for 2 h, then half was lyophilized. An aliquot of the water condensate was diluted in Aquafluor (New England Nuclear) and counted in a scintillation counter. To determine acid production, the other half of the incubation mixture was acidified to pH 2 with hydrochloric acid and extracted three times with 0.5 ml of ethyl acetate each time. The ethyl acetate layer was washed with saturated aqueous sodium chloride, dried, and transferred in acetone to thin layer silica plates. The plates were developed with chloroform–methanol–formic acid (98:2:1, v/v), and the region corresponding to pregnolic acid was extracted. The amount of acid is expressed as percent of total radioactive metabolites (including unmetabolized DOC), or as nmol of steroid formed.

Protein was determined by the method of Kalkar[11] or Lowry *et al.*[12].

RESULTS

Tritium exchange and pregnolic acid synthesis in liver of various species

In human and hamster liver, the exchange of tritium in [21-^{3}H]-DOC with water is due to the activity of an isomerase that catalyzes the interconversion of 17 ketol (20-oxo-21-hydroxymethyl) and 17-aldol (20-hydroxy-21-aldehyde) forms of the corticosteroid side chain [5, 13]. A survey of tritium exchange activity with [21-^{3}H]-DOC as substrate and cytosols prepared from livers of other mammalian species was made, based on the assumption that the exchange is related to isomerase in these, as well. The values obtained are tabulated in Table 1. Highest activity was found in mouse liver, which was three times more active than pig liver, the next most active source.

Table 1. Isomerase activity and pregnolic acid formation in livers of representative animals

Liver source	Isomerase (%)	Pregnolic acid formation (%)
Mouse	59.1	43.6
Pig	20.0	21.5
Rabbit	15.0	13.1
Sheep	12.0	15.0
Hamster	11.6	8.8
Dog	11.5	4.9
Rat	4.6	6.9

Each incubation vessel contained 0.1 ml of 100,000 × *g* supernatant of 20% liver homogenate from male animals, 20 μl of ^{3}H/^{14}C DOC (3.46 nmol, ^{3}H/^{14}C = 9.2) and 1.88 ml of 0.025 M Tris–HCl buffer, pH 8.0. A blank containing no enzyme was run in parallel. Isomerase activity is expressed as the per cent of tritium at C-21 that appeared in water after 2 h at 37°C. Pregnolic acid was measured directly after separating it from other metabolites on thin layer chromatograms as described under Methods, and is expressed as percent of total ^{14}C containing metabolites.

Table 2. Subcellular distribution of tritium exchange activity

Fraction	Activity Expt 1	Expt 2
	% of added tracer	
Homogenate	7.6	55
Nuclear	2.2	
Mito	1.3	17.0
Micro	0.7	
Cytosol	8.4	59.0
Mito + Cytosol	8.5	60.3
Micro + Cytosol	8.5	56.7

Each incubation vessel contained 0.05 ml of homogenate or subcellular fraction. Nuclear = 600 *g* sediment; Mito = 10,000 *g* sediment from nuclear supernatant; Micro = 105,000 *g* sediment from mito supernatant; cytosol = 105,000 *g* post-microsomal supernatant. Each fraction was reconstituted to the original volume of homogenate with 0.25 M sucrose. Other components were: 10 μl of [4-^{14}C, 21-^{3}H]-DOC (1.73 nmol, ^{3}H/^{14}C = 9.2) and 0.89 ml of 0.025 M Tris HCl, pH 8.0. Isomerase activity was measured as exchange of tritium at C-21 with the water of the medium in 2 h at 37°C. A blank with no added protein fraction gave a value of tritium exchange that was less than 0.1% of added tracer. The values shown represent the percentage of tritium at C-21 that underwent exchange. In Expt 1, a "low activity" C57BL/6j mouse was used; in Expt 2, a "high activity" DBA/2 mouse was used.

The end product of the oxidation of the steroid side chain is a hydroxy acid, which is produced from the aldol. The acid formed from DOC in hamster and human livers was shown to be 20-hydroxy-4-pregnen-21-oic acid [4, 14], to which we give the trivial name, pregnolic acid. The acids formed in the other species listed in Table 1 were chromatographically identical to pregnolic acid and are presumably the same acid. There was generally close correspondence between tritium exchange activity and acid formation in all cases.

Subcellular location of enzyme activity in mouse liver

Tritium exchange, or isomerase activity, was located predominantly in the cytosol fraction of mouse liver. The two experiments presented in Table 2 show the distribution of enzyme in a "low activity" (expt 1) and a "high activity" (expt 2) preparation. In both cases, cytosol had a slightly greater tritium exchange rate than the unfractionated homogenate. The lower activity of the homogenate was not due to inhibition by microsomes or mitochondria. A minor amount of tritium exchange remained with the particulate fractions. This may be due to absorbed enzyme, or to distinct enzymes associated with subcellular components. The sum of the activities of the individual subfractions was greater than that of the tissue homogenate before fractionation.

