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CORTICOSTEROID SIDE CHAIN OXIDATIONS—II. METABOLISM OF 20-DIHYDRO STEROIDS AND EVIDENCE FOR STEROID ACID FORMATION BY DIRECT OXIDATION AT C-21

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Summary—Rabbits have been injected with 4-^{14}C-labelled progesterone, deoxycorticosterone and corticosterone and the corresponding 20β-^{3}H-reduced steroids (20-dihydro steroids) in order to compare the influence of oxidation at C-20 on the excretion of steroid acids. Both 20β-reduced progesterone and deoxycorticosterone were extensively oxidized at C-20 and metabolized to 20-oxo-21-oic acids devoid of tritium. A small proportion of the acidic metabolites of [20β-^{3}H]dihydro deoxycorticosterone retained tritium. By contrast the majority of the metabolites of [20β-^{3}H]dihydro corticosterone were tritiated and [11β,20β-^{3}H]-dihydroxy-4-pregnene-3-one-21-oic acid was identified as a major acidic metabolite. These results indicate that the presence of a 11β-hydroxyl in 20β-dihydro corticosterone inhibits oxidation at C-20 and provides evidence for the direct oxidation of this corticosteroid at C-21 in this species.

INTRODUCTION

Oxidation of the corticosteroid side chain to 20-hydroxy-21-oic acids has been shown to occur in several mammalian species, including the human [1–3]. An enzymatic isomerization mechanism has been proposed whereby the ketol side chain rearranges to an aldol (20α/β-hydroxy-21-aldehyde [4], an epimerase converts the 20β- to the 20α-epimer [5] and an aldehyde dehydrogenase oxidizes the aldol to the 20-hydroxy-21-oic acid [6]. The *in vivo* metabolism of [21-^{3}H]cortisol to [20-^{3}H]hydroxy steroid acids by the human with retention of approx. 50% of the tritium was consistent with the isomerization mechanism [7]. However, the human has also been reported to excrete mainly 20-oxo-21-oic acid metabolites of deoxycorticosterone (DOC) [8] and liver preparations gave a mixture of both 20-oxo and 20-hydroxy steroid acids with the former predominating [9]. We recently compared the excretion of radiometabolites of 21-tritiated DOC, 11-deoxycortisol, corticosterone and cortisol in the rabbit [10], which resembles the human in excreting mainly 20-oxo-21-oic acid metabolites of DOC devoid of tritium, whereas corticosterone was metabolized to both 20-oxo and 20-hydroxy steroid acids, the latter retaining tritium that had presumably isomerized from C-21. If the isomerization pathway to 20-hydroxy acids is operative in the rabbit 20-oxo-21-oic acid metabolites of DOC might result from further oxidation of the 20-hydroxy acids. We therefore synthesized [20β-^{3}H]dihydro steroids to determine whether they would block the isomerase pathway *in vivo* and thereby reduce the excretion of steroid acids. Unexpectedly we found an increased excretion of steroid acid metabolites of 20-dihydrocorticosterone. The present paper reports studies on a number of 20β-tritiated dihydro steroids, and their 4-^{14}C-labelled parent compounds to determine the influence of steroid structure on steroid acid formation by the rabbit.

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Abbreviations: Progesterone (P); deoxycorticosterone (DOC); cortisol (F); corticosterone (B); 20α/β-dihydro P (DHP), 20α/β-hydroxy-4-pregnene-3-one; 20β-dihydro DOC (DHDOC), 20β,21-dihydroxy-4-pregnene-3-one; 20β-dihydro B (DHB), 11β,20β,21-trihydroxy-4-pregnene-3-one; 20β-dihydro-DHB-21-al, 11β,20β-dihydroxy-4-pregnene-3-one-21-aldehyde; 20β-DHB-21-oic acid, 11β,20β-dihydroxy-4-pregnene-3-one-21-oic acid.

EXPERIMENTAL

Synthesis of labelled steroids

[4-^{14}C]20α-dihydro progesterone (DHP). [4-^{14}C]P (25 μCi) was dissolved in 4 drops of ethanol and reduced in 0.1 M potassium

phosphate buffer pH 7.4 (total volume 5 ml) with *Streptomyces hydrogenens* (0.8 mg) supplied by Dr J. C. Orr (Memorial University of Newfoundland [11]). NADH (5 mg) was added and the reaction terminated after 2 h at 37°C by extraction with ethyl acetate (2 × 10 ml). The extract was washed with saturated sodium chloride solution, evaporated under a nitrogen stream and applied to a TLC plate (Anasil, OF, 20 × 20 cm). Development with chloroform–ethyl acetate (6:4) resolved [4-^{14}C]20α-DHP (R_f 0.48), which had the same mobility as the authentic standard, from P (R_f 0.70). Purity was confirmed by HPLC on a C_{18} column developed with 65% methanol. Yield 44%.

