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CORTICOSTEROID SIDE-CHAIN OXIDATIONS—I. STRUCTURAL EFFECTS ON THE EXCRETED ISOTOPE RATIOS OF 4-^{14}C- AND 21-^{3}H-LABELLED CORTICOSTEROID METABOLITES IN RABBIT URINE

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Summary—The metabolic fates of 4-^{14}C- and 21-^{3}H-labelled corticosteroids have been investigated in the rabbit by analysis of the normalized isotope ratios of neutral and acidic metabolites excreted in the urine. Isotope ratios of excreted radioactivity declined in the order cortisol (F) > corticosterone (B) > 11-desoxycortisol (S) > deoxycorticosterone (DOC). Steroid acids, isolated in alumina fraction C, represented 19.0, 15.0, 9.7 and 2.7% of the doses of DOC, B, S and F, respectively, and the isotope ratios declined in the order F > B > S > DOC. HPLC of steroid acid methyl ester derivatives indicated generally low isotope ratios for DOC and S steroid acids, consistent with complete side-chain oxidation to 20-oxo-21-oic acids and/or 17-carboxylic acids. Several B metabolite methyl esters peaks also exhibited low isotope ratios, but both B and F metabolites gave methyl esters that retained significant tritium consistent with the presence of 20-hydroxysteroid acids. The 21-hydroxysteroid metabolite fractions had isotope ratios of F = S > B > DOC. HPLC showed that 20-oxo (tetrahydro) metabolites of B and F had reduced isotope ratios unlike the C-20 reduced (hexahydro) metabolites of DOC and S. It may be concluded that the metabolic fate of the corticoid side-chain in the rabbit is dependent on the steroid structure and may result in the excretion of both 20-oxo and 20-hydroxysteroid acids.

INTRODUCTION

The availability of [21-^{3}H]corticosteroids, which can be synthesized by reduction of the 21-dehydrosteroids with sodium borotritiide [1], has provided considerable insight into the metabolic fate of the corticosteroid side-chain. Previously such knowledge was confined to reduction at C-20 and side-chain cleavage, but as summarized in Fig. 1 several new metabolic pathways have been deduced by Monder and Bradlow [2–4] in a series of elegant studies. Both 20-hydroxy and 20-oxo-21-oic acids have been identified, the proportions being dependent on the particular steroid and/or species studied. Characteristically, 20-hydroxysteroid acids are found to retain about half the original tritium consistent with the isomerization of tritium from the C-21 to C-20 position, whereas 20-oxo steroid acids are devoid of tritium. Exchange of the labile C-21 tritium for proton may also occur with reconstruction of the original corticoid side-chain. These reactions have been formulated from several *in vivo* studies on the human [4, 5] and with the isolated liver enzymes from the hamster [6], mouse [7] and human [8]. It has not yet been definitively determined to what extent the rabbit resembles these species in the metabolic formation of steroid acids. We have reported that the New Zealand white rabbit oxidizes both progesterone (P) and deoxycorticosterone (DOC) to 20-oxo-21-oic acids that are excreted primarily in the urine [9]. The rabbit differs from the above species in having a particularly active hepatic microsomal 21-hydroxylase (cytochrome *P*-450; *P*45011C5) that converts P to DOC [10, 11]. *In vitro* [12, 13] and *in vivo* [14] studies have also implicated

Abbreviations: DOC (deoxycorticosterone) 21-hydroxy-4-pregnene-3,20-dione; B (corticosterone), 11β,21-dihydroxy-4-pregnene-3,20-dione; S (11-desoxycortisol), 17α,21-dihydroxy-4-pregnene-3,20-dione; F (cortisol), 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; HHDOC, 3α,20β,21-trihydroxy-5β-pregnane; THB, 3α,11β,21-trihydroxy-5β-pregnan-20-one; HHS, 3α,17α,20β,21-tetrahydroxy-5β-pregnane; THF, 3α,11β,17α,21-tetrahydro-5β-pregnan-20-one; cortolic acid, 3α,11β,17α,20α/β-tetrahydroxy-5β-pregnan-21-oic acid; cortolonic acid, 3α,17α,20α/β-trihydroxy-5β-pregnan-11-one-21-oic acid.

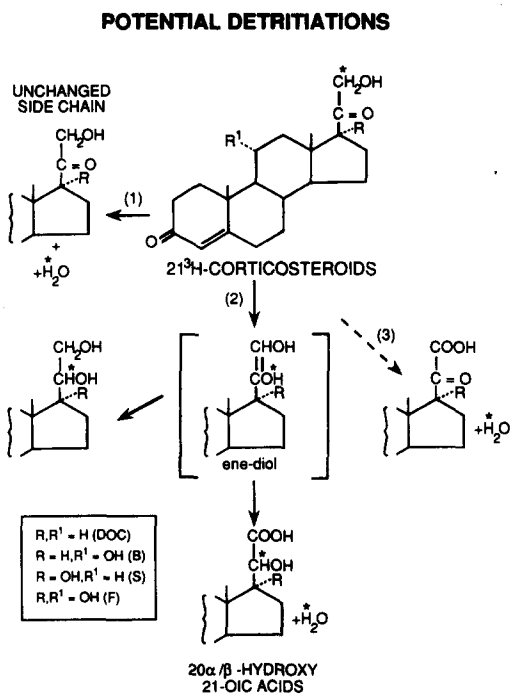

Fig. 1. Summary of potential corticosteroid side-chain detritiations adapted from previous publications [2, 3].

