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FORMATION AND METABOLISM OF TETRAHYDRODEOXYCORTICOSTERONE BY HUMAN FECAL FLORA

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SUMMARY

Incubation of deoxycorticosterone (DOC) with human fecal flora results in the formation of a variety of products depending on the experimental conditions. Fecal flora, diluted 10^1 to 10^7, reduced DOC (16 μg/ml) to THDOC which was further metabolized to 3α-pregnanolone. Small amounts of 3α-pregnanolone, in turn, were transformed to pregnandione and 3β-pregnanolone. Another structure, tentatively identified as 20ξ,21-dihydroxy-5ξ-pregnan-3 one (metabolite X) was often formed in yields of 5–10% in the early phases of incubation. These findings were corroborated in experiments using purified THDOC and purified pregnanolone as substrates.

Clostridium paraputrificum reduced DOC and progesterone in ring A in yields of 80%. Many nonsporing anaerobes also reduced DOC in ring A but in yields of less than 8%. Some strains of anaerobic organisms transformed DOC to metabolite X which appeared to be an endproduct. Organisms responsible for 21-dehydroxylation have not yet been isolated.

INTRODUCTION

21-Dehydroxylation, a feature of metabolism of corticoids in humans, appears to be restricted to those corticoids which undergo biliary excretion. Ring A—saturated and conjugated metabolites derived from DOC* and compounds A and B by hepatic metabolism are excreted into the bile [1–3]. DOC has been shown to be a precursor of urinary 3α,20α-dihydroxy-5β-pregnane glucuronide [4, 5] and compounds A and B are precursors of urinary 3α,20α-dihydroxy-5β-pregnan-11 one glucuronide [1–3]. Although information concerning the biliary excretion of aldosterone is lacking, this corticoid is the precursor of urinary 3α-hydroxy-5β-pregnan-(11β,18S)(18S,20α)-dioxide glucuronide [6, 7]. On the other hand, metabolites of cortisol are not significantly excreted in bile [8, 9], and Fukushima and Gallagher[10] have demonstrated that cortisol does not undergo 21-dehydroxylation.

Biliary steroids are metabolized by bacterial enzymes in the gut of many mammalian species. This was conclusively demonstrated by comparison of steroids excreted in the feces of germfree rats with those in feces from conventional rats [11–15], together with studies of metabolism of steroids in mixed cultures of fecal and coecal flora of rats [16–19]. Details of the bacterial metabolism of steroids in various animal species were recently reviewed by Taylor[20]. The ensuing metabolites may be excreted in the feces or returned to the liver for further metabolism or conjugation. They may then be passed on to the blood for renal excretion, or they may be returned to the bile, undergo several additional enterohepatic circulations and be further metabolized. There is no clear understanding of what determines the routes of excretion of a particular steroid. These routes of excretion vary from steroid to steroid and from species to species.

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 [21]. 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 the bile.

* Systematic names of the steroids referred to in the text by their trivial names or abbreviations are as follows: Deoxycorticosterone (DOC), 21-hydroxy-4-pregnene-3,20 dione; tetrahydrodeoxycorticosterone (THDOC), 3α,21-dihydroxy-5β-pregnan-20 one; 3α-pregnanolone, 3α-hydroxy-5β-pregnan-20 one; 3β-pregnanolone, 3β-hydroxy-5β-pregnan-20 one; pregnanedione, 5β-pregnane-3,20 dione; compound A, 21-hydroxy-4-pregnene-3,11,20 trione; compound B, (corticosterone) 11β,21-dihydroxy-4-pregnene-3,20 dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20 dione.

Dehydroxylation: substitution of a hydroxy group by a hydrogen atom.

We have recently described [22] the conditions under which mixed cultures of human fecal flora 21-dehydroxylate DOC. A number of metabolic products were formed but their inter-relationship is unknown. The experiments reported here were designed to elucidate the metabolic pathways of DOC by human fecal flora and pure bacterial cultures. In addition to its intrinsic value, clarification of the metabolic pathways should also provide answers of importance to the isolation in pure culture of 21-dehydroxylating organisms.

MATERIALS AND METHODS

Media

BHIC broth: Dehydrated brain heart infusion broth (Baltimore Biological Laboratories; Becton, Dickinson & Co., Cockeysville, MD) was made up according to the instructions of the manufacturer. It was supplemented with 0.5 g cysteine HCl and 1 g $NaHCO_3$ per l. medium, distributed in 200 ml amounts in 500 ml Erlenmeyer flasks, and sterilized at 121°C for 20 min. Studies of the kinetics of the metabolism of DOC were done in 1600 ml medium in a 2000-ml flask with a low set side arm and a magnetic stirrer.