[4-^{14}C]20β-DHP. [4-^{14}C]P (10.8 μCi) was reduced with 20β-hydroxysteroid dehydrogenase (20β-HSD) (20 U; Boeringer-Mannheim, U.S.A.) and NADH (1 mg) in 1 ml of 0.1 M sodium phosphate buffer, pH 6.0. After incubation overnight the reaction mixture was extracted as above and the [4-^{14}C]20β-DHP isolated by TLC in chloroform–ethyl acetate (7:3). The product had the same mobility as the 20β-DHP standard on TLC (R_f 0.34) and HPLC. Yield 58.6%.

[4-^{14}C]20β-Dihydro DOC. [4-^{14}C]DOC (5 μCi) was reduced with 20β-HSD as above. Yield 89.8%.

[4-^{14}C]20β-Dihydro B-21-al. [4-^{14}C]Corticosterone (B) (10 μCi) was oxidized to [4-^{14}C]21-dehydro B with cupric acetate [12]. The aldehyde was isolated by TLC in benzene–ethanol (9:1) and had the same mobility as the authentic standard (R_f 0.47). Yield was 53.9%. Reduction with 20β-HSD as above gave the 20β-reduction product (R_f 0.23) that was resolved from the unreacted 21-dehydro steroid (R_f 0.40) by TLC in benzene–ethanol (9:1). Yield was 33%. 20β-HSD activity is not inhibited by the 11β-hydroxyl group in C-21-aldehydes [13].

20β-Tritiated steroids

The following steroids (0.2 mmol) were selectively reduced at C-20 with sodium borotritiide (12.5 mCi; sp. act. 600 mCi/mmol) in ice-cooled dimethyl formamide–methanol (2:1) essentially as described by Oh and Monder [14]. Steroids were incubated for 2 h, the products purified by TLC and characterized by comparison with the authentic standards on TLC and HPLC.

P gave [20β-^{3}H]dihydro P. Yield 68%; sp. act. 138.8 Ci/mol.

DOC gave [20β-^{3}H]dihydro DOC. Yield 67.1%; sp. act. 96.3 Ci/mol.

B gave [20β-^{3}H]dihydro B. Yield 62.1%; sp. act. 96.3 Ci/mol.

[4-^{14}C]DOC (58.5 mCi/mmol), [4-^{14}C]P (60 mCi/mmol), [1,2-^{3}H]P (57 Ci/mmol) and [^{3}H]sodium borohydride (600 mCi/mmol) were purchased from NEN Research Products (Du Pont Canada Ltd.) and [4-^{14}C]B (50 mCi/mmol) and [1,2-^{3}H]B (36 Ci/mmol) from Amersham (Canada Inc.).

Synthesis of 11β,20α/β-dihydroxy-4-pregnene-3-one-21-oic (20α/β-DHB-21-oic) acids

A mixture of the 20α/β-DHB-21-oic acids was prepared as described by Han *et al.* [15] by alkaline rearrangement of the 21-dehydro B intermediate which was synthesized from B by oxidation with cupric acetate [12]. The acids were purified by TLC in chloroform–methanol–formic acid (96:4:1) (R_f 0.19) and on a silica gel column (Prepacked; size B) developed with the same solvent mixture. The steroid acids were eluted in approx. 450 ml of the solvent mixture. [4-^{14}C]20β-DHB-21-oic was prepared under the same conditions and purified by TLC. The 20β-DHB-21-methyl esters were resolved and purified from the 20α-epimers by HPLC.

The methyl ester derivatives were prepared by heating the dry acids with 14% boron trifluoride in methanol (0.5 ml; Sigma Chemical Co., U.S.A.) in a stoppered container at 60°C for 3 min. Cold sodium bicarbonate (8% aqueous solution; 1 ml) was added and methyl esters extracted into ethyl acetate (2 × 5 ml), washed with saturated sodium chloride solution and evaporated in a nitrogen stream. Methanolic solutions were used for HPLC.

Injection of rabbits

New Zealand White rabbits were housed in metabolic cages and urine collected every 24 h after injection of steroids into the marginal ear vein. Steroids were dissolved in 0.6 ml ethanol and diluted with 0.4 ml saline immediately before injection. The dose injected and the isotope ratio were determined after subtraction of the residual radioactivity in the syringe and vial. A rabbit was also injected i.p. with Ketoconazole (a gift from Janssen Pharmaceutica, Ontario, Canada) as previously described [16] except that the Ketoconazole was dissolved in 1 ml of 1 N HCl and diluted with 5 ml saline. The rabbit was injected for two consecutive days with Ketoconazole (25 mg/kg body wt) and with the labelled steroids on the fourth day. A noticeable

side effect of the Ketoconazole treatment was the clarification of the urine which had a bright yellow coloration.