cytochrome *P*-450 activity in the further oxidation of DOC to give 20-oxo-21-oic acids. Such a mechanism would be an alternative to the isomerization pathway characterized by Bradlow and Monders groups in several species [2, 3]. However, there is some evidence that the latter pathway may also be operative in the rabbit since rabbit liver cytosol has the capacity to oxidize DOC to a 20-hydroxy acid [7]. The rabbit thus appears to have the capacity to oxidize the corticosteroid side-chain to both 20-oxo and 20-hydroxysteroid acids by at least two pathways. To what extent these pathways may occur *in vivo* is difficult to assess precisely. In the present study we have used corticosteroids labelled specifically with 4-^{14}C and 21-^{3}H in an attempt to provide an overview of the metabolic fate of different corticosteroid side-chains and in particular to determine the influence of steroid structure on steroid acid formation by the rabbit.

EXPERIMENTAL

Steroids

[21-^{3}H]Corticosteroids were synthesized by reduction of the corresponding 21-dehydrosteroid [15] with sodium borotritriide [1]. The tritiated steroids were purified by TLC (Anasil OF; 250 μm; 20 × 20 cm) in benzene–ethanol mixtures (85:15 to 95:5 v/v) and eluted with methanol. The steroids had the following specific activities: [21-^{3}H]-deoxycorticosterone (26.5 Ci/mol); [21-^{3}H]corticosterone (25.8 Ci/mol); [21-^{3}H]11-desoxycortisol (52.3 Ci/mol); and [21-^{3}H]cortisol (30.3 Ci/mol). ^{14}C-Labelled steroids were obtained from the following sources: [4-^{14}C]-deoxycorticosterone (60 mCi/mmol), [4-^{14}C]11-desoxycortisol (50 mCi/mmol) and [4-^{14}C]-cortisol (60 mCi/mmol) from Dupont Canada Ltd; and [4-^{14}C]corticosterone (52 mCi/mmol) from Amersham.

Injections

New Zealand white rabbits were injected via the marginal ear vein with the following steroid mixtures dissolved in 60% ethanolic saline. The injected dose was calculated by subtraction of radioactivity remaining in the syringe and vial. The indicated number of rabbits received the following doses: deoxycorticosterone, 17.56 μCi ^{3}H/1.66 μCi ^{14}C (3 rabbits; ^{3}H/^{14}C 10.58); corticosterone, 42.69 μCi ^{3}H/12.19 μCi ^{14}C (3 rabbits, ^{3}H/^{14}C 3.50); cortisol, 54.3 μCi ^{3}H/9.44 μCi ^{14}C (3 rabbits; ^{3}H/^{14}C 9.44); and 11-desoxycortisol, 17.58 μCi ^{3}H/1.63 μCi ^{14}C (2 rabbits; ^{3}H/^{14}C 10.78).

Solid phase extraction (Sep paks)

Urine was collected during each 24-h period over 3 days from rabbits housed in metabolic cages with access to food and water. The urine was centrifuged and aliquots (5–25 ml) processed on Sep paks (C_{18}; Waters), primed as recommended by the manufacturer. After percolation of the urine, the cartridge was washed with water (2 × 5 ml) and eluted with methanol to resolve tritiated water and steroids. The combined aqueous phases were processed twice and the methanol eluates combined.

Glusulase hydrolysis

Methanol eluates from the Sep paks were evaporated under nitrogen, the residues dissolved in 0.2 M sodium acetate buffer (pH 5.0; 10 ml) and the mixtures hydrolysed for 24 h at 37°C with Glusulase (900 U/ml of β-glucuronidase and 200 U/ml of sulphatase). The incubates were again percolated through Sep paks and the steroids eluted with methanol.

Alumina adsorption chromatography

The methanol eluates from the Sep paks were evaporated under nitrogen, dissolved in

ether–ethanol (3:1 v/v; 4 ml) and applied to a 5 g alumina column packed in benzene. The columns were eluted with ether–ethanol (3:1 v/v; 15 ml), ethanol (15 ml), 50% aqueous ethanol (25 ml) and 25.0 ml each of 0.1 and 1.0 M sodium acetate buffer, pH 5.0. 5-ml Fractions were collected and aliquots (0.2 ml) were removed and counted. This procedure resolves 21-deoxysteroids eluted with ether–ethanol (3:1) (fraction A), 21-hydroxysteroids (50% ethanol; fraction B) and steroid acids (0.1 M sodium acetate buffer, pH 5.0; fraction C). Before hydrolysis steroid glucuronides were eluted with 1.0 M buffer (fraction D) and after hydrolysis unidentified metabolites remained in this fraction [16, 17].

