Metabolism of Deoxycorticosterone by Human Fecal Flora

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21-Dehydroxylation, a feature of metabolism of corticoids in humans, was observed in mixed cultures of fecal flora of normal individuals on a Western diet. The model substrate, 11-deoxycorticosterone (DOC), was metabolized to 3α-21-dihydroxy-5β-pregn-20-one (THDOC), 3α-hydroxy-5β-pregn-20-one (pregnanolone), and to two unidentified structures, metabolites X and Y. DOC was not metabolized in all media supporting growth of fecal flora. Conversion required an initial pH between 6.0 and 8.0. 21-Dehydroxylation occurred within 4 days of incubation in media inoculated with 10^1 toilet suspension/ fecal dilutions, containing obligate anaerobes only, DOC was converted to metabolite X and sometimes also to metabolite Y. The yield of pregnanolone was related to the promptness with which the specimen was processed, to the presence of cysteine in the medium, and to the concentration of substrate (optimum, 16 to 64 μg of DOC per ml). The yield of THDOC was related to the delay in the processing of the specimen, the concentration of substrate (maximum at 256 μg/ml), and aeration of the culture. Pure cultures of aerobic organisms of fecal origin either failed to metabolize DOC or converted it to metabolite X. Pure cultures of fecal anaerobes converted DOC to metabolite X and sometimes also to metabolite Y. Neither THDOC nor pregnanolone was produced by pure cultures.

21-Dehydroxylation, a feature of metabolism of corticosteroids in humans, appears to be restricted to those corticoids which undergo biliary excretion. Ring A-saturated and -conjugated metabolites derived from 11-deoxycorticosterone (DOC) (Fig. 1), 11-dehydrocorticosterone (compound A), and corticosterone (compound B) by hepatic metabolism are excreted into bile (17, 25, 29). DOC has been shown to be a precursor of urinary 3α,20α-dihydroxy-5β-pregnane glucuronide (3, 21), and compounds A and B are precursors of urinary 3α,20α-dihydroxy-5β-pregn-11-one glucuronide (6, 27). Although information concerning the biliary excretion of aldosterone is lacking, this corticoid is the precursor of urinary 3α-hydroxy-5β-pregn-11-β,18S (18S, 20α)-diox-ide glucuronide (23, 24). On the other hand, metabolites of cortisol are not significantly excreted in bile (19, 30), and Fukushima and Gallagher (13) have demonstrated that cortisol does not undergo 21-dehydroxylation.

The end products of metabolism of corticoids in humans are excreted mainly in the urine, whether or not they undergo enterohepatic circulation. Fecal excretion is of quantitatively minor importance. Thus, most of the biliary corticosteroids are reabsorbed. Since the structures of steroids in bile differ from those in urine and feces, biliary steroids must be metabolized prior to excretion, probably in the intestine (26).

In the rat, steroids are extensively metabolized in the gut. Comparison of the steroids excreted in the feces of germfree rats with those in feces from conventional rats (7, 8, 14-16), together with studies of metabolism of steroids in mixed cultures of fecal or cecal flora of rats (1, 10-12), have demonstrated that the intestinal flora of the rat can convert steroids into a wide variety of metabolites. Such conversions include hydrolysis of conjugates and 21-dehydroxylation of corticoids (11). Microorganisms responsible for 21-dehydroxylation have not yet been identified. However, several laboratories have reported the isolation of organisms capable of metabolizing bile acids from the feces of humans (18), rabbits (2), and rats (28). These organisms were classified as Bacteroides and
**Lactobacillus.** They reductively 7-dehydroxylate cholic, allocholic, and chenodeoxycholic acids.

The role of the intestinal flora of humans in metabolizing steroids remains almost completely unexplored. Intestinal contents obtained from the ileostomy of a colectomized patient removed the 21-OH group from 3β,21-dihydroxy-5α-pregnan-20-one (9). Based on this observation and the experiments in rats it seems reasonable to postulate that the urinary 21-dehydroxy metabolites of DOC, compounds A and B, and aldosterone arise through bacterial metabolism in the gut during the course of enterohepatic circulation. Further support for this hypothesis may be derived from the apparent inability of mammalian tissue to carry out 21-dehydroxylation and from the lack of urinary 21-dehydroxylated metabolites of cortisol, the metabolites of which are not excreted in bile.