Isomerase activities of individual mouse liver homogenates and cytosols showed considerable variability. Values ranged from 110 to 263 pmol of tritium exchanged per 2 h per mg of protein when total [21-^{3}H]-DOC was initially 1 nmol. The differences between animals is due to genetically determined effects on enzyme level [14], and possibly to the presence of inhibitors in the cytosol. When cytosols were passed through Sephadex G-25 columns to remove small molecules, the active high molecular weight effluent was found to have increased activity. Table 3 shows the results for low activity (I), intermediate activity (II) and high activity (III) preparations. Addition of the low molecular weight fraction (fr 7 + 8) back to the enzymes did not reestablish the lower activity. It is not clear, therefore, whether passage through the column did in fact remove an inhibitor or

Table 3. Effect on isomerase activity and acid formation of passage of cytosol through Sephadex G-25 column

Enzyme source	I		II		III	
	Isomerase (d.p.m. × 10^{-4}/ mg protein)	Acid formed (nmol/mg protein)	Isomerase (d.p.m. × 10^{-4}/ mg protein)	Acid formed (nmol/mg protein)	Isomerase (d.p.m. × 10^{-4}/ mg protein)	Acid formed (nmol/mg protein)
Cytosol	0.82	0.071	1.46	0.114	3.30	0.209
Cytosol + (fr 7 + 8)	0.87	0.081	1.46	0.105		
G-25 effluent	1.34	0.044	2.32	0.118	3.62	0.059
G-25 effluent + (fr 7 + 8)	1.38	0.048	2.32	0.107		
(fr 7 + 8)	0	0	0	0		
Boiled cytosol*	0	0	0	0		

* Cytosol, 0.5 ml, from DBA/2 mice (Runs II and III) or C57BL/6 mice (run I) was transferred to a Sephadex G-25 (coarse) column (V_0 = 3 ml; V_e = 8 ml) and eluted with 0.025 M Tris–HCl, pH 8. One ml fractions were collected. Fraction 3 contained the enzyme. Fractions 7 and 8 (fr 7 + 8) contained the low molecular weight components, and had no protein. Cytosol was diluted to give the same protein concentration as the G-25 effluent (5.4 mg protein/ml). Total protein in each incubation was 1.33 ± 0.30 (SD) mg/ml. Concentration of steroid was 0.654 nmol per mg protein (63,600 d.p.m. ^{3}H/nM steroid). Incubation was in 0.02 M Tris, pH 8.0, for 2 h at 37°C. Final volume was 1.0 ml. To prepare boiled cytosol, two ml of cytosol were heated at 100°C for 3 min in a boiling water bath.

activated the enzyme in some other way. The differences in activities of the preparations illustrated were not due to inhibition that was restricted to the preparation with lowest activity, for gel filtration increased activities both in preparations I and II about 1.6-fold. A cytosol that was initially highly active (preparation III) showed little or no change in activity after passage through Sephadex G-25.

Steroid acid formation, in contrast with isomerase activity, did not increase after gel filtration. Acid production either was unchanged (cytosol II) or decreased (cytosols I and III).

Effect of heating

Tritium exchange and acid synthesis were not diminished when cytosol was heated at 50°C for 10 min. At 55°C, 50% of the initial acid forming activity was lost in 10 min. In this time, the detritiating activity decreased by 30%. At 100°C, all activities were abolished within 3 min.

Attempts to separate tritium exchange and acid formation

According to a published model [1], the activities associated with isomerase and acid formation are linked. That is, pregnolic acid formation cannot occur unless isomerase first generates the aldol precursor, although isomerase activity can persist in the absence of acid formation. It should therefore be possible to demonstrate isomerase in the absence of acid forming activity, but it should not be possible to have acid synthesis without isomerase.

Tritium exchange activity was fractionated with ammonium sulfate as shown in Fig. 1. Both detritiation and acid forming activity were precipitated in the range of 60–80% ammonium sulfate. About 34% of the soluble tritium exchange activity precipitated with 55% ammonium sulfate with little detectable acid formation. Most of the detritiation (62.6%) and steroid acid forming (61.3%) activities precipitated between 55 and 80% ammonium sulfate. The broad distribution in the ammonium sulfate fractions of the tritium exchange activity suggests that this is heterogeneous in the mouse liver. When the combined fractions precipitated between 55 and 80% ammonium sulfate was passed through a Sephacryl-S-200 gel filtration column, the isomerase activity emerged in a narrow symmetrical band (Fig. 2) together with pregnolic acid synthesizing activity. Chromatography resulted in a loss of isomerase activity, for despite the substantial separation of protein during development of the column, specific activity of the emerging isomerase was 989 pmol per mg protein compared to 1170 pmol per mg protein placed on the column.

