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EXCHANGE OF HYDROGEN AT C-21 DURING DEHYDROXYLATION OF DEOXYCORTICOSTERONE BY MIXED CULTURES OF HUMAN FECAL FLORA

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SUMMARY

Deoxycorticosterone labeled at C-4 with ^{14}C and at both positions at C-21 with ^{3}H was incubated with mixed cultures of human fecal flora. 3α-Hydroxy-5β-pregnan-20-one, 3β-hydroxy-5β-pregnan-20-one and 5α-pregnane-3,20-dione were isolated and found to contain both isotopes in varying ratios. In every case, more than half of the tritium was lost. These experiments show that the hydrogen at C-21 has a complex role in 21-dehydroxylation by the flora of the gut.

INTRODUCTION

21-Dehydroxylated metabolites of aldosterone [1, 2], corticosterone [3], 11-dehydrocorticosterone [4] and 11-deoxycorticosterone (DOC) [5, 6] are excreted in human urine. We have recently demonstrated that mixed cultures of human fecal flora can 21-dehydroxylate DOC [7]. The major 21-deoxysteroid produced from DOC by such cultures is pregnanolone, although small amounts of the 3β epimer of pregnanolone and 5β-pregnane-3,20-dione are also formed [8].

In addition, fecal flora from man, rat and rabbit are capable of dehydroxylating bile acids. For example, 7α-dehydroxylating organisms of fecal origin have been described by Midtvedt and Norman[9], by Bokkenheuser *et al.*[10], and by Hattori and Hagakawa[11]. Further, it appears likely that fecal flora of rabbit can 12-dehydroxylate bile acids since lithocholic acid in the rabbit feces must be derived from biliary cholic acid, as chenodeoxycholic acid is not present in rabbit bile in appreciable amounts [12]. Reductive dehydroxylation of steroids and bile acids has not been demonstrated in mammalian tissue, but rather seems confined to the flora of the gut. Little is known of the mechanisms by which this reduction proceeds. The purpose of the experiments described here was to examine the role of the C-21 hydrogens in dehydroxylation. DOC labeled with ^{14}C at 4 and with ^{3}H about equally at both positions on C-21 was incubated with mixed cultures of fecal flora, the 21-deoxy products were isolated and their isotopic ratios were determined. The $^{3}H/^{14}C$ ratios of the products were lower than that of the precursor in every case, indicating that in the course of the 21-dehydroxylation of DOC, tritium was lost from C-21.

EXPERIMENTAL

Materials and general methods

Reagent grade solvents were used as supplied except for methanol, which was technical grade. Chromatography and determination of radioactivity were carried out as previously described [13, 14]. Deoxycorticosterone-[^{14}C] was purchased from New England Nuclear Corp., Boston, and 21-[^{3}H]-DOC [15] was the generous gift of Dr. Carl Monder, Research Institute of the Hospital for Joint Diseases, Mount Sinai School of Medicine, New York. Both steroids were at least 97% pure by isotopic dilution analysis.

Incubation. The incubation of DOC with fecal flora was carried out in the manner already reported [7]. Briefly, 50 ml brain heart infusion broth (Difco Laboratories, Inc., Detroit, Michigan) was sterilized (121°C, 20 min) in 100 ml bottles with screw caps fitted with rubber diaphragms. The medium was cooled and 0.8 mg carrier DOC mixed with the labeled steroids described above was added, given a concentration of DOC of 16 $\mu g\ ml^{-1}$ medium. The medium was inoculated with 0.25 ml of 10^{-3} suspension of freshly voided feces from a healthy donor on a Western diet, and the culture was incubated at 37°C for 7 days.

Isolation of products. The cultures were extracted and the extracts chromatographed using the heptane–ethylene chloride: 90% ethylene glycol–10% water gradient [7]. The 3α-pregnanolone eluted from this system was rechromatographed on 15 g Celite in heptane–methylcellusolve. 3α-Pregnanolone was eluted in about 6 HBV. The radioactivity eluted from the gradient ahead of 3α-pregnanolone was also rechromatographed in heptane–methylcellusolve. Variable amounts of pregnanedione and 3β-pregnanolone were eluted in 2 and 5 HBV respectively.

Determination of isotopic ratios. Authentic unlabeled steroid was added to the corresponding steroid eluted from heptane–methylcellusolve. Several recrystallizations were carried out and the isotopic ratios in both crystals and mother liquors were determined. The differences in ratios among the final mother liquor, the final crystals and the next to final crystals did not exceed $\pm 2\%$.