Processing of urine

Urine was centrifuged to remove sediment before being processed on Sep paks (Waters, U.S.A.) and hydrolyzed with Glusulase as previously described [10]. Alumina adsorption column chromatography was also carried out as previously described [17] to sequentially elute 21-deoxysteroids (A) with ether–ethanol (3:1), 21-hydroxysteroids (B) with 50% aqueous ethanol, and steroid acids in fractions C and D with 0.1 and 1.0 M sodium acetate buffer, pH 5.0, respectively. [20β-^{3}H]Hydroxy acidic metabolites were eluted with 1.0 M sodium acetate buffer (fraction D) which greatly assisted the present investigation. In a previous study [17] we have reported the effective resolution of the 21-deoxy and 21-hydroxy steroid metabolites of P and DOC. Thus, although we recognize that the alumina adsorbent is not completely inert and may catalyse certain chemical reactions, it has proved useful for the resolution of the steroid metabolites.

HPLC

Steroid acids and methyl ester derivatives were chromatographed on a C_{18}-Ultrasphere ODS column (4.5 mm × 25 cm; Perkin-Elmer, U.S.A.) developed isocratically at a flow rate of 1 ml per min. Acids were run in methanol–water (60:40) containing 1% phosphoric acid [15] and methyl esters in methanol–water mixtures (60–65% methanol). Conditions were as previously described [10, 18]. Briefly, 1 ml fractions of the effluent were collected in 5 ml mini vials with a Pharmacia fractomette collector and the radioactivity was measured after addition of PCS scintillation fluid (4 ml; Formula 963; Du Pont, Canada Ltd.). The u.v. absorption of internal standards was detected with a Beckman variable wavelength detector at 254 nm.

Normalized isotope ratios

The normalized $^3H/^{14}C$ ratios, designated N, were obtained by dividing the ratios of the products by the ratios of the injected dose. This allows for the comparison of different injected doses. An isotope ratio of 1.0 indicates equivalent metabolism of both the tritiated and ^{14}C-labelled precursor steroids.

RESULTS

Rabbits were injected with mixtures of isotopically labelled steroids in order to determine the influence of reduction at C-20 on the metabolic fate of the side chains of P, DOC and B. The results are collated in Tables 1–5 under the following topics. Table 1 compares the excretion of radioactivity in urine collected in the 48 h period after injection of the indicated steroid mixtures. Table 2 shows the extent of *in vivo* detritiation and the excretion of tritiated water as determined by solid phase extraction on Sep paks. Table 3 gives the proportions of neutral and acidic metabolites estimated by solvent partition and Table 4 the group fractionation of metabolites into 21-deoxy (A), 21-hydroxy (B), and steroid acid fractions (C and D) on alumina adsorption columns. The corresponding alumina chromatograms are shown in Figs 1 and 2. Table 5 compares the normalized isotope ratios of the alumina fractions. To simplify discussion the results are given for each set of steroids injected.

P metabolites

[1,2-^{3}H]P vs [4-^{14}C]20α-DHP. Metabolism of the [4-^{14}C]20α-DHP was remarkably similar to [1,2-^{3}H]P as indicated by the normalized isotope ratios and the proportions of acidic metabolites excreted. The normalized ratio of 1.25 indicated that P was a marginally better precursor of steroid acids. These acids were eluted primarily in the alumina fraction C (N = 0.97–1.00) and shown to be mainly 20-oxo compounds by analysis of the methyl ester derivatives by HPLC on a C_{18} reverse phase column (results not shown). A major peak (N = 1.62) accounted for 62.7% of the tritiated acids and 48% of the

Table 1. Excretion of radioactivity in urine collected for 48 h after injection of steroid mixtures

Steroids injected	Dose (%)		
	^{3}H	^{14}C	N
Progesterone			
[1,2-^{3}H]P	89.1	87.1	1.01
[4-^{14}C]20α-DHP	76.0	76.1	1.01
[20β-^{3}H]DHP	13.3	75.3	0.18
[4-^{14}C]20β-DHP	16.7	66.5	0.25
Deoxycorticosterone			
[20β-^{3}H]DHDOC	31.7	97.7	0.33
[4-^{14}C]DOC			
Corticosterone			
[20β-^{3}H]DHB	57.9	77.2	0.76
[4-^{14}C]B	78.7	87.5	0.90
[1,2-^{3}H]B	81.4	73.3	1.10
[4-^{14}C]20β-DHB-21-al			

N represents the normalized isotope ratio. Each result comes from a different rabbit.

Table 2. Solid phase extraction of steroid radiometabolites and tritiated water from urine

Steroids injected	% Of radioactivity in urine					
	Methanol eluate			Water eluate		
	^3H	^{14}C	N	^3H	^{14}C	N
	Progesterone					
[1,2-^{3}H]P	91.4	91.7	1.00	Negl.	Negl.	—
[4-^{14}C]20α-DHP	95.9	97.6	1.00	Negl.	Negl.	—
[20β-^{3}H]DHP	58.8	100.6	0.07	36.3	2.1	1.97
[4-^{14}C]20β-DHP	43.7	94.0	0.09	45.5	3.0	2.99
	Deoxycorticosterone					
[20β-^{3}H]DHDOC	92.0	91.0	0.33	6.2	3.8	0.53
[4-^{14}C]DOC						
	Corticosterone					
[20β-^{3}H]DHB	83.9	89.6	0.72	5.9	1.9	2.39
[4-^{14}C]B	82.5	84.7	0.82	6.6	2.0	2.91
[1,2-^{3}H]B	93.9	87.0	1.19	Negl.	Negl.	—
[4-^{14}C]20β-DHB-21-al						

^{14}C-acids. Specific reduction with 20β-HSD produced a change in mobility on HPLC to that of a more polar compound, indicating the presence of a 20-oxo function in the original methyl esters. A second minor peak (5.5% tritium/9.9% ^{14}C; N = 0.69) did not move on reduction with the enzyme suggesting it contained steroid acids already reduced at C-20.