The peak ammonium sulfate activity was passed through a Sephadex G-100 column (2.5 cm × 76 cm, ascending flow), eluting with 0.01 M sodium phosphate, pH 7.0. This resulted in neither further purification of isomerase or of pregnolic acid synthesis. Both activities came out at the same position.

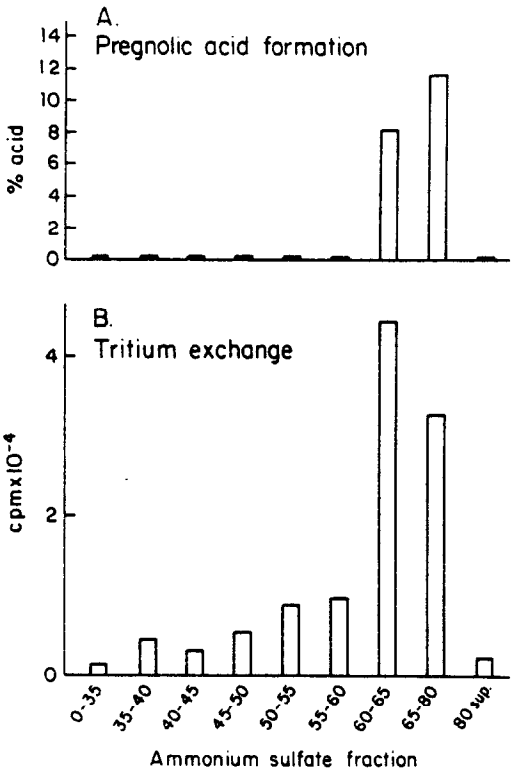

Fig. 1. Tritium exchange and pregnolic acid synthesis in ammonium sulfate fractions of mouse liver cytosol. Concentration of ammonium sulfate is expressed as per cent of saturation at 4°C. Panel A. Acid formation expressed as per cent of [4-^{14}C, 21-^{3}H]-DOC converted to pregnolic acid in 2 h at 37°C. Panel B. Tritium exchange expressed as c.p.m. of tritium appearing in water after 2 h at 37°C, when initial 21-^{3}H was 22.7 × 10^4 c.p.m.

Passage of the most active fraction from the G-100 column through a DEAE-cellulose column resulted in no purification of isomerase, and the total loss of acid forming activity.

Identification of the acid metabolite

Incubation of 2 nmol of [4-^{14}C, 21-^{3}H]-DOC with cytosol in 0.01 M Tris, pH 8.0, for 2 h at 37°C, resulted in the formation of a ^{14}C labeled product that contained a diminished amount of tritium. When the incubation mixture was extracted into ethyl acetate, a ^{14}C labeled component was left behind in the aqueous phase. This was extracted by ethyl acetate only after acidification of the aqueous solution to pH 2 with hydrochloric acid. This component was chromatographed on silica gel coated thin layer plates. Its mobility corresponded to that of authentic pregnolic acid in chloroform–methanol–formic acid (90:10:1, by vol.), R_f = 0.38. In the chromatographic profile shown in Fig. 3, pregnolic acid accounted for 36.2% and DOC represented 37.3% of the total activity. The remaining activity was located at positions coinciding with 5α pregnanetriol, 5β-pregnanetriol, and other positions as yet unidentified.