PR broth: 45 ml prereduced BHIC in 60 ml bottles was kindly donated by the Scott Laboratories, Inc., Fiskeville, RI.

Supplemented BHIC: To 100 ml BHIC were added yeast extract (Difco Laboratories, Inc.) 0.75 g, Tween 80 (Rohm & Haas, Philadelphia, PA) 0.1 g, celite filtered supernatant of tomato juice (Sacramento) 10 ml, and phosphate salts to a molarity of 0.1 and a pH of 6.9. The medium was distributed in 50 ml amounts in 100 ml rubber stoppered vials and sterilized.

SPB (18 ml supplemented peptone broth, in closed tubes) and thiogylcollate broth were purchased from Becton, Dickinson & Co., Cockeysville, MD.

EMB plates (eosin methylene blue agar) and CNA plates (colistin nalidixic agar) were prepared from the corresponding dehydrated media purchased from the Difco Laboratories, Inc.

BAP (sheep blood agar plates) were obtained from Scott Laboratories, Inc.

pH. pH was measured on a Beckman Zeromatic II.

Shake cultures. Cultures were incubated aerobically in flasks on a reciprocating shaker at 100 oscillations/min, 38 mm stroke.

Sources of micro-organisms

(a) *Fecal flora.* Fecal samples from healthy adults on the usual Western diet were collected in stool cups under ordinary atmospheric conditions. Processing began within 30 min of defecation [22]. Using syringes the specimens were decimally diluted in SPB.

(b) *Pure cultures isolated in our laboratories.* Diluted fecal material was streaked on EMB, CNA, and BAP. EMB and CNA were incubated aerobically at 37°C for 24 h. Different colonies were picked, identified by conventional bacteriological technique, and checked for ability to metabolize steroids. The BAP was incubated anaerobically in Gas Pak jars (Becton, Dickinson & Co.) at 37°C for 48 h. Different colonies were picked and tested for gaseous requirements. Facultative anaerobes were identified as described above; non-sporing, obligatory anaerobes were identified by Dr. L. V. Holdeman, Virginia Polytechnic Institute, Blacksburg, VA, according to the methods developed in her laboratories [23]. To recover spore forming organisms, a 10^{-2} suspension of feces in thioglycollate was left at 4°C for 7 days and then placed in a water bath at 100°C for 5 min. For germination 0.5 ml of the suspension was transferred to PR and incubated at 37°C for 2 days. The culture was streaked on BAP, and incubated in Gas Pak jars at 37°C for 2 days; morphologically different colonies were picked and examined for ability to metabolize steroids. Relevant strains were identified by Dr. L. V. Holdeman.

(c) *Bacterial strains received from collections.* The following scientists kindly donated cultures from their collections. Dr. L. V. Holdeman: 13 pure strains from jejunum of man representing more than 95% of the flora in that area. Dr. V. L. Sutter, V. A. Hospital, Los Angeles: *Lactobacillus acidophilus.* And Dr. V. M. Young, Baltimore Cancer Research Center, Baltimore, MD: 3 unspeciated strains of *Lactobacillus* from gingiva and throat. Three strains of *Lactobacillus* were purchased from American Type Culture Collection (ATCC).

Solvents.

The solvents were reagent grade except for methanol which was technical grade. Solvent systems are listed in Table 1.

Labeled steroids.

[1,2-^{3}H]-DOC and [1,2-^{3}H]-progesterone were purchased from New England Nuclear Corp., Boston, MA. The steroids were at least 97% pure by isotopic dilution analysis. Prior to incubation the carrier was mixed with radioactive steroid to 2×10^6 c.p.m./culture.

Table 1. Solvents for chromatography

System	Components (volumetric ratio)
A*	Gradient: heptane–ethylene dichloride
	Ethylene glycol–water 9:1 v/v (s)
B	Isooctane–benzene 85:15 v/v (m)
	propylene glycol (s)
C	Heptane–methylcellusolve

* 5 g of celite containing 2.5 ml of water were packed into the column before the 15 g of celite containing 7.5 ml of ethylene glycol–water 9:1 [26]. The apparatus for generation of the gradient and the characteristics of the gradient have been described previously [27].

s = stationary phase.

m = mobile phase.