HPLC

Steroid methyl esters were prepared by heating the dried steroid acid metabolites with boron trifluoride–methanol (Sigma Chemical Co.; 1.0 ml) in a hot water bath for 2–4 min. The methyl esters were extracted with ethyl acetate and the metabolite profiles compared by HPLC on a C_{18} ODS reverse phase column (Perkin Elmer, 4 mm × 25 cm) equipped with a guard column (Brownlee Ltd). Columns were developed with methanol–water mixtures (60–65%) under isocratic conditions. Eluent fractions (1.0 ml) were collected in 5-ml mini vials (Beckman) and the ^3H and ^{14}C content was measured after addition of 4 ml PCS-scintillation fluid (Formula 963, Dupont Canada Ltd) in a Beckman liquid scintillation counter. 21-Hydroxysteroids, isolated in fraction B from alumina columns were analysed by HPLC on a straight phase silica gel column developed with an isopropyl–hexane gradient as previously described [18].

Nonlabelled reference steroids were chromatographed as above and fractions collected in glass tubes. Aliquots were spotted on TLC plates and the steroids detected by spraying with sulphuric acid–ethanol (1:1 v/v) and heating at 110°C for 10 min.

Table 1. Excretion of radioactivity

Steroid	Rabbit No.	Dose[a] (%) ^3H	Dose[a] (%) ^{14}C
DOC	1	20.6	83.0
	2	20.4	85.0
	3	18.5	78.7
B	4	56.6	68.2
	5	60.6	86.8
	6	56.2	79.4
S	7	40.8	72.1
	8	55.8	91.1
F	9	58.6	66.8
	10	55.7	57.7
	11	69.8	72.4

[a]Means of radioactivity excreted in urine collected for 48 h (B; F) and 72 h (DOC; S), respectively.

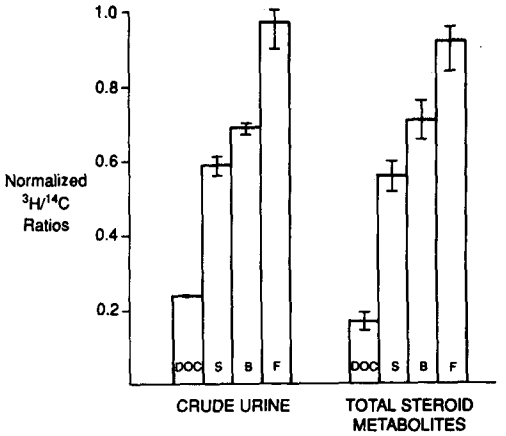

Fig. 2. Normalized isotope ratios of crude urines before and after passage through Sep paks to resolve tritiated water from steroid radiometabolites.

Normalized $^3H/^4C$ ratios

Normalized $^3H/^4C$ ratios were calculated by dividing the experimentally determined ratios by the corresponding dose ratios injected into the rabbits. An unchanged isotope ratio would give a normalized ratio of 1.0.

RESULTS

Recovery of injected radioactivity

The excretion of ^{14}C-labelled metabolites of the four corticosteroids is shown in Table 1. The majority were excreted during the first 24-h period following injection of 4-^{14}C- and 21-^3H-labelled B and F, or in the second 24-h period with DOC and S. The recovery of tritium was

Table 2. Detritiation of 4-^{14}C- and 21-^3H-labelled corticosteroids assessed in crude urine processed on Sep paks

Steroid	Rabbit No.	Radioactivity in urine (%) ^3H Methanol	^3H Water	^{14}C Methanol	^{14}C Water
DOC	1	67.0	29.1	97.9	2.7
	2	72.0	24.1	87.2	9.3
	3	68.0	20.4	83.4	4.0
B	4	98.2	0.9	90.1	5.1
	5	88.2	5.9	89.4	5.1
	6	93.0	8.3	89.8	7.0
S	7	81.4	7.1	82.8	4.2
	8	89.5	5.1	89.8	2.9
F	9	92.6	2.7	86.0	2.3
	10	96.0	2.8	96.0	2.4
	11	92.8	5.0	94.9	4.4

Water fractions contained the combined spent urines and water washes after two passes through each Sep pak. Steroids were eluted in 5 ml methanol.

lower than for ^{14}C with all four corticosteroids and particularly pronounced with those rabbits injected with DOC. Tritium, but not ^{14}C, continued to be excreted for up to 3 weeks after injection.

The normalized $^{3}H/^{14}C$ ratios of the crude urines (Fig. 2) showed a trend from DOC (mean 0.24), to cortisol (mean 0.97; range 0.90–1.0) with S (mean 0.59; range 0.59–0.61) and B (mean 0.69; range 0.68–0.70) intermediary.

Distribution of tritium

Aliquots of the crude urine were processed on Sep paks to separate tritiated water from the tritiated steroid metabolites. Rabbits injected with 4-^{14}C- and 21-^{3}H-labelled DOC excreted urine that contained the greatest proportion of tritiated water (20.4–29.1%) (Table 2). Figure 2 shows that the normalized isotope ratios of the steroid metabolites separated on Sep paks parallel those obtained with the crude urine.