[20β-^{3}H]DHP vs [4-^{14}C]20β-DHP. To follow the metabolic fate of the reduced P side chain more precisely [20β-^{3}H]DHP was given to rabbits admixed with [4-^{14}C]20β-DHP. The lability of the C-20 tritium was immediately evident from the low excretion (13.3–16.7%) of the injected dose of tritium compared to ^{14}C (Table 1). The low normalized isotope ratios of the crude urines (0.18 and 0.25) also indicated that a 75–82% loss of the 20β-tritium had occurred during metabolism of [20β-^{3}H]DHP. A further 36.3–45.5% of the tritium in the urine was shown to be present as tritiated water by solid phase extraction (Table 2). The low isotope ratios in the acidic fractions isolated by solvent partition (0.03–0.04; Table 3) and by alumina chromatography (Table 4) also show that considerable oxidation had occurred at the C-20β-position. This was consistent with the metabolic fate of 20α-DHP as deduced from HPLC analysis of the steroid acid metabolites.

DOC metabolites

[20β-^{3}H]DHDOC vs [4-^{14}C]DOC. Significant oxidation at the 20β-position of DHDOC was also detected. The excreted normalized isotope ratio of 0.33 for the crude urine indicated a 67% loss in tritium as compared to the dose ratio. The majority of the urinary radioactivity was, however, still attached to the steroid metabolites (Table 2). Both steroids were metabolized to steroid acids. Alumina chromatography (Fig. 1; Table 4) showed that the majority of the ^{14}C-acids (20.1% of dose) were eluted in fraction C with a further 6.7% in fraction D. By contrast tritiated acids were negligible in fraction C

Table 3. Solvent partition of neutral and acidic metabolites

Steroids Injected	% Of radioactivity in urine					
	Neutral fraction			Acidic fraction		
	^3H	^{14}C	N	^3H	^{14}C	N
	Progesterone					
[1,2-^{3}H]P	13.0	22.7	0.60	52.0	42.5	1.25
[4-^{14}C]20α-DHP	21.6	31.0	0.69	49.6	41.0	1.22
[20β-^{3}H]DHP	24.2	24.5	0.08	17.8	52.8	0.03
[4-^{14}C]20β-DHP	15.5	21.6	0.13	11.6	51.8	0.04
	Deoxycorticosterone					
[20β-^{3}H]DHDOC	21.8	18.4	0.39	52.5	54.8	0.36
[4-^{14}C]DOC						
	Corticosterone					
[20β-^{3}H]DHB	6.9	39.1	0.12	58.8	24.8	1.66
[4-^{14}C]B	4.2	31.1	0.14	31.5	17.1	1.85
[1,2-^{3}H]B	42.0	6.0	8.4	41.4	68.8	0.72
[4-^{14}C]20β-DHB-21-al						

Table 4. Alumina column fractionation

Steroids injected	Isotope	Dose (%) 21-Deoxysteroid (A)[a]	21-Hydroxysteroid (B)	Steroid acid (C)	Steroid acid (D)	Total[b]
		Progesterone				
	^{3}H	6.5	5.4	37.4	7.9	89.3
[1,2-^{3}H]P	^{14}C	9.8	9.2	31.6	8.2	96.4
[4-^{14}C]20α-DHP	^{3}H	6.9	7.4	36.5	4.3	87.5
	^{14}C	10.2	11.6	30.8	4.3	85.2
	^{3}H	0.9	1.8	0.9	0.6	102
[20β-^{3}H]DHP	^{14}C	6.1	8.9	26.2	5.3	93.2
[4-^{14}C]20β-DHP	^{3}H	1.2	2.0	1.2	0.7	103
	^{14}C	6.1	9.0	25.9	5.2	100
		Deoxycorticosterone				
[20β-^{3}H]DHDOC	^{3}H	0.1	4.8	0.2	6.8	51.0
[4-^{14}C]DOC	^{14}C	2.3	9.9	20.1	6.7	73.7
		Corticosterone				
	^{3}H	0.04	4.4	0.9	22.5	64.5
[20β-^{3}H]DHB	^{14}C	2.0	18.2	14.4	12.1	82.5
[4-^{14}C]B	^{3}H	0.06	7.4	0.9	34.2	84.5
	^{14}C	0.85	27.8	17.3	10.5	103
[1,2-^{3}H]B	^{3}H	—	30.0	16.7	6.7	85.6
[4-^{14}C]20β-DHB-21-al	^{14}C	—	4.7	6.7	26.0	72.4

[a]Alumina column fraction designations as in Figs 2 and 3.
[b]Total recovery of radioactivity from the columns.