[1,2-^{3}H]-THDOC was prepared in our laboratories by biosynthesis from DOC as previously described [22]. Briefly, 2000 ml BHIC with 500 mg DOC and 2×10^9 c.p.m. were seeded with 10 ml 10^{-3} fecal suspension and incubated at 37°C for 7 days. Extracted steroids were fractionated by partition chromatography on celite columns as previously described [22]. The combined THDOC fractions were further purified by t.l.c. on 250 μm 20 × 20 silica gel plates using a solvent system isooctane–ethyl acetate–acetic acid (5:25:0.2 by vol.). The resulting product was identified as THDOC by t.l.c. (R_F values, blue tatrazolium reaction, U.V. absorption) and the I.R. spectrum.

[1,2-^{3}H]-Pregnanolone was prepared in a similar fashion except for the following modifications. (a) The concentration of DOC in the medium was 16 μg/ml [22]; (b) following partition chromatography on system A (Table 1), the fractions containing pregnanolone were combined and rechromatographed on system B. The resulting crystalline product was identified as 3α-pregnanolone by t.l.c. and IR spectra of the steroid and its acetate [24].

Incubation of steroids

Methanolic solutions of steroids were added to the sterilized media to a concentration of 16 μg steroid per ml medium [22] and 0.5% methanol (v/v). The media were then seeded with appropriately diluted fecal flora, or with suspensions of pure, young bacterial cultures in the proportion 20:0.1. The cultures were incubated at 37°C.

Separation of steroids

Extraction, partition and t.l.c., determination of radioactivity, and quantitation of yield, were done as previously described [22].

Identification of steroids

The isolated steroids were identified as previously described [22]. Briefly, R_F values (3β-pregnanolone: 0.46 ± 0.02; 3α-pregnanolone: 0.41 ± 0.02; metabolite X: 0.36 ± 0.02; THDOC: 0.28 ± 0.03), blue tetrazolium reaction, and absorption of U.V. light were determined on a t.l.c. plate in our solvent system. 3α-Pregnanolone and 3β-pregnanolone were crystallized and identified by comparison of their infrared spectra with those of authentic steroids [24]. THDOC, resisting crystallization, was dissolved in methylene chloride and the I.R. spectrum was compared with that of an authentic sample. Pregnandione was identified by co-crystallization of labeled metabolite with unlabeled carrier to afford the same specific activity in successive crystals and the final mother liquor residue.

RESULTS

(I) *Bacterial metabolism of DOC*

Our previous work [22] shows that DOC is metabolized to THDOC, to 3α-pregnanolone, and to an unidentified product designated metabolite X.

Kinetics. 1600 ml BHIC with DOC (16 μg/ml) was seeded with 8 ml 10^{-3} fecal suspension and incubated at 37°C. Samples were collected after 18 and 24 h, and then at daily intervals. Results from a typical experiment are shown in Fig. 1. Within 18 h DOC was metabolized to THDOC (65%) and metabolite X (10%). The recovery rate of labeled steroids was 75%. As the concentration of THDOC began to decrease after 24–48 h of incubation, pregnanolone appeared. Over the next 5-6 days, the yield of pregnanolone increased to about 70%, with a corresponding decline in the concentration of THDOC. The yield of metabolite X remained essentially unchanged at 10%. Sometimes pregnanolone was observed as early as the 2nd day and in yields as high as 60–70%. However, regardless of its time of appearance or its rate of production, pregnanolone was always formed later than THDOC.

Metabolite X. Since metabolite X occurred frequently in earlier studies with anaerobic cultures [22], and appeared in low but significant yields in the above experiments, efforts were made to identify the steroid. Its polarity was similar to that of DOC; it was blue tetrazolium negative and the I.R. spectrum revealed the presence of a carbonyl group. On the basis of this evidence the structure 20ξ,21-dihydroxy-5ξ-pregnan-3 one was tentatively assigned. Its I.R. spectrum differed from that of the 5β,20β isomer. Thus the stereochemistry at 5 and 20 remains to be firmly established.

3β-Pregnanolone. Usually a small peak, more mobile than 3α-pregnanolone, was eluted from system A, often as a shoulder on the 3α-pregnanolone peak. Rechromatography of the combined fractions from both peaks on system B clearly revealed the presence of two compounds: 3α-pregnanolone and a faster moving component identified as 3β-pregnanolone (Fig. 2).