As described in Results, some common intestinal aerobic and anaerobic organisms failed to metabolize DOC to THDOC and pregnanolone. The same compounds, however, were metabolized by mixed fecal flora of humans. The experiments reported here were designed to elucidate the factors influencing the metabolism of biliary corticoids by mixed fecal flora in vitro. Although Δ4-3-ketosteroids are not found in bile in significant amounts, DOC was chosen as the model substrate. It is closely related to the naturally occurring biliary steroids, commercially available, easy to detect, and can be radioisotopically labeled.

**MATERIALS AND METHODS**

**Media.** Dehydrated Todd-Hewitt broth and brain heart infusion (BHI) broth, were obtained from Difco Laboratories, Inc., Detroit, Mich. Reconstituted media were sterilized by autoclaving at 121°C for 20 min and stored at 4°C for no longer than 48 h before use.

THC broth: Dehydrated Todd-Hewitt broth (30 g), 1 g of cysteine hydrochloride, and 2 g of NaHCO₃ were dissolved in 1 liter of distilled water, distributed in suitable aliquots, and sterilized.
THCM broth: THCM broth was reconstituted in the same way as THC broth. A 1-cm layer of mineral oil, degassed by previous autoclaving, was poured on top of the distributed media prior to sterilization.

BHIC broth: Dehydrated BHIC broth (37 g) and 0.5 g of cysteine hydrochloride were dissolved in 1 liter of distilled water, distributed, and sterilized.

Chopped meat: Chopped meat (5 g) boiled in 0.025 N NaOH was added to 50 ml of BHIC and sterilized.

P. Preparations of steroid metabolizing organisms. BHI broth was kindly donated by Scott Laboratories Inc., Fiskeville, R.I.

The following ready-made media were purchased in amounts of 50 ml per bottle: tryptic soy broth (TSB), thioglycollate, and BHIC from Scott Laboratories; thiol broth and thiol broth with 15% sucrose from Difco Laboratories.

pH. pH was measured on a Beckman Zeromatic II pH meter using a glass electrode.

Shake cultures. To determine the tolerance of the steroid-metabolizing organisms toward oxygen, cultures were incubated in flasks on a reciprocating shaker at 100 oscillations/min (38-mm stroke). During incubation a stream of air (50 ml/min) was blown onto the surface.

Subjects. Donors of fecal material consumed the usual Western diet. Feces were collected in stool cups under ordinary atmospheric conditions. Processing was started within 30 min of defecation.

Solvents. The solvents were reagent grade except for methanol, which was technical grade.

Preparation of steroids. 11-deoxycorticosterone was purchased from New England Nuclear Corp. The steroid was at least 97% pure by isotopic dilution analysis. Prior to incubation the carrier was mixed with radioactive steroid to give 2 x 10^6 counts/min per culture regardless of the amount of carrier added to the medium.

Incubation of steroids. An appropriate methanolic solution of steroid was added to the sterilized medium to give the desired concentration of steroid and 0.5 to 1.0% methanol (vol/vol). The media were inoculated with decanted fecal fluids in the proportion 20:1 and incubated at 37 C for 4 to 25 days. The ability of pure bacterial strains to metabolize DOC was tested by seeding BHI and PR with 0.1 ml of 48-h culture for each 20 ml of medium. Anaerobic media were inoculated with syringe and needle to prevent oxygen from entering the vials. Vents were not used. All media were incubated at 37 C for 7 days.

Extraction of steroids. At the end of the incubation, the culture was poured into 10 volumes of methanol while stirring. After standing overnight, this mixture was filtered and the filtrate was evaporated to the aqueous residue under vacuum. The residue was diluted to 200 ml with water and extracted with 3 x 100 ml of methylene dichloride. The combined extracts were washed first with 1 N sodium hydroxide until the washes were colorless and then with distilled water until the washes were neutral. The organic phase was dried over sodium sulfate; 0.5 ml of ethylene glycol was added, and the solvents were evaporated under vacuum. The oily residue was then subjected to chromatography.