Group fractionation of radiometabolites

Methanol eluates from the Sep paks were evaporated and the steroid metabolites hydrolysed with Glusulase to effect cleavage of possible steroid glucuronides and sulfates. Figure 3 shows the group fractionation of radiometabolites on alumina adsorption columns developed under similar conditions. The designation of the four groups as 21-deoxysteroids (A), 21-hydroxysteroids (B), steroid acids (C) and fraction D, is based on previous studies with DOC metabolites [16].

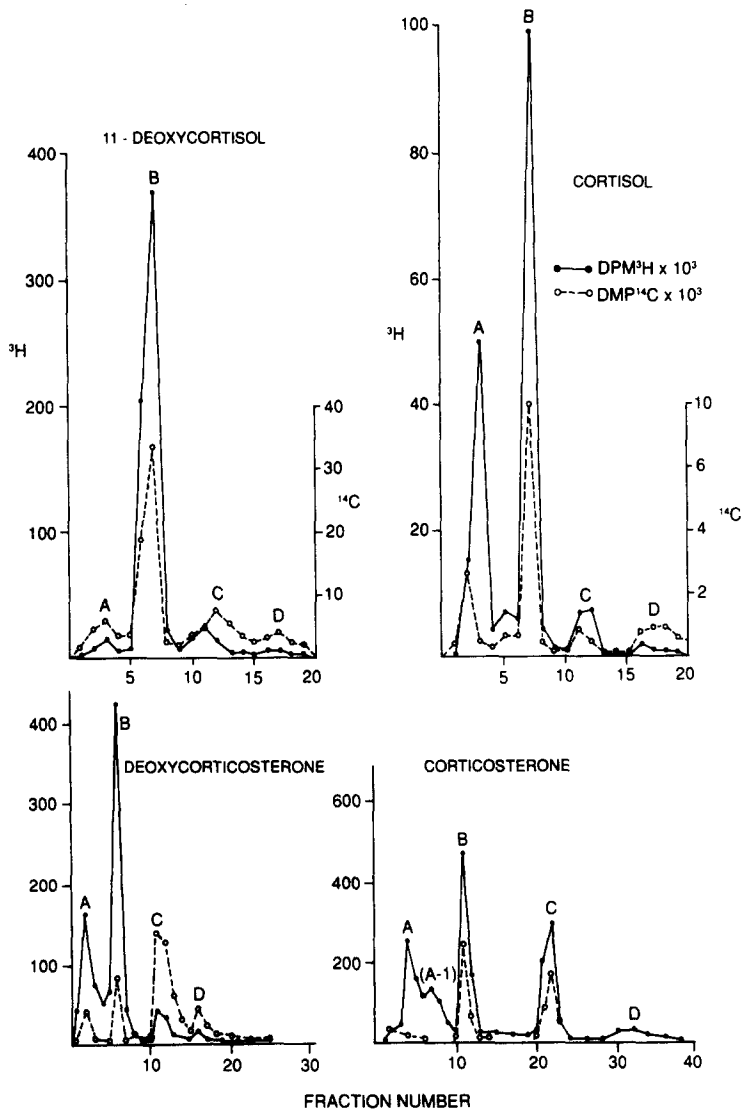

Fig. 3. Comparison of the alumina column chromatograms of the metabolites of the four 4-^{14}C- and 21-^{3}H-labelled corticosteroids. The metabolites were hydrolysed with Glusulase and chromatographed on 5 g alumina columns, except for corticosterone (20 g column).

Table 3. Group fractionation of 4-^{14}C- and 21-^{3}H-labelled corticosteroid metabolites

Isotope	Steroid	Dose[a] (%) 21-Deoxy steroids	21-Hydroxy steroids	Steroid acids	Fraction[b] D
^{3}H	DOC	2.4	5.5	0.67	0.54
		1.6–3.9	4.5–6.1	0.28–0.90	0.37–0.63
	B	10.5	15.5	6.3	0.73
		7.0–15.7	10.4–18.8	3.5–8.8	0.4–1.0
	S	1.1	18.1	2.4	0.79
		1.1–1.2	15.4–20.8	1.9–2.4	0.7–0.87
	F	14.9	26.2	2.9	1.5
		10.8–19.9	24.4–27.5	23–3.5	1.3–1.6
^{14}C	DOC	4.9	12.7	19.1	11.4
		2.0–10.2	10.4–17.2	11.7–24.7	8.2–16.5
	B	3.9	19.8	15.0	7.9
		2.4–6.1	16.1–26.4	13.6–17.3	6.2–9.9
	S	3.0	18.7	9.7	4.0
		1.6–4.4	16.2–21.2	8.3–11.0	2.7–5.3
	F	8.1	27.6	2.7	7.3
		5.9–10.7	27.1–28.4	2.0–3.7	6.8–8.3

[a]Means and ranges of 3 (DOC; F) and 2 rabbits (B; S), respectively.
[b]Steroids were eluted in fractions A–D from typical alumina columns after Glusulase hydrolysis.