Pregnandione. The first three fractions eluted from system A usually contained radioactivity. Upon rechromatography on system C a steroid identified as pregnandione was separated.

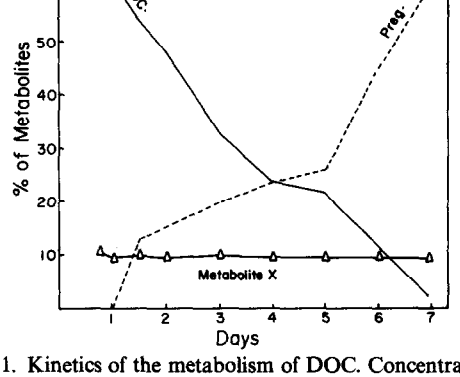

Fig. 1. Kinetics of the metabolism of DOC. Concentration of substrate: 16 μg DOC/ml; media: BHIC 1600 ml; inoculum: 8 ml 10^{-3} fecal suspension.

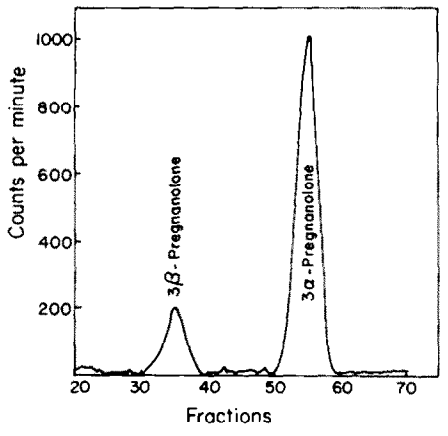

Fig. 2. Separation of 3α-pregnanolone and 3β-pregnanolone on a celite column using isooctane: benzene (85:15, v/v), as the mobile phase and propylene-glycol as stationary phase.

Organisms metabolizing DOC. Conversion of DOC to THDOC or pregnanolone is not a function of the most common aerobic or anaerobic fecal bacteria [22]. However, our experiments clearly demonstrated that converting organisms must be present in the fecal flora.

(a) Clostridia. Of four *Clostridium* species recovered from the fecal flora, three did not metabolize DOC (PR medium) and one ring-A reduced DOC quantitatively to THDOC. The presence of the 21-OH group was not necessary for ring-A reduction since the culture also transformed progesterone to pregnanolone. Dr. L. V. Holdeman identified the organism as *Clostridium paraputrificum.*

(b) Lactobacillus. Since *Lactobacillus* species grew poorly in conventional media, supplemented BHIC (pH 6.9) was used in metabolic experiments. Not only did it support good growth of *Lactobacilli*, but it also permitted 21-dehydroxylation of DOC by mixed fecal flora. *L. acidophilus*, *L. arabinosus* (ATCC No. 8014), *L. caseii* (ATCC No. 9595), *L. caseii* (ATCC No. 7469) and 3 unspeciated *Lactobacillus* strains from gingiva and throat, failed to metabolize DOC. Only *L. leichmanii* yielded 1.8-2.9% THDOC but no pregnanolone.

(c) *Human jejunal flora.* Since biliary steroids enter the duodenum, we speculated that bacteria representative of the upper gut might be enzymatically equipped to metabolize DOC. Thirteen strains, representing 95% of the jejunal flora, were tested in PR. None of the organisms metabolized DOC to pregnanolone, but THDOC in yields of 1–8% was obtained from *Eubacterium aerofaciens*, *Eubact. aerofaciens III*, *Bacteroides ruminicola brevis*, *Bacteroides fragilis distasonis*, *Bifidobacterium adolescentis*, *Bifidobact. longum*, *Peptostreptococcus I*, *Coccus EC* (similar to *Peptostrep. productus*) and from *Streptococcus mitis*. *Bacteroides fragilis vulgatus* did not produce THDOC, but formed two other unidentified metabolites in low yields. The metabolic activity of *L. acidophilus* and *L. leichmanii*—both members of the jejunal flora -was described above.

(d) *Mixtures of bacterial strains.* Metabolism of DOC to pregnanolone could conceivably be the joint effort of two or more bacterial species, the individuals of which showed little or no metabolic activity. To test this hypothesis, a non-metabolizing *E. coli*, two non-metabolizing *Streptococci*, and a *Bacteroides* species "Q" (Holdeman) transforming DOC to metabolite X, were grown together in various combinations in PR and BHIC. No synergistic effect on steroid metabolism was observed.