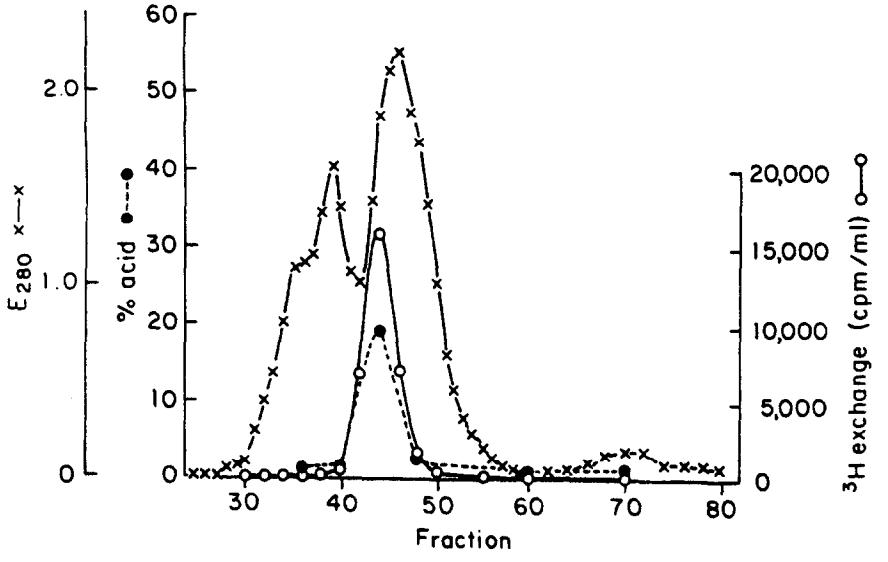

Fig. 2. Fractionation of the combined 55–80% ammonium sulfate sediments on Sephacryl S-200. Fractions of 5.3 ml were collected. ×——×, protein; o——o, tritium exchanged with water; ●——●, pregnolic acid

The major radioactive acid metabolite was converted to the methyl ester with diazomethane. This derivative migrated with known synthetic acid ester on silica gel plates in three solvent systems–chloroform–methanol, 98:2, v/v (R_f = 0.17); ethyl acetate, (R_f = 0.67); chloroform–acetone, 80:20, v/v (R_f = 0.50).

Effects of prolonged incubation on tritium exchange and pregnolic acid synthesis

In the chromatogram shown in Fig. 3, pregnolic acid represented 72% of the metabolites of DOC which were formed after 2 h of incubation. The remainder were neutral steroids. The effects of cytosol which had previously been filtered through Sephadex G-25 on isomerase and pregnolic acid synthesis was now determined. Two levels of enzyme were used. Starting with 7.8×10^4 d.p.m. of ^{3}H (6.5×10^4 d.p.m./nmol; $^3H/^{14}C$ = 2.23), the transfer of tritium to water after 24 h of incubation was 81.5 and 93.5% of the total steroid for 10 and 50 µl enzyme preparation, respectively. Acid production was 3 and 26% for 10 µl and 50 µl of cytosol. These results, with those of Fig. 3, show that the formation of acid stops within 2 h, but that the tritium exchange process persists as the exchange of tritium with the medium

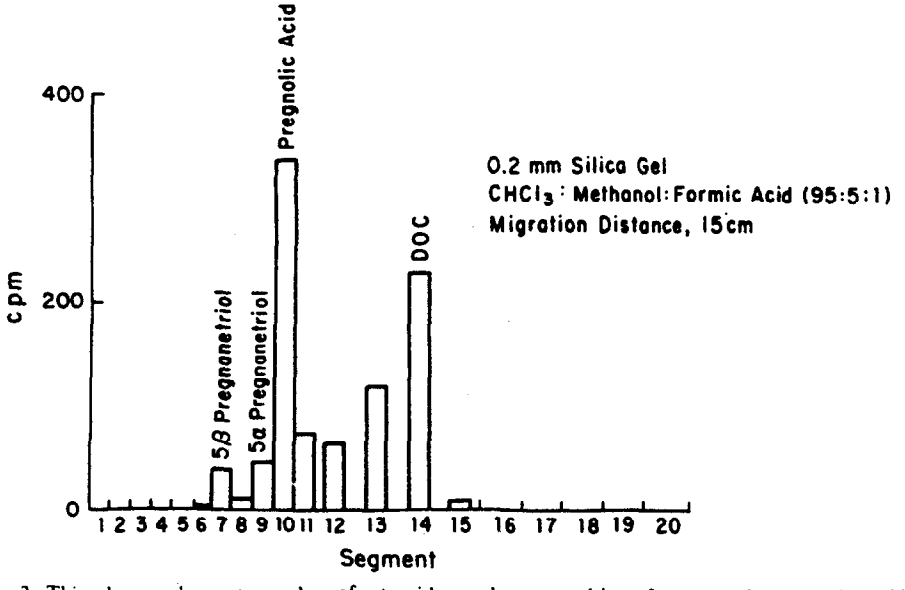

Fig. 3. Thin layer chromatography of steroid products resulting from incubation of [4-^{14}C, 21-^{3}H]-DOC with mouse liver cytosol.