The "21-deoxysteroid" fraction also contained the products of corticosteroid side-chain cleavage. The parent steroids, DOC, B, S and F were all eluted in the 21-hydroxysteroid fraction (B). Table 3 summarizes the proportions of radioactivity recovered in the four groups and Fig. 4 the corresponding normalized isotope ratios. The results will be considered separately under each steroid group for convenience.

Steroid acids (fraction C)

The radiometabolites of the four corticosteroids that eluted in alumina fraction C were considered to be acidic since they stayed at the origin when chromatographed on thin layer plates with benzene–ethanol mixtures of increasing polarity. Based on the recovery of ^{14}C, DOC was the best precursor with up to 24.7%

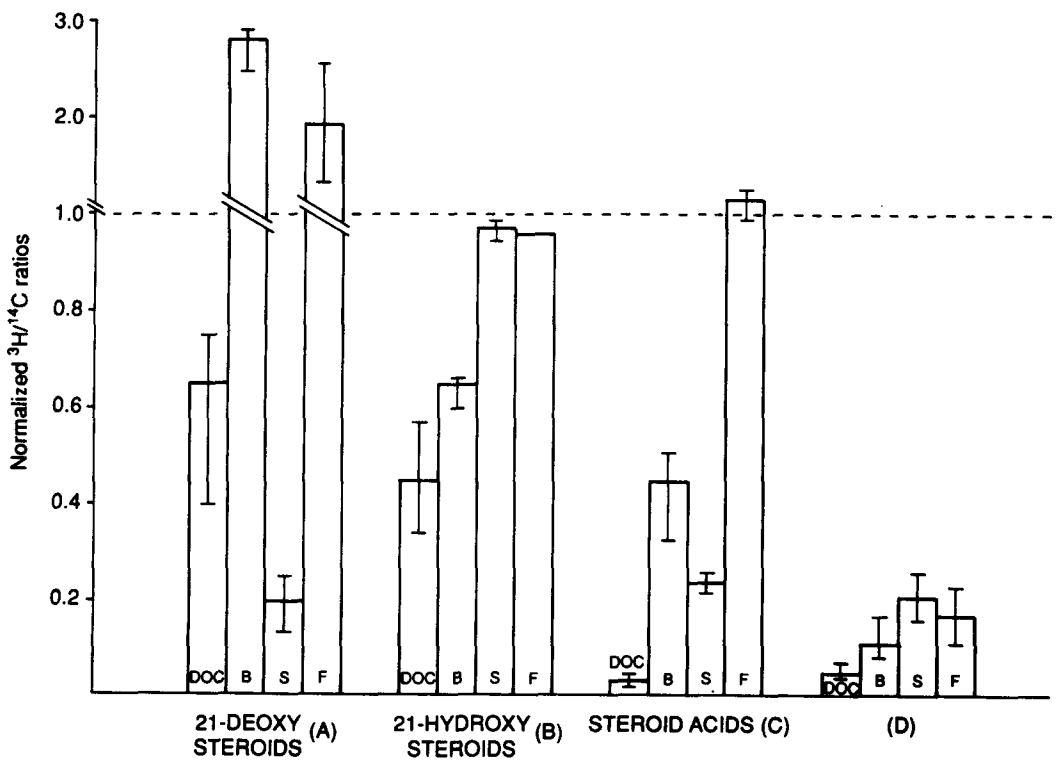

Fig. 4. Normalized isotope ratios of four corticosteroid metabolite groups resolved by alumina column chromatography.

of the dose being excreted in this fraction. The other corticosteroids showed a decline in the order DOC > B > S > F with only a mean of 2.7% of the [^{14}C]F dose recovered in this fraction. By contrast, the highest loss of tritium occurred with the DOC steroid acids which contained only 0.67% of the [21-^{3}H]DOC dose. These results were reflected in the isotope ratios plotted in Fig. 4 with DOC steroid acids retaining negligible tritium (mean isotope ratio 0.03; range 0.02–0.04). The isotope ratios of the B and S fraction C acids were also significantly reduced, whereas the isotope ratios of the F metabolites averaged 1.13 (range 0.96–1.27). This was spuriously high since, as shown below, the methyl esters had lower normalized ratios.

Fraction C methyl esters

The methyl esters of the fraction C metabolites were analysed by HPLC. The complex spectra and normalized isotope ratios of the major radioactive peaks are shown in Fig. 5. Further studies are underway to characterize the major metabolites, but the indicated spectra demonstrate the variability in isotope ratios. The majority of the DOC methyl esters had consistently low isotope ratios, possibly indicative of the presence of 20-oxo-21-oic and/or