(0.2% of the dose) whereas 6.8% of the dose was eluted in fraction D with 1 M sodium acetate buffer. This resulted in a normalized ratio of 0.44 for the latter compared to 0.01 in fraction C (Table 5). The alumina chromatogram scans in Fig. 1 also contrast the 20β-DHDOC metabolite patterns with those of P and 20β-DHP. It is evident that the 20β-DHDOC chromatogram differs in that a greater proportion of tritiated acids are recovered in fraction D. This suggested that fraction D may contain 20β-tritiated 20-hydroxy steroid acids. Another unusual feature was the low recovery of tritium from the alumina columns with the 20β-DHDOC experiments. A typical, reproducible, elution resulted in only 51–73.7% recovery from the urine of two rabbits (Table 4). Further elution with 1 M buffer and the collection of up to 80 fractions gave a quantitative recovery of tritium but no distinct peak of radioactivity was eluted. This would suggest that some tritium was displaced from the metabolites during chromatography.

The potential elution of 20β-hydroxy-21-oic acids in alumina fraction D was confirmed by reduction of a 20-oxo steroid acid fraction of DOC (isolated from fraction C) with 20β-HSD. This resulted in a shift in mobility and its elution in alumina fraction D.

B metabolites

[20β-^{3}H]DHB vs [4-^{14}C]B. It was reasoned that oxidation at C-20 would be impeded by the 11β-hydroxyl function of 20β-DHB. Furthermore, blocking the C-20 position would be expected to inhibit isomerization of the corticosteroid side chain which has been shown to result in the formation of 20-hydroxy-21-oic acids in several species [2]. In the present study it was immediately evident that significant retention of tritium had occurred since the crude urines had normalized isotope ratios of 0.76–0.90 (Table 1). 20β-DHB gave a greater yield of total acids than B and more 20-hydroxy acids as indicated by N values of 1.66 and 1.85 for the solvent partitioned acids isolated from two rabbit urines. The tritiated acids of 20β-DHB were eluted from alumina columns primarily in fraction D with 1 M buffer [22.5–34.2% of dose (Table 4); N = 1.84–3.23; Table 5]. The acidic metabolites of B were eluted primarily in fraction C (14.4–17.3% of dose; N = 0.06–0.11). A further 10.5–12.1% of the dose of ^{14}C was eluted in fraction D primarily as a small peak in fractions 16–18 whereas the tritiated acids were eluted as a broad peak in fractions 17–30. The structures of these ^{14}C-acids have not been determined.

Effect of Ketoconazole treatment. The metabolism of [20β-^{3}H]DHB vs [4-^{14}C]B was compared in the same rabbit before and after treatment with Ketoconazole, a cytochrome *P*-450 inhibitor. No significant differences were observed in the excretion of radioactivity or in the fractionation of metabolites into neutral and acidic fractions by solvent partition or alumina