Chromatography. Partition chromatography supported on celite was used for the separation and quantitation of steroids as previously described (5, 22). The column was packed with 10 g of celite moistened with 5 ml of water (31), followed by 30 g of celite mixed with ethylene glycol-water (9:1, vol/vol). The column was developed first with 100 ml of heptane. The gradient, generated in an apparatus consisting of two connected cylinders of equal cross-sectional area, was then started. The mixing chamber was initially charged with 500 ml of heptane and the reservoir with an equal weight of ethylene dichloride (300 ml).

(ii) Thin-layer chromatography. Thin-layer chromatography was performed on Bakersflex silica gel with fluorescence indicator (no. 1B2F) in a solvent system consisting of iso-octane-ethyl acetate-acetic acid (5:25:0.2, vol/vol/vol). The plates were examined under ultraviolet light at 254 and 360 nm (a, b-unsaturated carbonyl), after blue tetrazolium spraying (b-ketol) and after spraying with sulfuric acid-ethanol (50:50, vol/vol; nonspecific demonstration of steroids on a chromatogram). The plates were viewed under ultraviolet light at 254 and 360 nm.

RESULTS

DOC was the model steroid chosen for this study. The products identified upon incubation of DOC with fecal microflora from normal...
subjects were pregnanolone, THDOC, and unmetabolized DOC, along with several unidentified products. Progesterone was not detected in any of the experiments. The reduction of ring A was stereo-specific for the 3α-hydroxy and A/B cis ring fusion. Other isomers of reduction of ring A were not found.

**Duration of incubation.** Media containing DOC (200 μg/ml) were inoculated with a 10−⁶ suspension of feces from three normal subjects. About 54% of the DOC was converted to THDOC (Table 1) within 4 days. Extension of the incubation to 25 days did not influence the result materially. Small quantities of pregnanolone were observed after 10 days of incubation, and the yields increased to about 5% after 25 days. Approximately 6% of the DOC was recovered unchanged. DOC was not metabolized in the absence of microbial growth.

**Media.** Table 1 also shows that the yield of metabolites was essentially the same whether we employed BHIC broth with 0.05% cysteine hydrochloride or THC broth with 0.1% cysteine hydrochloride. The presence of a layer of mineral oil to exclude oxygen from the medium made no difference in the yields of any of the products (inoculum: 1 ml of a 10−⁶ suspension).

Of other media tested, chopped meat, PR, and TSB also supported growth of 21-dehydroxylating organisms. In contrast, 21-dehydroxylation was not observed in thioliol broth, thiol broth with 15% sucrose, thioglycolate broth, or in BHI without cysteine.

**Aerobic conditions.** To determine if fecal flora metabolize DOC under conditions more aerobic than employed above, BHIC and TSB (200 ml per 500-ml Erlenmeyer flask) with DOC (16 μg/ml) were inoculated with a fecal suspension (10−⁶). The experimental cultures were incubated on a shaker at 37 C for 7 days. The control cultures were neither shaken nor exposed to a stream of air. In aerated cultures, DOC was quantitatively metabolized in BHIC, but 17% remained unmetabolized in TSB.

**Table 1. Yield of metabolites after various periods of incubation**

| Subject | Days of incubation (Days) | Media (200 ml; 200 μg of DOC per ml)* |
|---------|---------------------------|--------------------------------------|
|         |                           | BHIC | THDOC | Preg | DOC | THDOC | Preg | DOC | THDOC | Preg | DOC | THDOC |
| A       | 0                         | 100  | 0     | 0    | 100 | 0     | 0    | 100 | 0     | 0    | 100 | 0    |
| B       | 4                         | 0    | 12    | 55   | 0    | 15   | 32   | 0    | 5    | 55   |
| C       | 4                         | 0    | 11    | 55   | 0    | 6    | 65   | 0    | 16   | 49   |
| A       | 10                       | 1    | 5     | 57   | 2    | 4    | 53   | 0    | 9    | 58   |
| B       | 10                       | 1    | 3     | 60   | 1    | 2    | 55   | 0    | 5    | 59   |
| C       | 10                       | 1    | 10    | 54   | 1    | 0    | 48   | 3    | 4    | 62   |
| A       | 25                       | 7    | 5     | 43   | 3    | 5    | 42   | 0    | 4    | 52   |
| B       | 25                       | 5    | 6     | 47   | 7    | 5    | 43   | 3    | 5    | 54   |
| C       | 25                       | 2    | 5     | 48   | 5    | 1    | 48   | 4    | 5    | 52   |

* Inoculum: 1 ml of a 10−⁶ fecal suspension. Media were in 500-ml Erlenmeyer flasks with screw caps.