A mixed culture of 10^8 organisms each of *E. coli* (facultative anaerobe; does not metabolize DOC), *Cl. paraputrificum* (anaerobe; reduces DOC to THDOC), and *B. fragilis* (anaerobe; converts DOC to metabolite X) was incubated with DOC. Under both aerobic (BHIC) and anaerobic (PR) conditions DOC was reduced to THDOC and to metabolite X. If *E. coli* was eliminated from the bacterial mixture, there was no growth in the BHIC. Thus, *E. coli* modified the BHIC to permit growth of the obligatory anaerobes with subsequent transformation of DOC. 21-Dehydroxylation was not observed.

(II) Bacterial metabolism of THDOC

A series of experiments was conducted to determine if THDOC could be an intermediate between DOC and pregnanolone.

Kinetics. 1600 ml BHIC with THDOC (16 μg/ml) was seeded with 8 ml 10^{-3} fecal suspension. Incubation, sampling and analysis were done as described for DOC. Results from a typical experiment are shown in Fig. 3. The conversion of THDOC to pregnanolone began within a few hours and was completed in less than 48 h. Neither metabolite X nor any other metabolite was observed. On occasions the transformation took 3–4 days, but the product was always the same. Rechromatography on system B of

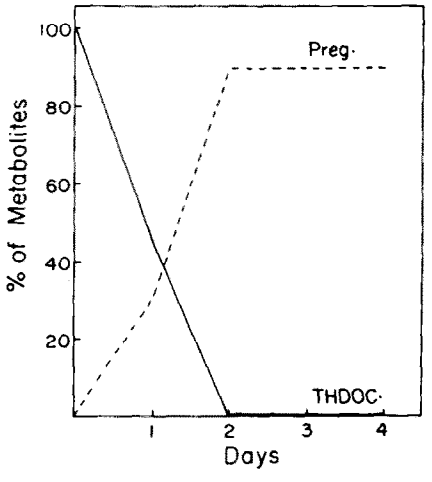

Fig. 3. Kinetics of the metabolism of THDOC. Concentration of substrate: 16 μg THDOC/ml; media: BHIC 1600 ml; inoculum: 8 ml 10^{-3} fecal suspension.

the product revealed the presence of both 3α-pregnanolone and 3β-pregnanolone.

Progressively diluted fecal suspensions. To determine the concentration of 21-dehydroxylating organisms in fecal flora, decimally diluted suspensions were seeded on aerobic (BHIC) and anaerobic (PR) media with THDOC, 16 μg/ml. After incubation for 7 days at 37°C the steroids were extracted and identified. Table 2 shows that the number of aerobic and anaerobic bacteria per gram feces was 10^6 and $>10^9$ respectively. Moreover, THDOC was 21-dehydroxylated in BHIC by fecal suspensions up to 10^{-6}, but was not completely metabolized in PR.

Shake cultures. Since the above experiments suggested that 21-dehydroxylating organisms might be aerobic, BHIC with THDOC (16 μg/ml) was seeded with 10^{-3} and 10^{-6} fecal suspensions and incubated aerobically on a shaker. Pregnanolone was not formed under these conditions. THDOC was metabolized to an unidentified, blue tetrazolium positive, product with a greater mobility than THDOC. Conventionally incubated cultures (controls) yielded pregnanolone.

Pure cultures. THDOC was not metabolized by the following recently isolated organisms: 10 strains of *E. coli*, *Lactobacillus acidophilus*, *L. arabinosus* (ATCC No. 8014), *L. caseii* (ATCC No. 9595), *L. species* (Young No. 2424), *Bact. fragilis*, *Cl. paraputrificum*, or by an unseparated mixture of fecal clostridia. A mixture of 7 enterococci transformed THDOC to an unknown metabolite with the same characteristics as described under shake cultures.

(III) *Bacterial metabolism of pregnanolone*

To determine if 3α-pregnanolone undergoes further metabolism, 200 ml BHIC with purified 3α-pregnanolone (16 μg/ml) was seeded with 1 ml 10^{-3} fecal suspension, incubated and extracted. Approximately 70% of the 3α-pregnanolone was recovered unchanged, but 15% of the substrate had been converted to pregnandione and 10% to 3β-pregnanolone.