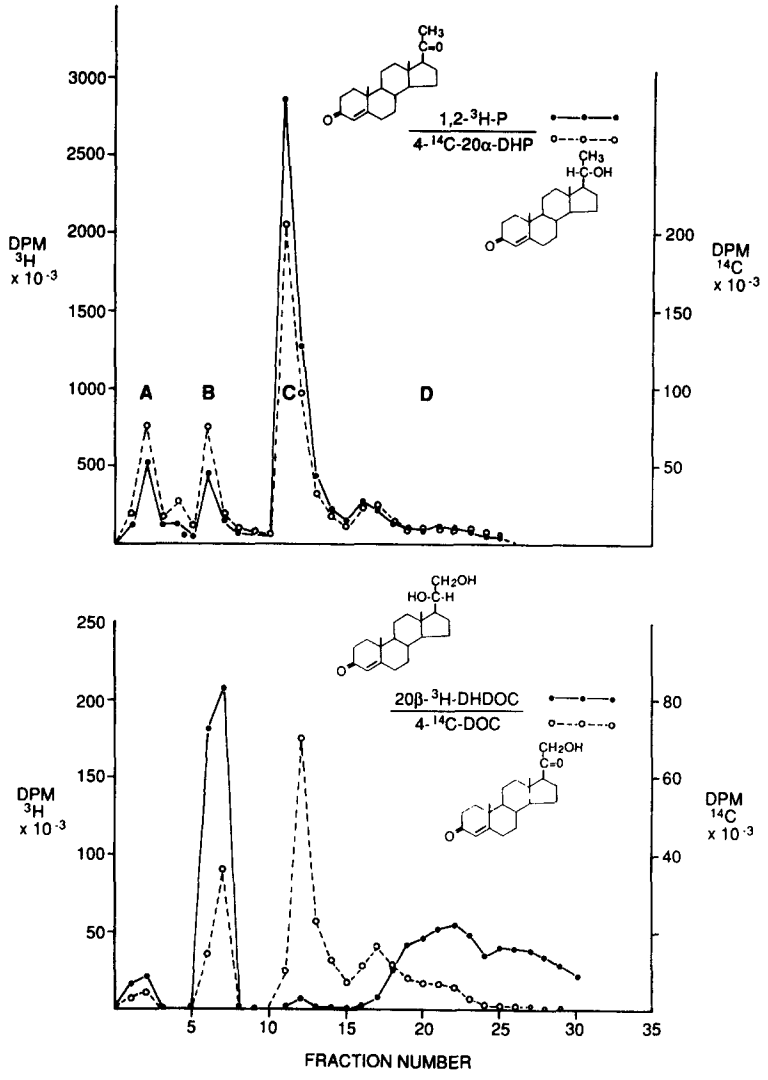

Fig. 1. Alumina column chromatograms of Glusulase hydrolyzed metabolites. Fractions A–D were eluted with ether–ethanol (3:1), 50% aqueous ethanol, 0.1 and 1.0 M sodium acetate buffer, pH 5.

column chromatography. The normalized ratios for the solvent partitioned acidic metabolites excreted in the urine before and after Ketoconazole treatment were 1.86 and 1.96, respectively. Corresponding values for alumina fraction D metabolites were 3.19 and 2.95, respectively.

Table 5. Normalized isotope ratios[a] of steroid fractions from alumina columns

Steroids injected	21-Deoxysteroids (A)	21-Hydroxysteroids (B)	Steroid acids (C)	Steroid acids (D)
		Progesterone		
[1,2-³H]P	0.67	0.58	1.27	0.97
[4-¹⁴C]20α-DHP	0.68	0.63	1.21	1.00
[20β-³H]DHP	0.12	0.18	0.03	0.10
[4-¹⁴C]20β-DHP	0.19	0.23	0.04	0.12
		Deoxycorticosterone		
[20β-³H]DHDOC	0.04	0.49	0.01	0.44
[4-¹⁴C]DOC				
		Corticosterone		
[20β-³H]DHB	0.02	0.24	0.06	1.84
[4-¹⁴C]B				
[1,2-³H]B	0	6.3	2.5	0.42
[4-¹⁴C]20β-DHB-21-al				

[a]Normalized ratios (N) were calculated by dividing the $^3H/^{14}C$ ratios of the isolated fractions by the dose ratios.

These results were comparable with those previously shown in Tables 4 and 5 and show that Ketoconazole did not inhibit the metabolism of [20β-^{3}H]DHB to steroid acids.

[1,2-^{3}H]B vs [4-^{14}C]20β-DHB-21-al. Since direct oxidation of the primary alcohol group at C-21 appeared to be implicated in the oxidation of 20β-DHB to acids it was of interest to test the 21-aldehyde as a potential intermediate. The [4-^{14}C]20β-DHB-21-al was synthesized and its metabolism compared with that of [1,2-^{3}H]B. The results shown in Tables 1–5 and Fig. 2 support the contention that the 21-aldehyde may be an intermediate since it gave a comparable yield of acids to the 20β-DHB. However, the alumina fraction D elution patterns of the 20-DHB-al and 20-DHB metabolites (Fig. 2) were not identical.

Characterization of 20β-DHB-21-oic acid as a major metabolite of 20β-DHB. HPLC of the methyl ester derivatives of the tritiated acidic metabolites isolated from alumina fraction D showed two metabolite peaks (Fig. 3). The major peak co-chromatographed with the standard 11β,20β-dihydroxy-4-pregnene-3-one-21-methyl ester (RT 16.1). The less mobile peak (RT 22.0) was not characterized but had the mobility of a 5-reduced steroid. Solvent partitioned acids were purified on a larger scale by chromatography on a prepacked EM silica gel column developed with chloroform–methanol–formic acid (96:4:1) [14]. The major tritiated steroid fraction eluted after about 450 ml of solvent (N = 13.2). HPLC of the acid fraction on a C_{18} column developed with methanol–water (6:4) containing 1%