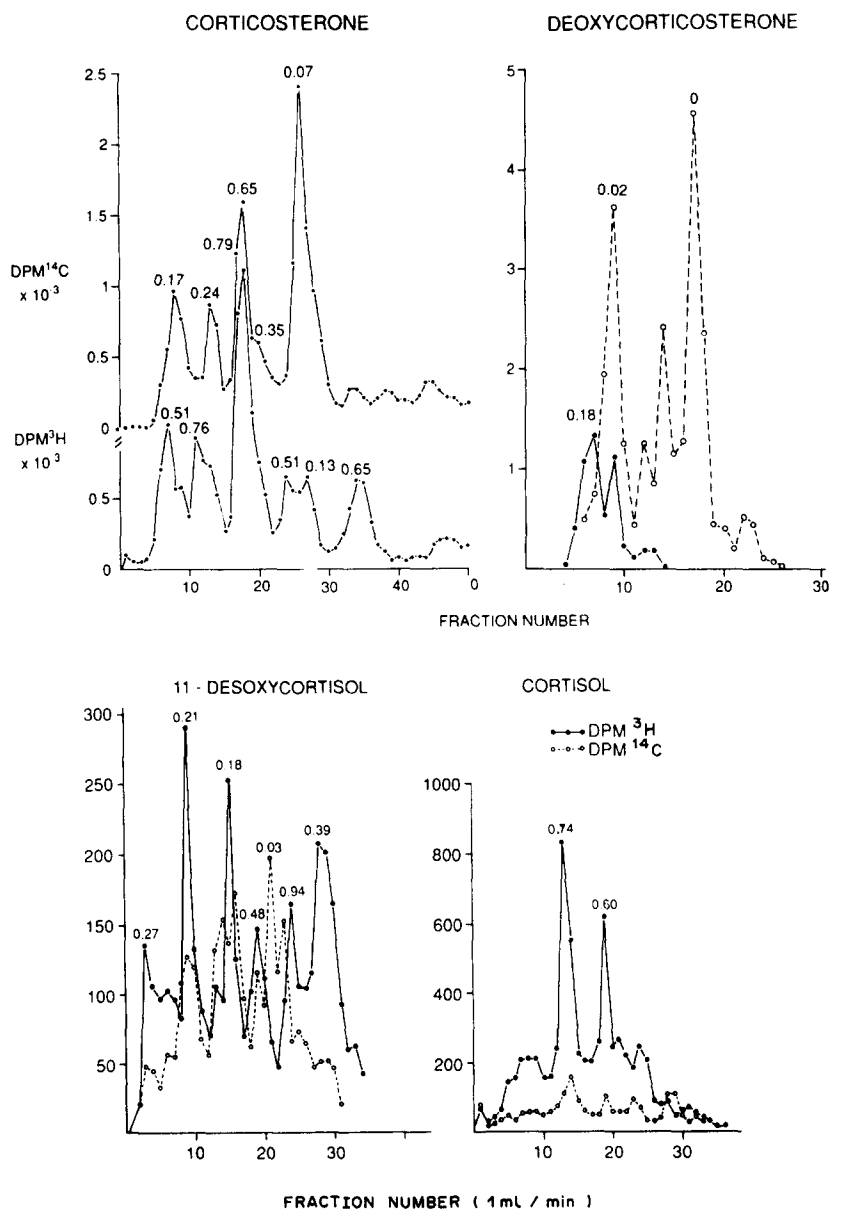

Fig. 5. HPLC spectra of methyl ester derivatives of corticosteroid metabolites isolated in alumina fraction C. Steroids were chromatographed on a C_{18} ODS column isocratically with 60, 65 and 70% methanol for F, B and S and DOC, respectively. Numbers are normalized ratios.

17-carboxylic acid methyl esters. The presence of a 20-oxo function was confirmed by the specific reduction with 20β-hydroxysteroid dehydrogenase which produced a shift in the mobility of the major peak to a lower retention time. HPLC resolved methyl esters of corticosterone that exhibited both high (0.65) and low (0.07) normalized isotope ratios. Retention of tritium may indicate the presence of a 20-hydroxy-21-oic acid. The desoxycortisol methyl ester spectra were highly complex, particularly when compared to cortisol. Although the cortisol fraction C metabolite group isolated from the alumina column had a mean isotope ratio near 1.0, the two methyl ester peaks resolved by HPLC had lower ratios consistent with a loss of 26–40% of tritium.

21-Hydroxysteroids (alumina fraction B)

The 21-hydroxysteroid fraction showed a decline in their isotope ratios in the order DOC > B > S = F. This represented a mean loss of tritium of 55, 35, 3 and 4%, respectively. HPLC of the 21-hydroxysteroid metabolites on a straight phase silica column (Figs 6 and 7) revealed a somewhat different situation. The results fell into two groups. The 11-deoxysteroids, DOC and S (Fig. 6) gave a major peak with the same mobility as hexahydro DOC and hexahydro S, respectively. The isotope ratios showed that no loss of tritium had occurred. B and F both gave major peaks with the same mobilities as the tetrahydro reduced steroids. The normalized ratios showed that these compounds had lost 64 and 20% of the tritium, respectively. The excretion of tetrahydro reduction products of B and F probably reflect the inhibitory effect of the 11β-hydroxy function on reduction at C-20.