DISCUSSION

The steroid metabolites. Except for metabolite X, the major metabolites encountered in our experiments were identified. Metabolite X appears to be a 20ξ,21-dihydroxy-5ξ-pregnan-3 one. Since all other metabolites recovered in these experiments were 5β, it is apparent that the organisms lack significant amounts of a C-21-steroid-5α-reductase. Thus it is very likely that the configuration of C-5 is β. The I.R. spectrum of metabolite X differed from that of the 5β,20β isomer; the most probable structure, therefore, is 20α,21-dihydroxy-5βpregnan-3 one.

Pathways. Our experiments demonstrate that in aerobically incubated BHIC mixed fecal flora metabolize DOC to pregnanolone *via* the intermediate THDOC (Fig. 4). This conclusion is based on two observations. First, DOC was invariably metabolized to THDOC before pregnanolone appeared. Second, purified THDOC was quantitatively converted to pregnanolone. However, one cannot draw the conclusion that ring-A reduction is a prerequisite for 21-dehydroxylation. It is conceivable that ring-A reducing organisms in the mixed culture are the faster growing organisms and convert DOC to THDOC before the 21-dehydroxylating organisms have multiplied sufficiently to influence the metabolic products.

On the other hand it is clear that the 21-OH group is not needed for the ring-A reduction as demonstrated in the experiments using progesterone as substrate.

Three 21-deoxy steroids are formed from DOC or THDOC, namely 3α-pregnanolone, 3β-pregnanolone and pregnandione. 3α-THDOC is the precursor for 3α-pregnanolone, whose oxidation by bacterial flora to pregnandione was demonstrated. Further, the conversion of 3α-pregnanolone to 3β-pregnanolone was also demonstrated. It is likely that this epimerization proceeds *via* oxidation of the alcohol at C-3 followed by reduction of the resultant ketone. There is an alternative possibility for the formation of 3β-pregnanolone. It could be derived from small amounts of 3β-THDOC produced from DOC along with the major metabolite 3α-THDOC, although the presence of 3β-THDOC has not been demonstrated.

In PR medium a number of *Bacteroides* strains converted DOC to yet another product: metabolite X [22], a compound which was also observed in low and constant yields by mixed fecal flora in BHIC. This product was not available in sufficient quantities to permit further metabolic experiments. However, the fact that the yield changed little during a prolonged incubation (Fig. 1), indicates that it is resistant to further bacterial metabolism, which is in agreement with the findings by Eriksson *et al.* [16].

Modifying factors. The outcome of the *in vitro* metabolism of DOC is influenced by a variety of factors. The metabolism will take place in certain media but not in others and within the pH range 6–8 [22]. The concentration of the substrate is decisive [22]. The gaseous environment also plays a role in the

Table 2. Metabolism of THDOC by progressively diluted fecal suspensions

Log_{10} fecal suspension	Media*		Metabolites (%)	
	BHIC	PR	THDOC	Pregnanolone
−3	+†	+	0	75
−6	+	+	0	83
−7	NG‡	+	82	0
−8	NG	+	76	0
−9	NG	+	81	0

* Supplemented with THDOC, 16 μg/ml. Inoculum 1 ml fecal suspension.

† + = growth.

‡ No growth. The first dilution without bacterial growth is indicative of the number of aerobic organisms per gram feces.