* Metabolites. Preg, Pregnanolone.

* Yield in percent.
(Table 2). THDOC was the principal metabolite, with higher yields in BHIC than in TSB. Small amounts of pregnanolone were produced in BHIC. In control cultures, DOC was quantitatively 21-dehydroxylated in BHIC, whereas 9% was recovered as THDOC from TSB.

**pH.** BHIC media with initial pH ranging from 5.0 to 10.0 were inoculated with a 10^-4 fecal suspension (Table 3). Bacterial growth was luxuriant, and DOC (16 μg/ml) was quantitatively 21-dehydroxylated at initial pH between 6.0 and 8.0. At pH values 5.0, 9.0, and 10.0, growth was scanty and DOC was not metabolized. pH decreased during incubation, but a final pH as low as 5.6 did not interfere with the 21-dehydroxylation of DOC.

**Concentration of substrate.** BHIC with various concentrations of DOC were incubated for 7 days with 1 ml of a 10^-1 to 10^-7 fecal suspension from a normal subject. Figure 3 shows that the concentration of substrate influenced the production of metabolites. At 256 μg of DOC per ml of medium, close to saturation, THDOC was the principal metabolite and only small amounts of DOC and pregnanolone were recovered. At progressively lower concentrations of DOC, the yield of THDOC decreased whereas that of pregnanolone increased to a maximum at a substrate level of 64 μg/ml. At still lower concentrations of substrate, the yield of pregnanolone decreased concomitantly with the appearance of an unknown metabolite.

**Dilution of fecal inoculum.** In an attempt to distinguish between the metabolism of DOC by a mixture of aerobic and anaerobic organisms on one hand and obligatory anaerobes on the other, both BHIC and PR were seeded with progressively diluted fecal suspensions from a normal donor. After 7 days of incubation, the cultures were examined for aerobic and anaerobic organisms by routine techniques employed in clinical laboratories. A typical example of this kind of experiment is recorded in Table 4. BHIC supported growth of fecal suspensions 10^-1 to 10^-7. The cultures consisted of both aerobic and anaerobic organisms. DOC was 21-dehydroxylated by cultures up to 10^-7 suspensions. The last BHIC vial with growth (10^-7) usually yielded metabolite X, but on occasions small amounts of pregnanolone were recovered.

**PR medium supported growth of fecal suspensions to 10^-18 to 10^-11. Cultures 10^-1 to 10^-7, containing both aerobic and anaerobic organisms, 21-dehydroxylated DOC. As the cultures changed to obligatory anaerobic flora at 10^-7, the metabolism of DOC switched from preg-
Three metabolites were stored for 4 h or longer. THDOC was the main metabolite from media containing 128 μg of DOC per ml, regardless of the duration of storage of feces. Although not shown in Table 4, essentially similar results were obtained with 10⁻⁷ fecal suspensions whether incubated with 16 or 128 μg of DOC per ml.

**Pure cultures.** Pure cultures of aerobic organisms from human feces were isolated and identified with conventional techniques. Obligatory anaerobes were recovered from anaerobically incubated blood agar plates streaked with 10⁻⁴ to 10⁻¹⁰ fecal suspensions. Speciation was done by L. Holdeman, Virginia Polytechnic Institute, Blacksburg, Va. The strains were incubated with DOC (16 μg/ml) for 1 week. Whereas neither pregnanolone nor THDOC was produced, two other metabolites, X and Y, were recovered (Table 6). Some of the aerobic organisms converted DOC to metabolite Y. Others