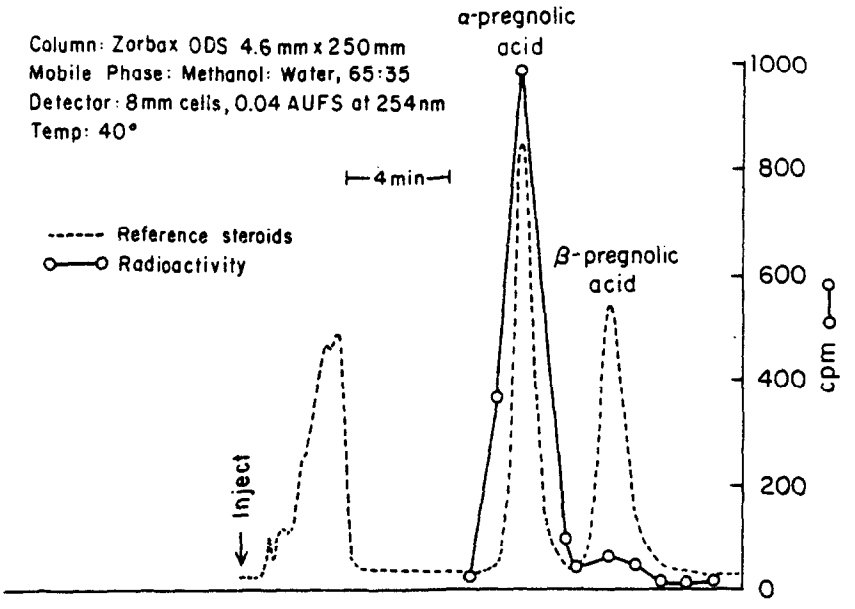

Fig. 4. Reverse phase high performance liquid chromatography of *p*-bromphenacyl esters of pregnolic acid. Column was an octadecyl silyl bonded phase, 25 cm long and 4.6 mm i.d. Mobile phase was methanol–water (35:65, v/v). Peak A 20α epimer Peak B, 20β epimer, absorbance at 250 mm; ○ c.p.m.

approaches completion. With 10 μl of cytosol, 82–90% of the DOC was recovered with a loss of 90% of the tritium from the side chain, which was otherwise unaltered. The chromatographic analysis of the 50 μl cytosol incubation was more complex. Of the total [^{14}C]-steroid recovered, 10% of the label was more polar than pregnolic acid: 26% was pregnolic acid; 24% migrated to a position between the acid and [^{14}C]-DOC and 34% was recovered as [^{14}C]-DOC.

Stereochemistry of the hydroxy acid side chain

The stereochemistry of the side chain of pregnolic acid is determined by the asymmetry of the chiral center at C-20. The chirality may be either 20R (20α) or 20S (20β). The stereoisomers were readily separated by high performance liquid chromatography using the procedure described by Farhi and Monder[10]. The *p*-bromphenacyl derivatives of the ^{14}C labeled acid product synthesized by the mouse enzyme was chromatographed on a "phenyl" column with methanol–water (65:35, v/v) as developing solvent. The distribution of radioactivity superimposed over the known standards are shown in Fig. 4. In this experiment 94.4% of the total was 20α.

Kinetic constants

The rates of tritium exchange and pregnolate synthesis with cytosol and [4-^{14}C, 21-^{3}H]-DOC were measured and evaluated after graphing reciprocal velocity vs the reciprocals of DOC concentration. The K_M values for isomerase and acid synthesis were 4.3×10^{-5} M and 8.0×10^{-5} M, respectively. V_{max} for isomerase was 20.9 nmol/mg protein/h, and for acid synthesis was 7.7 nmol/mg protein/h.

Effect of enzyme concentration

The effect of cytosol volume on tritium exchange and acid synthesis is shown in Fig. 5. Substrate was present at 10^{-5} M and 10^{-4} M. Rates of both acid synthesis and tritium exchange were linear with enzyme level over the entire range studied with the higher level of steroid. At low levels of steroid (10^{-5} M) the rate of oxidation diminished with increasing amount of enzyme because there was not enough substrate to maintain first order kinetics throughout the incubation. Under all conditions, the extent of tritium exchange exceeded the extent of acid formation.

Stereochemistry of tritium exchange

The [21-^{3}H]-DOC is stereochemically asymmetric at C-21 and exists in two chiral forms: (21S)-[21-^{3}H]-DOC and [21R-^{3}H]-DOC. Table 4 shows that the isomerase exchanged the tritium from the 21S isomer with water at a faster rate than the 21R isomer. In 2 h, the per cent of total tritium exchanged was 44 and 17% for 21S and 21R epimers, respectively. The retention of tritium in the acid product was 18% for (21S)-[21-^{3}H]-DOC, and 31% for (21R)-[21-^{3}H]-DOC. The more rapid exchange with water of (21-S)-[21-^{3}H]-DOC than the 21R epimer resulted in a lower ^{3}H/^{14}C ratio in the acid product. The predominantly 20α stereochemistry of the product is again shown in these experiments. The ratios of 20α–20β epimer were 7:1 in both cases.