Alumina fractions A and D

Radiometabolites eluted with ether–ethanol (alumina fraction A) have been shown to contain 21-deoxysteroid metabolites of DOC [16, 18] but it could also contain the products of corticosteroid side-chain cleavage derived from F and S. The high isotopic ratios observed with the F and S metabolites eluted in this fraction however, initially suggested retention of tritium, but this was found to be spurious since on evaporation of the alumina fractions up to 90% of the tritium proved volatile. The residue from the cortisol metabolites gave a major peak (N = 0.56) on HPLC indicating some retention

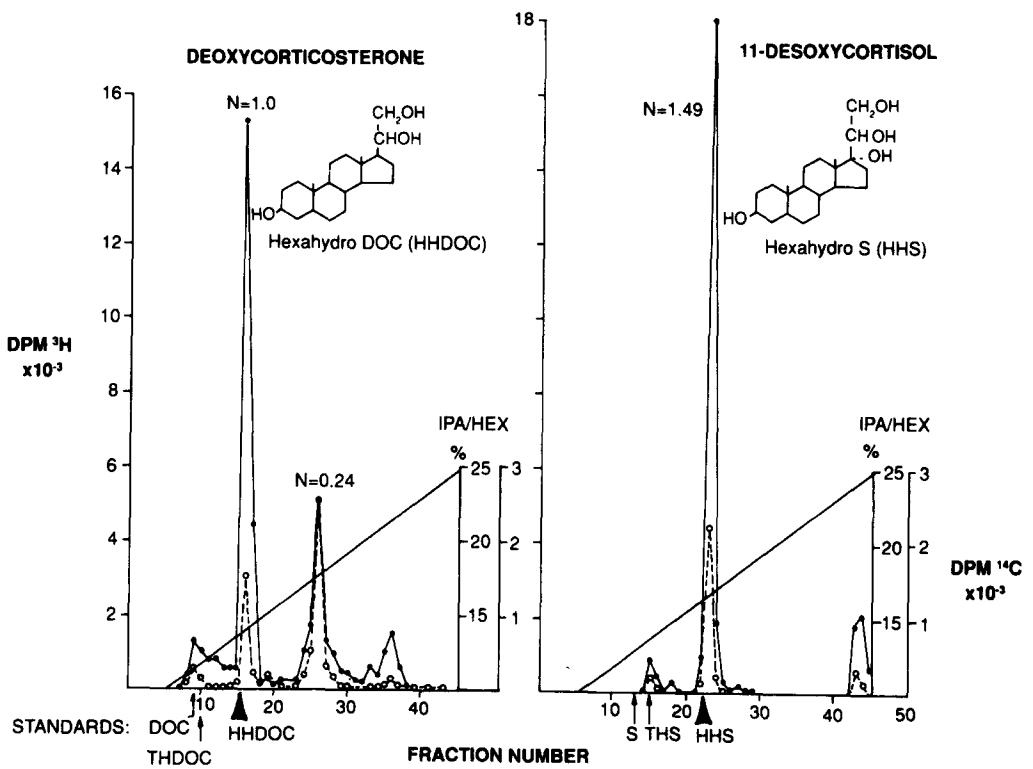

Fig. 6. HPLC spectra of the 21-hydroxysteroid metabolites of DOC and S isolated from alumina fraction B. Metabolites were chromatographed on a Chromegabond diol silica gel column with isopropyl alcohol–hexane gradients. N = normalized isotope ratio. Arrows indicate the elution of HHDOC and HHS standards chromatographed separately.