| Log₁₀ fecal suspension | Organisms | Media (50 ml)* | Organisms | Media (50 ml)* |
|------------------------|-----------|---------------|-----------|---------------|
| Aérobies               | Anaérobies| Pregnanolone  | DOC       | X             |
|                        |           |               |           | Pregnanolone  |
|                        |           |               | DOC       | X             |
| -1.0                   | +         | +             | -         | V*            |
| -3.0                   | +         | +             | -         | V             |
| -6.0                   | +         | +             | -         | V             |
| -7.0                   | +         | +             | -         | V             |
| -7.5                   | +         | +             | V         | +             |
| -8.0                   | -         | +             | NG*       | NG            |
| -8.5                   | -         | +             | NG        | NG            |
| -9.0                   | -         | +             | NG        | NG            |

* DOC: 16 μg/ml; inoculum: 0.25 ml fecal suspension; incubation: 7 days. Growth occurred in PR up to log -11, but DOC was not metabolized.

* V, Variable.

* ND, Not done.

* NG, No growth.

**Table 4. Metabolism of DOC by progressively diluted fecal suspensions**

**Table 5. Survival of DOC-metabolizing bacteria in feces stored aerobically at room temperature**

| Metabolites | DOC (μg/ml)* |
|-------------|--------------|
|             | 0.5 h | 4 h | 24 h | 0.5 h | 4 h | 24 h |
| DOC         | 0*    | 5    | 4    | 0    | 4   | 5    |
| THDOC       | 0     | 30   | 30   | 46   | 40  | 43   |
| Pregnanolone| 51    | 15   | 17   | 5    | 0   | 0    |
| Unknown X   | 0     | 7    | 23   | 3    | 2   | 0    |

* BHIC: 200 ml; inoculum: 1 ml of a 10⁻³ fecal suspension; incubation: 7 days.

* Hours of storage.

* Yield in percent.
TABLE 6. Metabolism of DOC by pure cultures

| Bacterial strains       | Media* | Metabolites          |
|-------------------------|--------|----------------------|
|                         |        | DOC X Y               |
| *Escherichia coli       | BHIC   | + - +                 |
| Enterococcus sp.        | BHIC   | + - +                 |
| Klebsiella sp.          | BHIC   | + - +                 |
| Proteus mirabilis       | BHIC   | + - +                 |
| Staphylococcus aureus   | BHIC   | + - +                 |
| Bacteroides fragilis*   | PR     | - + +                 |
| Bacteroides fragilis    | PR     | - + +                 |
| subsp. ovatus           | PR     | - + +                 |
| Bifidobacterium         | PR     | (+) + -               |
| adolescentis            |        |                      |

* BHIC: 200 ml; DOC, 16 µg/ml; inoculum, 1 ml of 24-h broth culture; incubation 7 days. PR: 50 ml; DOC and incubation as for BHIC; inoculum, 0.25 ml of 24-h broth culture.

* Subspecies unidentifiable.

did not metabolize DOC. All anaerobic strains converted DOC to metabolite X and two also produced metabolite Y.

*Escherichia coli* and *Enterococcus* incubated in PR failed to metabolize DOC.

**DISCUSSION**

The results of our experiments provide further evidence in support of the concept that intestinal microflora participate in the metabolism of steroids originating in the host. The human intestine, like the intestine of rodents, harbors microorganisms capable of 21-dehydroxylation of corticoids in vitro. These organisms are probably responsible for the 21-dehydroxylation of corticoids observed in humans in vivo (6, 21, 24). Thus, the presence of 21-dehydroxylated metabolites of corticoids in human urine can be explained by postulating that, after hepatic metabolism, conjugation, and biliary excretion, the biliary steroids undergo deconjugation and bacterial 21-dehydroxylation in the intestine, reabsorption, possibly further metabolism such as reduction of the 20-ketone, conjugation, and finally urinary excretion. If it is true, as these experiments imply, that the intestinal flora are responsible for 21-dehydroxylation of corticoids in humans, it follows that hepatic metabolites of aldosterone must be excreted in bile. Experiments specifically designed to determine whether metabolites of aldosterone do indeed undergo biliary excretion would provide a further test of our hypothesis.