Comparison of corticosterone and DOC

Table 5 shows that corticosterone was also a substrate for tritium exchange and acid formation.

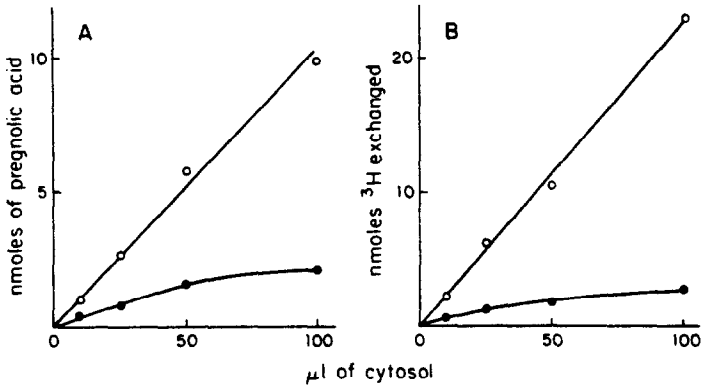

Fig. 5. Effect of cytosol level on [4-^{14}C, 21-^{3}H]-DOC metabolism. Panel A. pregnolic acid formed; Panel B. tritium exchange. ●——● 1×10^{-5} M DOC, O——O 1×10^{-4} M DOC.

though as we had found with human and hamster liver enzymes, DOC was more rapidly metabolized. The metabolism of corticosterone to 11β-hydroxy-pregnolic acid was sensitive to the amount of enzyme used. The relative activity of corticosterone was 24% of DOC for isomerase and 20% of DOC for acid formation.

DISCUSSION

We have shown that corticosteroids are oxidized to hydroxy acids by enzyme prepared from human and hamster liver [4, 15]. The extention of our studies to the mouse was prompted by our finding that mouse liver was particularly active, and oxidized the model substrate, 11-deoxycorticosterone, to hydroxy acid to a far greater extent than hamster or other animal livers did. We therefore decided to perform a more detailed study of the mouse liver anticipating that, if the activity was indeed as high as it appeared, and the enzymes corresponded in properties with those we had previously prepared, then this source would be an excellent one to use for purification.

The literature on corticosteroid metabolism in mice is not extensive. One of the difficulties encountered in attempting studies in either the mouse or the rat is that the path of excretion of metabolites is primarily through the gastrointestinal tract. This presents serious problems of interpretation, since one cannot be certain that the alterations observed are not due to bacterial action in the intestine [16, 17]. The problem of enterohepatic recycling adds another burden,

Table 4. Stereochemistry of tritium exchange

	DOC ^{3}H/^{14}C	Pregnolic acid ^{3}H/^{14}C	Tritium exchange (%)	Stereochemistry at C-20 (%α)	(%β)
(21S)-[4-^{14}C, 21-^{3}H]-DOC	3.60	0.65	44	88	12
(21R)-[4-^{14}C, 21-^{3}H]-DOC	4.65	1.43	17	87.5	12.5

Table 5. Tritium exchange and 21-oic acid synthesis: comparison of [4-^{14}C, 21-^{3}H]-corticosterone (B) and 11-deoxy [4-^{14}C, 21-^{3}H]-corticosterone (DOC) as substrate

Cytosol (ml)	Tritium exchange B (%)	DOC (%)	B/DOC	21-oic acid B (%)	DOC (%)	B/DOC
0.1	7.3	20.6	0.24	4.4	21.7	0.20
0.2	11.2	—		7.5	—	

Cytosol from two male DBA/2 mice were incubated with 0.02 M Tris, pH 8.0 and Corticosterone (38 μCi ^{3}H/μmol, ^{3}H/^{14}C = 3.67, 10×10^4 d.p.m.^{3}H) or 11-deoxycorticosterone (37 μCi ^{3}H/μmol, ^{3}H/^{14}C = 2.3, 7.8×10^4 d.p.m.^{3}H) in 0.01 ml of ethanol. Final volume was 1.0 ml. Tritium exchange was measured after 2 h at 37°C. Steroid acids were isolated by thin layer chromatography. Values of acids are expressed as per cent of total ^{14}C recovered from the plate.

which many workers feel it is better to avoid. The polar metabolites isolated from fetal mouse livers after the injection of tritiated corticosterone into the mothers appear to be neutral tetra and hexahydro metabolites [18]. In both prenatal and postnatal mice, unidentified polar products in livers represent a significant fraction of the total metabolites [18–20]. The products, which under one set of conditions collectively amounted to 70% of the total metabolites [19], were not identified, but could have contained glucosiduronides of neutral reduced metabolites or acids. In all *in vitro* studies with liver preparations the only products of corticosterone sought and found were the neutral reduced metabolites, and the production of these depended on the availability of NADPH, which was in part under genetic control [21, 22].