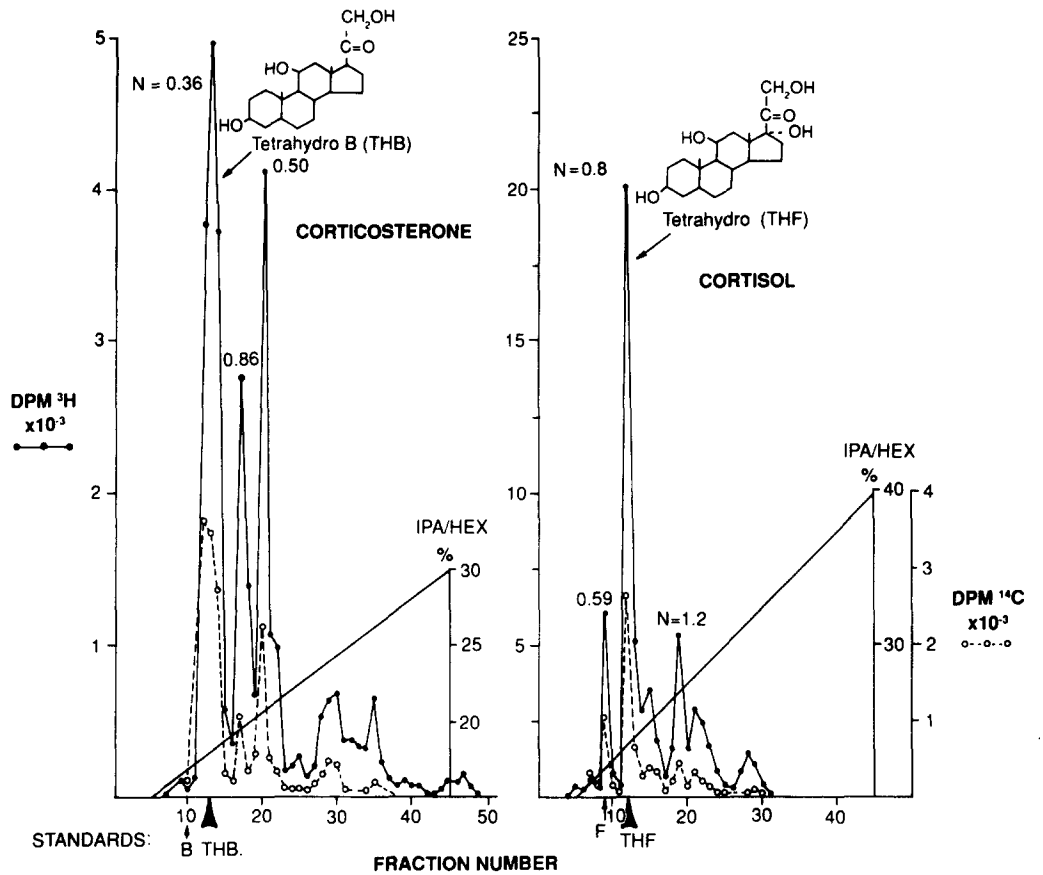

Fig. 7. HPLC spectra of B and F 21-hydroxysteroid metabolites chromatographed as indicated in Fig. 6. Arrows indicate the elution of THB and THF standards run individually.

of tritium in this "21-deoxysteroid" fraction. Loss of tritium on evaporation of alumina fractions was only observed with the F and B metabolites eluted in alumina fraction A. Since these metabolites had previously been tritiated, as judged by their elution from Sep paks, it must be concluded that some detritiation occurred during chromatography on alumina.

Steroid glucuronides were eluted in fraction D before hydrolysis. After hydrolysis the radiometabolites eluted in fraction D were acidic and had variable normalized isotope ratios but generally <0.5.

DISCUSSION

It is evident from the present study that there is considerable lability of the corticosteroid side-chain during its *in vivo* metabolism by the rabbit. In this respect it resembles previous studies on the human [2] and other species [3]. Administration of 4-^{14}C- and 21-^{3}H-labelled DOC to both the human [5] and the rabbit resulted in loss of tritium and the apparent excretion of 20-oxo-21-oic acids. By contrast, the human excreted primarily 20-hydroxy-21-oic acid metabolites of cortisol which retained considerable tritium in the side-chain. Previous studies have shown that cortisol [4, 20] is a better precursor of steroid acids than DOC [5, 20] in the human. By contrast DOC was the best precursor of steroid acids in the rabbit, which accounted for up to 24.7% of the dose, whereas cortisol was the weakest precursor (maximum of 3.7% of the dose). It has also to be recognized that this may be an underestimation in the rabbit since it is based on acids isolated in alumina fraction C and does not take into account the acidic metabolites in fraction D. Corticosterone was the second most efficient precursor of acids (17% of the dose), after DOC. A previous study [20] with 1,2-tritiated steroids also found that the human excreted significant levels of acid metabolites of corticosterone (14.9%) but this was not confirmed by Bradlow *et al.* [2]. In the rabbit it appears that the presence of a 11β-hydroxyl group, particularly in association with a 17α-hydroxyl group, decreases side-chain oxidation to 21-oic acids. HPLC of the methyl esters showed clearly that

the majority of the acidic metabolites of DOC and S had lost most of their tritium. This could be due to oxidation to 20-oxo-21-oic acids or side-chain cleavage. A major acidic metabolite of DOC had the same mobility as a 20-oxo-21-methyl ester standard on HPLC and was eluted with a lower retention time after reduction with 20β-hydroxysteroid dehydrogenase. Evidence of 20-hydroxy-21-oic acids in the spectra of the four corticosteroids under examination was obtained by the retention of tritium as indicated by an isotope ratio in the region of 0.5. The HPLC spectra of the methyl ester derivatives of both B and F metabolites contained peaks with significant retention of tritium. A methyl ester derivative of a major B metabolite (N = 0.65) had a mobility close to that of a standard 20α-hydroxy-21-methyl ester derivative of corticosterone.

The 21-hydroxysteroid fractions also showed the effects of steroid structure on detritiation of the side-chain. HPLC analysis showed that 20-oxo (tetrahydro) compounds were the major metabolites of B and F, whereas DOC and S gave mainly C-20 reduced hexahydro metabolites. The presence of hexahydro metabolites of DOC and S may indicate that these steroids, unlike B and F which possess 11β-hydroxyl functions, are substrates for reduction at C-20. Alternatively, the hexahydro metabolites might be derived via isomerisation of the C-21 tritium to the C-20 position with reduction of the 20-hydroxy-21-aldehyde intermediate, as proposed elsewhere [2]. The metabolism of DOC and S to mainly 20-oxo acids may be due to direct oxidation at C-21 and/or further metabolism of an intermediate 20-hydroxy acid, presumably produced by the isomerase reaction [3], by a 20α-hydroxysteroid dehydrogenase. The presence of the 11β-hydroxyl function in F and B would be inhibitory in this situation. Further studies underway on the *in vivo* metabolism of 20-dihydrosteroids to steroid acids would support the involvement of both 20-hydroxysteroid dehydrogenase activity and direct oxidation at C-21 [21].

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