Oxidation of pregnanolones. Twenty mg of doubly labeled pregnanolone with carrier prepared as described in the previous paragraph were dissolved in 0.1 ml pyridine. Ten mg CrO_3 in 0.5 ml pyridine was added and the oxidation was allowed to proceed at room temperature overnight. The reaction mixture was poured into water and the products extracted 3 times with methylene chloride. The extracts were combined, washed first with 0.1 N HCl, then with saturated $NaHCO_3$ until the washes were alkaline and finally with water until the washes were neutral. The organic phase was dried over Na_2SO_4 and evaporated. The residue was chromatographed on heptane–methylcellusolve and pregnanedione was eluted. The pregnanedione was recrystallized to constant isotopic ratio as described above.

RESULTS

The isotopic ratios found in the products of microbial dehydroxylation of DOC are presented in Table 1. The isotopic ratios of 3α and 3β pregnanolone were the same as those of their chromic acid oxidation product, 5β-pregnanedione. This indicates that the isotopic ratios in the pregnanolones are the true ratios and that no tritium was lost from the steroid during the oxidation. Such loss could have resulted from enolization at C-21 occurring during oxidation or work up of the product. Further, if tritium had been metabolically transferred from 21 and reintroduced at C-3, a loss of tritium would have occurred during the oxidation.

The $^3H/^{14}C$ ratios were different from metabolite to metabolite and from experiment to experiment. Apparently, the loss of tritium is variable and may be subject to variations in conditions which inevitably occur from culture to culture. In every case however, more than half of the tritium was lost while at the same time some tritium was always retained.

Table 1. Isotopic ratios in metabolites of DOC

Experiment	$^3H/^{14}C$ Dione	3β (dione)	3α (dione)
I			0.28
II	0.08	Ins.	0.27 (0.26)
III	Ins.	1.2 (1.1)	0.94 (0.94)

The $^3H/^{14}C$ ratio in the DOC was 4.71.
Dione = 5β-pregnanedione; (dione) = 5β-pregnanedione derived from 3α or 3β-pregnanolone by oxidation with CrO_3; 3β = 3β-hydroxy-5β-pregnan-20-one; 3α = 3α-hydroxy-5β-pregnan-20-one; Ins. = Insufficient material isolated for determination of isotopic ratio.

In order to be certain that the microorganisms in the culture were responsible for the loss of tritium, the doubly labeled precursor, DOC, was incubated for 7 days at 37°C in sterile medium. There was no evidence of formation of any products from DOC and the recovered DOC had the same isotopic ratio as that incubated.

DISCUSSION

It is clear from the results presented above that 21-dehydroxylation of DOC proceeds in mixed cultures of fecal flora with exchange of hydrogen at position 21. Since more than half of the label was lost, both hydrogens must participate in the overall exchange. These results obscure any stereospecific exchange which may have occurred. The variable and incomplete loss of tritium indicates that the role of the 21-hydrogens in 21-dehydroxylation in mixed cultures is complex. The exchange of hydrogen may be integral to the mechanism of dehydroxylation, may occur by enolization before, concerted with, or after dehydroxylation, or may occur by oxidation prior to dehydroxylation. Further, at least some of the variability may be due to isotope effects.

The mechanism of 7-dehydroxylation of bile acids has been studied by Canonica *et al.*[16] and by Bergstrom *et al.*[17]. Canonica *et al.*[16] showed a loss of tritium from 7β[^{3}H]-labeled cholic acid upon 7-dehydroxylation in mixed cultures of human fecal flora. However, the loss was not complete, and somewhat less tritium had also been lost from the recovered cholic acid. Similar results were obtained with the epimer of cholic acid (7β-OH), although in this case more isotope had been lost from the recovered precursor than from the 7-deoxycholic acid. On the other hand, Bergstrom *et al.*[17] have demonstrated retention of tritium in deoxycholic acid isolated from rabbit bile following intraperitoneal injection of 7β-^{3}H-cholic acid. The label in the deoxycholic acid was shown to be at 7α by hydroxylation with rat liver. Thus, although the tritium at C-7 was retained during dehydroxylation, it underwent inversion of configuration. The different results may be due to the different experimental conditions used by these two groups. No exchange of hydrogen at 7 by enolization is possible during 7-dehydroxylation of bile acids, due to the lack of an adjacent ketone. However, in the case of both cholic acid and DOC, oxidation is possible at the position undergoing dehydroxylation.

Further experiments are necessary to clarify the role of exchange of hydrogen during dehydroxylation of both bile acids and steroids.

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