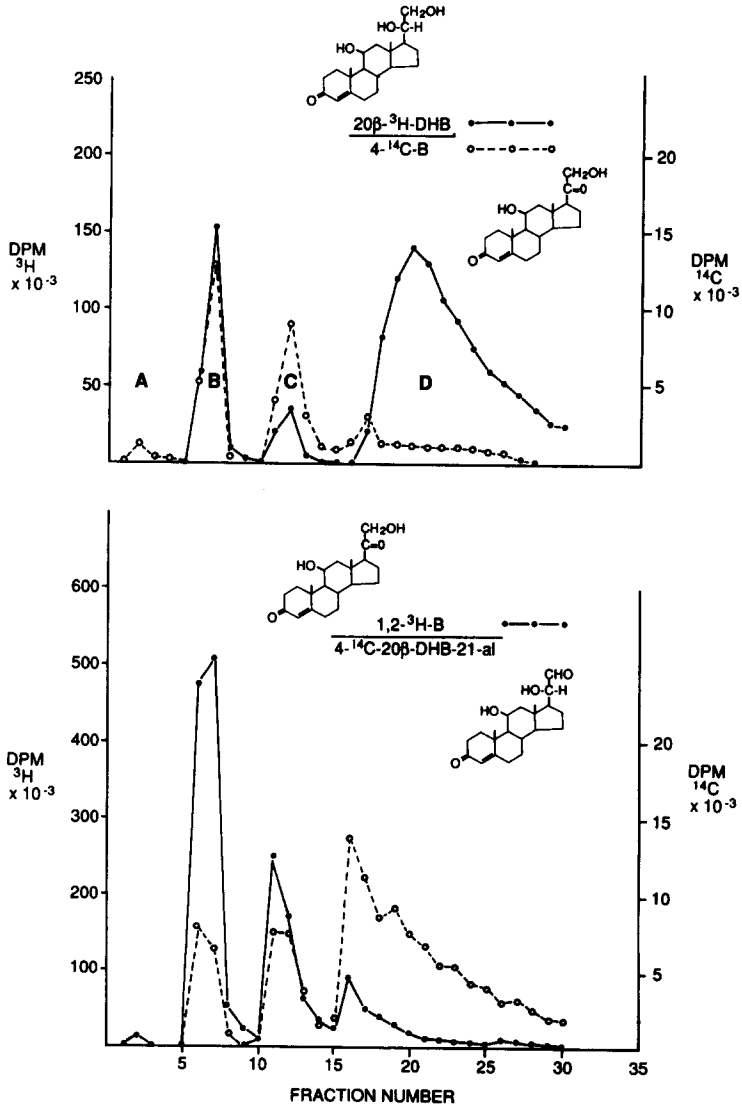

Fig. 2. Alumina column chromatograms developed as in Fig. 2.

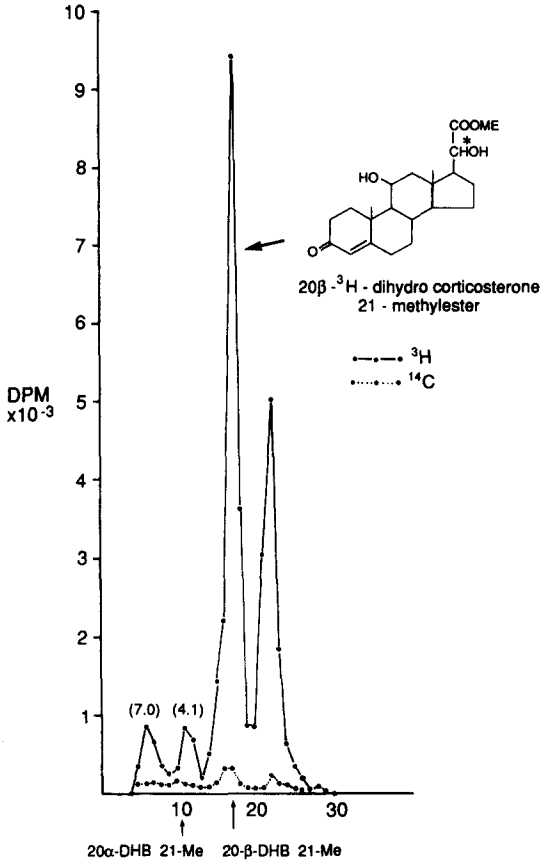

Fig. 3. HPLC chromatogram of methyl ester derivatives of alumina fraction D metabolites of [20β-^{3}H]DHB and [4-^{14}C]B. Arrows indicate the elution of internal standards detected by their u.v. absorption (254 nm). 1 ml fractions of the column eluate were collected and the radioactivity counted in a liquid scintillation counter.

phosphoric acid gave a single peak (fraction 14; flow rate 1 ml/min). This corresponded in mobility with the 20β-DHB-21-oic acid standard (RT 13.74) and was well separated from the 20α-epimer (RT 11.57). [4-^{14}C]20β-DHB-21-methyl ester was admixed with the isolated tritiated metabolite and the unlabelled standard. HPLC was carried out sequentially in methanol water (60:40; twice and 65:35; once). The internal standard was detected by its u.v. absorption at 254 mu and the corresponding fractions were collected, concentrated and rerun as above. The ^{3}H/^{14}C ratios of sequential runs were 47.7, 50.8 and 51.0, respectively.