In the papers cited, corticosteroids were converted to polar products whether they were administered *in vivo* or *in vitro*. In no case was there any attempt to identify all of the metabolites, so it is not possible to evaluate from the literature to what extent side chain oxidation played a role in metabolism.

In our investigation we have emphasized the oxidative metabolism of the ketol side chain. This is in contrast with the workers who focussed on reduction as evidenced by their concern with the role of NADPH as a cofactor. From the studies reported here, we concluded that mouse liver engages in extensive oxidation of 11-deoxycorticosterone and corticosterone to hydroxy acid metabolites. Oxidation of DOC in the preliminary studies accounted for 30–40% of the total recovered steroid. In further studies, the conversion under the same conditions ranged from 10 to 30%. We have traced the source of this variability to genetic factors [14, and in preparation].

The properties of the system resemble that of human and hamster liver preparations in most respects. In each case, tritium exchange activity was localized in the postmicrosomal supernatant fraction of the liver. In human and hamster liver we have identified this exchange reaction as a reflection of an isomerization that catalyzes the interconversion of a ketol and aldol side chain. We have interpreted the tritium exchange in mouse liver as being due to the same mechanism, but it must be emphasized that this assumption, though reasonable, is an extrapolation from our previous work.

Like the hamster and human preparations, the formation of acid decreased and was eventually abolished during fractionation, even though the isomerase activity was retained. The loss of acid formation was more rapid than tritium exchange. We interpret this to mean that isomerase is the most stable component of the overall pathway leading from ketol to aldol to hydroxy acid.

Incubation of the 21-tritiated DOC with low levels of enzyme preparations that catalyzed no acid formation permitted recovery of the DOC unaltered, but with no 21-^{3}H, as we have found previously for human and hamster preparations. These are properties we have identified with isomerase activity. Greater amounts of cytosol led to the formation of other products and the recovery of only one third of the original DOC unlabeled. This is not surprising, since the crude preparations with which we have been working contain a number of enzymes that metabolize steroids.

The stereochemistry of exchange of (21R) and (21-S)-[21-^{3}H]-DOC was the same as the hamster and human preparations. The exchange rate of the 21S epimer was faster than of 21R, as we have previously found [8]. In a detailed analysis, we have shown that tritium exchange with water in hamster liver occurs with the 21-S epimer, and that the 21-R form undergoes an inversion to 21-S catalyzed by an epimerase. Evidently similar mechanisms occur in mouse liver.

Two differences between the mouse and hamster systems emerge. In the mouse the stereochemical configuration of the side chain was predominantly 20α. The 20β epimer was a minor product. In contrast, both 20α and 20β epimers were formed as major products in man and hamster. Lindberg *et al.*[21] studied the neutral metabolites of tritium labeled corticosterone formed by liver homogenates of mice. What is significant in the context of our study is their finding that C-20 reduction proceeds almost exclusively to the 20α glycol. We, too, have found that side chain metabolism yields 20α hydroxy metabolites preferentially. We have proposed that 20 hydroxy acids and glycols are formed through a common 20 hydroxy-21-aldehyde intermediate. Reduction requires NADPH. These observations raise an important question. Is the NADPH dependent conversion of the ketol side chain to glycol direct, or does it require a prior rearrangement of the side chain to an aldol, as is necessary for hydroxy acid synthesis? We have presented evidence that both mechanisms operate in man [23, 24].

There is another striking way in which the mouse and hamster systems differ. When attempts were made to purify the mouse liver enzymes beyond the ammonium sulfate fractionation stage, further increase in specific activity was not achieved. We found, however, that the total recovered activity was greater after Sephadex G-25 filtration. This suggests that a low molecular weight inhibitor was removed or the enzyme is in some way activated. We have not yet been able to identify the postulated inhibitor or to reconstitute its effect from the low molecular weight fraction of the cytosol. This phenomenon requires further study.

In conclusion, the mouse liver is able to metabolize 11-deoxycorticosterone and corticosterone oxidatively to hydroxy acid metabolites, much as human and hamster livers do. There are differences between species but the similarities are sufficiently numerous to justify the use of mouse liver as enzyme source. It is likely that the pathway of steroid metabolism cata-

lyzed by these liver enzymes is a general one, though in each species quantitative distribution of products may differ.