The steroid chosen as the model substrate, DOC, does not in every respect represent the steroids which are present in bile. Unlike DOC, the biliary steroids are both reduced in ring A to tetrahydro derivatives and conjugated, mainly with sulfuric acid, glucuronic acid, or both (25). Incubation of DOC with the fecal flora yielded both 21-dehydroxylated and ring A-reduced products. Many of our experiments showed that the reduction of ring A proceeded independently of 21-dehydroxylation; for example, in high concentrations of DOC and in aerated cultures, THDOC was the main metabolite. Whether or not THDOC is a precursor for pregnanolone is unknown.

In addition to the above steroids, two unknown metabolites, X and Y, were observed. Unknown X was recovered from pure cultures of anaerobic organisms (Table 6) in yields of 30 to 90% and from cultures of fecal suspensions in yields of 5 to 30%. The yield increased with the storage of the fecal specimen (Table 5) and with the dilution of the substrate (Fig. 3). Unknown X had a chromatographic mobility similar to that of DOC, did not absorb at 250 nm, and did not reduce blue tetrazolium. The infrared spectrum showed characteristic absorption bands for both hydroxyl and carbonyl functions and confirmed the absence of an α,β-unsaturated ketone.

Unknown Y appeared irregularly, was unrelated to the duration of storage of the specimen, and was produced in yields of 5 to 19%. Unknown Y appeared in fractions 90 to 120 and was thus a highly polar structure probably possessing three hydroxyl groups.

Whether or not types and yields of metabolites are characteristic for the fecal flora of the individual, which in turn may be related to dietary habits (20), has not been established.

The experiments demonstrated that the types of metabolites produced were influenced by the concentration of substrate. Similar observations were made by Midtvedt and Norman (28), who studied the bacterial 7-dehydroxylation of cholic and chenodeoxycholic acid. The mechanism whereby the substrate exerts its controlling effect is unknown.

The types and yields of metabolites were unrelated to the concentration of fecal suspension below $10^{-7}$. The cultures consisted of mixed aerobic and anaerobic flora, and only these cultures yielded pregnanolone. Yields increased with increased freshness of the specimens, suggesting that 21-dehydroxylation bacteria are moderately sensitive to oxygen. Cultures of fecal suspension $10^{-4}$ to $10^{-8}$, containing anaerobes only, invariably yielded metabolite X and occasionally also metabolite Y, but never THDOC or pregnanolone. Our experiments did
not provide evidence for the contention that aerobic organisms participated actively in the 21-dehydroxylation of DOC. It seems more likely that the role of aerobes is to provide a suitable environment for the organisms that are actually responsible for the conversion.

It is noteworthy that cysteine hydrochloride was a component of most media supporting growth of 21-dehydroxylating organisms. TSB was the exception. However, due to the enzymatic hydrolysis of proteins employed in its preparation, cysteine may well be present in TSB. On the other hand, as exemplified by thioglycolate broth, the presence of cysteine is not enough to assure that a medium will support 21-dehydroxylation.

Organisms responsible for 21-dehydroxylation of DOC have not yet been recovered in pure cultures. A variety of bacteria (e.g., Enterobacteriaceae, Pseudomonas species, staphylococci, corynebacteria, etc.) (4) are capable of reducing the keto group. Other organisms, e.g., Bacillus putrificus, Clostridium parputrificum, and Streptomyces, are capable of reducing the double bond in ring A (4). Thus, the ring A reduction of DOC can conceivably be attributed to one or more of the above-mentioned organisms, all common members of the copro flora. We have been unable to find references to bacterial species with 21-dehydroxylating capacity. This reductive reaction was not observed with selected anaerobic strains grown with DOC in PR broth.

Most of the reactions reported, with the exception of cleavage of conjugates, are reductions. The lumen of the gut contains little oxygen, probably due to the metabolism of facultative anaerobes. The flora consists largely of obligate anaerobes. Under anaerobic conditions many facultative and obligate anaerobes satisfy their requirements for energy mainly by fermentation, a process which generates reducing equivalents. Generally, some metabolic intermediate acts as an electron acceptor and the reduced product is then excreted. When exogenous electron acceptors can be used, fermentation is rendered more efficient. Both ring A reduction and dehydroxylation are reactions in which the steroid is an exogenous electron acceptor.

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