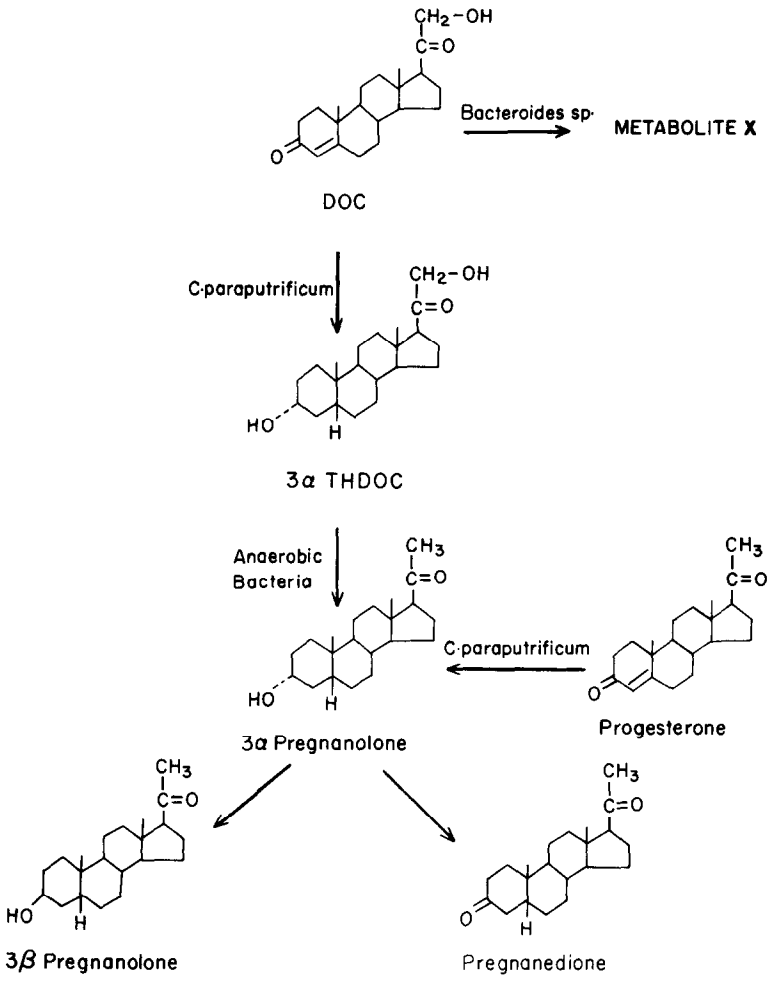

Fig. 4. Proposed pathways for the bacterial metabolism of DOC. All transformations were observed in experiments with mixed fecal flora. Bacterial species or families capable of performing specific conversions are also shown.

metabolism. For example, incubation of 10^{-3} fecal flora in BHIC resulted in high yields of pregnanolone which could be completely abolished by increased aeration of the culture such as incubation on a shaker [22]. On the other hand, if the medium and atmosphere were absolutely free of oxygen (PR), mixed fecal flora converted DOC to metabolite X sometimes together with pregnanolone in low yields. If the gas mixtures in PR were supplemented with 0.5 ml O_2 (2% O_2 in the atmosphere), pregnanolone was the major metabolite. The effect of the gaseous environment on the steroid metabolism is highly suggestive of an Eh dependent bacterial competition.

In other experiments we noted signs of bacterial collaboration. If *E. coli*, which cannot metabolize DOC, was grown in BHIC together with *Cl. paraputrificum* and *B. fragilis*, metabolic products characteristic for the two anaerobes were formed. When *E. coli* was removed from the bacterial mixture, BHIC was no longer a suitable medium for the anaerobes, and DOC was not metabolized. Since the two anaerobes grown together in PR form their typical metabolic products, it may be assumed that *E. coli* exerts its influence by providing a suitable Eh.

Pure cultures. Cl. paraputrificum, an organism known to transform DOC to THDOC [25] was recovered from feces. It reduced DOC to THDOC in yields of 85% and, in addition, it metabolized progesterone to pregnanolone in similar yields. THDOC in yields of less than 8% was produced by many nonsporing anaerobic species including *L. leichmanii*. The bacterial conversion of DOC to THDOC must take some time. THDOC, therefore, may not be available as substrate for 21-dehydroxylating organisms in the earliest growth phases of mixed cultures. This could explain the slow formation of pregnanolone often observed in cultures with DOC as the substrate.

Although organisms possessing 21-dehydroxylase have not yet been isolated in pure cultures, it seems reasonable to assume that the organisms are obligatory anaerobes with a comparatively high tolerance to oxygen. This is supported by the facts that preg-

nanolone is not formed in aerobic shake cultures, that conversion requires reduced media, and that the cultural manipulations can be done in an ordinary aerobic atmosphere.

CONCLUSION

In our previous experiments we used DOC as a model for 21-dehydroxylation by fecal flora. Since DOC is not present in significant amounts in bile, it was important to determine whether DOC was an appropriate model. In this report we have demonstrated that THDOC, which is present in bile as conjugates, also undergoes 21-dehydroxylation. Since mixed cultures also reduce DOC in ring-A the specificity of the 21-dehydroxylating organisms regarding the structures of ring-A is unknown. Consequently it seems appropriate to follow isolation of these organisms by determining 21-dehydroxylation of THDOC especially since the steroid can now be prepared in labeled form in good yield by *Cl. paraputrificum*.

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