DISCUSSION

In the present study, and the one preceding [10] we have attempted to simplify the analysis of steroid side chain oxidations by comparing the fates of steroids labelled specifically with tritium at C-20 or C-21, respectively. The previous study with 21-tritiated steroids [10] showed the isomerization of the side chains of B and cortisol to 20-hydroxy-21-oic acid metabolites. In this respect, the rabbit appeared to resemble several other mammalian species in which the "classical" isomerization pathway leading to 20-hydroxy-21-oic acids had been deciphered by Bradlow and Monder [1–3]. Human [9], hamster [19] and mouse [20] liver have been reported to contain isomerization complexes, termed "steroid acid synthetase complexes" which oxidize the corticosteroid side chain to both 20-oxo and 20-hydroxy-21-oic acids, with the aldol (20-hydroxy-21-aldehyde) as an intermediate. In the case of the human this results in the formation of a greater proportion of 20-oxo than 20-hydroxy acid metabolites of DOC [9]. We have recently isolated a similar "steroid synthetase complex" from rabbit liver cytosol [21] using the procedures developed by Monder's group [4] and found it to oxidize DOC predominantly to the 20α-hydroxy-21-oic acid (20α-DHDOC-21-oic acid). However, both the rabbit and the human excrete 20-oxo-21-oic acid metabolites of DOC [10]. The mechanism of 20-oxo acid formation by the enzyme complexes of these two species remains to be further elucidated. Neither enzyme complex required cofactors so 20-HSD activity does not appear to be involved.

In the present study we have attempted to follow the *in vivo* fate of the corticosteroid side chain with steroids specifically reduced at C-20 with sodium borotritiide. The P side chain is also oxidized to C-21 acids since rabbit liver contains an active hepatic cytochrome *P*-450 21-hydroxylase (*P*45011C5) [22, 23] that converts the P side chain to the ketol side chain of DOC. Both P and 20α-DHP were efficiently oxidized to 20-oxo acids indicating rapid 21-hydroxylation and oxidation of 20-DHP at C-20 with loss of tritium. Significant loss of tritium also occurred with 20β-DHDOC, though a higher proportion of the solvent partitioned acidic metabolites retained some tritium (N = 0.36 vs 0.03–0.04 for [20β-^{3}H]DHP). This study was the first to show that a significant proportion (6.8% of dose) of [20β-^{3}H]DHDOC acidic metabolites were eluted from alumina adsorption columns with 1 M buffer (fraction D; Fig. 2). The recovery of tritium in fraction C was only 0.2% of dose compared to 20.1% for ^{14}C. Reduction of the [^{14}C]DOC acids in fraction C with 20β-HSD produced a shift in mobility and their elution in fraction D. It therefore

appears that 20β-hydroxy steroid acids are more slowly eluted than the 20-oxo acids.

The excretion of acidic metabolites of [20β-^{3}H]DHB was of particular interest since it had been speculated that the presence of the 20-hydroxyl would prevent isomerization of the side chain to 20-hydroxy acids. Bradlow [1] has stated the requirement for a 20-oxo function in the corticoid side chain for acid formation by the human, presumably via the isomerization pathway, citing a paper on the metabolism of 20-dihydro compounds (Reichstein's E and U) which apparently did not give any acids though data was not provided to support this contention [24]. Unlike 20β-DHP or 20β-DHDOC, 20β-DHB with its 11β-hydroxyl function would be expected to be resistant to oxidation at C-20 [13]. However, 20β-DHB proved to be a better precursor of steroid acids than B and a [20β-^{3}H]acid ([11β,20β-^{3}H]dihydroxy-4-pregnene-3-one-21-oic acid) has been isolated and characterized. The excretion of a ring A unsaturated metabolite was fortuitous and facilitated its identification. It would seem likely that side chain oxidation precedes ring A reduction and that the combination of a 11β-hydroxy and the 20-hydroxy-21-oic acid side chain may inhibit ring A reduction. Thus, Monder [15] was able to isolate a 20α-DHB-21-oic acid metabolite of administered B from mouse liver.

In the present study the isolation of a 20β-hydroxy acid with the tritium still attached is evidence of a direct oxidation at C-21 and excludes the possibility of an isomerization mechanism being operative. Direct oxidation at C-21 could involve a cytochrome *P*-450 system since we have solubilized a cytochrome *P*-450 fraction from rabbit liver microsomes that oxidized DOC to a 20-oxo-21-oic acid (4-pregnene-3,20-dione-21-oic acid) [25]. Although the activity of this oxidase is low *in vitro* administration of Ketoconazole, a potent inhibitor of cytochromes *P*-450 [26], reduced the excretion of acidic metabolites of DOC by the rabbit by about 50% [16]. In the present study, Ketoconazole administration had no demonstrable effect on the metabolism of [20β-^{3}H]DHB to [20β-^{3}H]hydroxy-21-oic acids suggesting that cytochromes *P*-450 were not involved. Recently we have observed the direct oxidation of 20β-DHB to C-21-acids by rabbit liver cytosol [21]. Unlike the isomerization reaction NAD was required for acid formation. *In vivo* oxidation of [20β-^{3}H]DHB-21-al to 20β-hydroxy-21-oic acids in high yield provides further support for direct oxidation at C-21. Aldehyde dehydrogenases that oxidize the aldol (20-hydroxy-21-aldehyde) side chain to 20-hydroxy-21-oic acids have been isolated from human [27] and horse [6] liver cytosols.

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