REFERENCES

1. Monder C. and Bradlow H. L.: New frontiers in corticosteroid metabolism. *Recent Prog. Horm. Res.* **36** (1980) 345–400.
2. Senciall I. R. and Dey A. C.: Acidic steroid metabolites: Tritium transfer and the *in vitro* formation of 4-pregnene-3, 20-dione-21-oic acid by rabbit and rat hepatic and extra-hepatic tissues. *J. steroid Biochem.* **9** (1978) 1093–1097.
3. Bradlow H. L., Zumoff B., Monder C., Lee H.-J. and Hellman L.: Isolation and identification of four new carboxylic acid metabolites of cortisol in man. *J. clin. Endocr. Metab.* **37** (1973) 811–818.
4. Lee H.-J. and Monder C.: Oxidation of corticosteroids to steroidal carboxylic acids by an enzyme preparation from hamster liver. *Biochemistry* **16** (1977) 3810–3814.
5. Martin K. O., Oh S.-W., Lee H.-J. and Monder C.: Studies on 21-^{3}H-labeled corticosteroids: Evidence for isomerization of the ketol side chain of 11-deoxycorticosterone by a hamster liver enzyme. *Biochemistry* **16** (1977) 3803–3809.
6. Oh S.-W. and Monder C.: Synthesis of corticosteroid derivatives containing the 20β-ol-21-al side chain. *J. Org. Chem.* **41** (1976) 2477–2480.
7. Monder C. and White A.: Purification and properties of a sheep liver steroid 21-hydroxysteroid dehydrogenase. *J. biol. Chem.* **238** (1963) 767–774.
8. Orr J. C. and Monder C.: The stereospecificity of the enzymic reduction of 21-dehydrocortisol in man. *J. biol. Chem.* **250** (1975) 7547–7553.
9. Lewbart M. L. and Schneider J. J.: Glycolic acid metabolites of desoxycorticosterone. Assignment of configuration at C-20. *J. org. Chem.* **29** (1964) 2559–2566.
10. Farhi R. L. and Monder C.: Analyses of steroidal carboxylic acids by high pressure liquid chromatography. *Analyt. Biochem.* **90** (1978) 58–68.
11. Kalckar H. M.: Differential spectrophotometry of purine compounds by means of specific enzymes III. Studies of the enzymes of purine metabolism. *J. biol. Chem.* **167** (1947) 461–475.
12. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
13. Monder C., Martin K. O. and Bogumil J.: Presence of epimerase activity in hamster liver corticosteroid side chain isomerase. *J. biol. Chem.* **255** (1980) 7192–7198.
14. Monder C., Walker M. C. and Bradlow H. L.: Metabolic transformation of the corticosteroid side-chain: Enzymic and genetic studies in *Progress in Research and Therapeutic Application of Corticosteroids* (Edited by H. J. Lee). Heyden Philadelphia, (in press).
15. Martin K. O. and Monder C.: Oxidation of corticosteroids to steroidal-21-oic acids by Human liver enzyme. *Biochemistry* **15** (1976) 576–582.
16. Lowy J., Albepart T. and Pasqualini J. R.: ^{3}H-corticosterone metabolism in the rat. *Acta endocr., Copenha.* **61** (1969) 483–493.
17. Senciall I. R. and Thomas G. H.: Enterohepatic circulation of biliary (4-^{14}C) progesterone metabolites in the rabbit. *J. Endocr.* **48** (1970) 215–221.
18. Spain K. M., Kisieleski W. and Wood N. K.: Cleft palate induction: Quantitative studies of ^{3}H corticoids in A/Jax mouse tissues after maternal injections of cortisol. *J. Dent. Res.* **54** (1975) 1069–1077.
19. Burton A. F. and Jeyes C. L.: Corticosteroid metabolism in fetal and newborn mice. *Can. J. Biochem.* **46** (1968) 15–20.
20. Burton A. F. and Turnell R. W.: 11-dehydrocorticosteroids in tissues of mice. *Can. J. Biochem.* **46** (1968) 497–502.
21. Lindberg M., Shire J. G. M., Doering C. H., Kessler S. and Clayton R. B.: Reductive metabolism of corticosterone in mice: Differences in NADPH requirements of liver homogenates of males of two inbred strains. *Endocrinology* **90** (1972) 81–92.
22. Shire J. G. M., Kessler S. and Clayton R. B.: The availability of NADPH for corticosterone reduction in six strains of mice. *J. Endocr.* **52** (1972) 591–592.
23. Monder C., Zumoff B., Bradlow H. L. and Hellman L., Studies on the biotransformation of cortisol to the cortoic acids in man: Metabolism of 21-dehydrocortisol. *J. clin. Endocr. Metab.* **40** (1975) 86–92.
24. Monder C., Bradlow H. L. and Zumoff B. Acidic metabolites V isosteroids as intermediates in cortoic acid formation. *J. clin. Endocr. Metab.* **51** (1980) 312–315.