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21-DEHYDROXYLATION OF CORTICOIDS BY ANAEROBIC BACTERIA ISOLATED FROM HUMAN FECAL FLORA

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SUMMARY

Two human intestinal anaerobes, culture No. 116 and *Eubacterium lentum*, synthesize enzymes capable of metabolizing C-21 steroid hormones. 21-Dehydroxylase activity by culture No. 116 benefits from the presence of *Escherichia coli* or allied organisms in the medium; their effect appears to be a lowering of the Eh. The enzymes activity is independent of arginine and shows no sign of product inhibition. The enzyme, which is constitutive, functions at pH 6.5–7.3; its activity is unrelated to the 4-ene-3CO group but is inhibited by a C20-OH group. *E. lentum* (neotype), in addition to a 21-dehydroxylase, synthesizes a 3α-hydroxysteroid dehydrogenase active at Eh ± -150 mV, which explains the epimerization of 3α-pregnanolone observed in cultures of fecal flora. The metabolic alterations of deoxycorticosterone by cultures of fecal flora may be duplicated by *Clostridium paraputrificum* and *E. lentum*, whether acting in concert or sequentially.

INTRODUCTION

The importance of bacterial metabolism of biliary steroids has become more evident in recent years [1–3]. In many mammalian species, including man, the steroid metabolites are absorbed, conjugated, and eventually excreted in the urine. Thus, the structures of urinary and biliary steroids differ. For example, 11-dehydrocorticosterone [4] and corticosterone [5] undergo enterohepatic circulation and eventually are excreted in the urine as 3α, 20α-dihydroxy-5β-pregnan-11-one, a transformation involving the removal of the 21-OH group. In 1971 Eriksson and Gustafsson [6] demonstrated 21-dehydroxylation of 3β,21-dihydroxy-5α-pregnan-20-one with a slurry of human intestinal contents, and recently Bokkenheuser *et al.*[7, 8] reported that cultures of mixed fecal flora convert 11-deoxycorticosterone (DOC) to 3α-hydroxy-5β-pregnan-20-one (pregnanolone). The structures are shown in Fig. 4. The recent isolation of 21-dehydroxylating organisms from human fecal flora [8] provided an opportunity to determine some of the parameters controlling the formation and function of 21-dehydroxylase. The results are correlated with the molecular alterations of the biliary steroids in man.

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; dihydrodeoxycorticosterone (DHDOC), 21-hydroxy-5β-pregnane-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.

Dehydroxylation: substitution of a hydroxy group by a hydrogen atom (21-DOH).

Dehydrogenation: removal of 2 hydrogen atoms.

3α-hydroxysteroid dehydrogenase: 3α-DH.

MATERIALS AND METHODS

Media

Conventional bacteriological media were employed as previously described [7, 8]. The following special media were used: prereduced broth (*PR*, 45 ml Brain Heart Infusion broth supplemented with 0.05% cysteine HCl in 60 ml vials) from Scott Laboratories Inc., Fiskeville, R.I.; *PRA*, 45 ml PR to which was added 0.5 ml of a 60% autoclaved (121°C, 15 min) solution of arginine HCl in distilled water; *SPB* (18 ml supplemented peptone broth in closed tubes) and vials containing 50 ml thioglycollate broth from Baltimore Biological Laboratories, Division of Becton, Dickinson & Co., Cockeysville, MD.

Bacterial transformation of steroids was examined in PR and BHIC. Per liter of water *BHIC* consisted of: dehydrated brain heart infusion broth (37 g) from Baltimore Biological Laboratories; 0.5 g cysteine HCl; 1 g $NaHCO_3$; and 4 ml of 0.025% aqueous resazurin (J. T. Baker Chemical Co., Phillipsburg, NJ). For routine experiments, BHIC was distributed in 200 ml amounts in 500 ml Erlenmeyer flasks and sterilized at 121°C for 20 min. The kinetics of molecular transformation were studied by employing 1600 ml medium in 2000 ml flasks with low set side arms and a magnetic stirrer.

pH and Eh. These parameters were measured on a Beckman Zeromatic II. Eh measurements were done by a platinum electrode. The instrument was checked regularly with quinhydrone-saturated buffers

at pH 4 and 7. The readings were invariably within 10 mV of the theoretical values.

Sources of micro-organisms. a. Culture No. 116 is the first organism shown to synthesize 21-dehydroxylase. The organism was recovered in our laboratories from human feces and was found to be a gram positive, non-sporulating, obligate anaerobe; its taxonomic position is equivocal [9].

b. The neotype strain of *Eubacterium lentum* (VPI 0255), also known to elaborate a 21-dehydroxylase [9] was kindly supplied by Drs. L. V. Holdeman and W. E. C. Moore, Virginia Polytechnic Institute, Blacksburg, Virginia.

c. *Clostridium paraputrificum* was isolated in our laboratories [8].

d. Aerobic organisms (*Escherichia coli*, *Proteus*, etc.) were isolated from human stools and identified by conventional techniques.

Solvents. The solvents were reagent grade except for methanol which was technical grade. The solvent systems employed were described by Bokkenheuser *et al.*[8].

Steroids. The structures of appropriate steroids are shown in Fig. 4. [1,2-^{3}H]-deoxycorticosterone (DOC), purchased from New England Nuclear Corp., Boston, Massachusetts, was at least 97% pure by isotopic dilution analysis. [1,2-^{3}H]-tetrahydrodeoxycorticosterone (THDOC) and [1,2-^{3}H]-pregnanolone were prepared in our laboratories by biosynthesis, as previously described [8]. Unlabelled steroids were purchased from Steraloids, Inc., Wilton, NH. If not otherwise stated, carrier and radioactive steroids were mixed in methanol and added to the sterilized medium prior to incubation to give a concentration of 16 μg steroid per ml, 0.5% methanol (v/v), and 1–2 × 10^6 c.p.m. per culture [7, 8].

Incubation. Media supplemented with substrate were seeded with 24–48 h bacterial broth culture in the proportion 20:0.1. The cultures were incubated at 37°C.

Thin layer chromatography (*t.l.c.*) Five ml of culture were extracted with 5 ml methylene chloride for 1 min; the organic phase was dried over sodium sulfate and evaporated under nitrogen at 45°C. The dry residue was dissolved in 100 μl acetone and subjected to t.l.c. on Bakerflex silica gel with fluorescence indicator (no. 1B 2F; J. T. Baker Chemical Co.) as previously described [7, 8]. Quantitation of DOC and progesterone was often done from Bakerflex. The spots were located under U.V.-light, and the radioactive substance together with absorbent were scraped off and transferred to a vial. The steroids were eluted with 0.5 ml methanol and counted as previously described [7, 8].

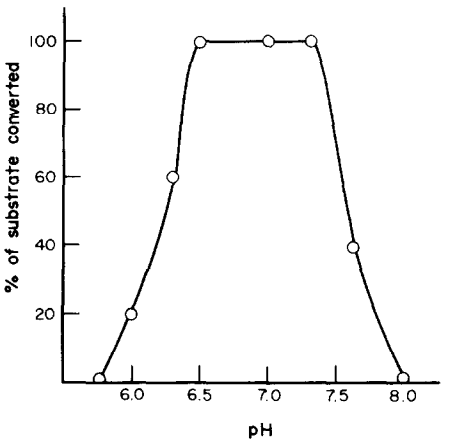

Fig. 1. 21-Dehydroxylation by culture No. 116 at different pH. Substrate: THDOC, 16 μg/ml.

Extraction, partition chromatography, quantitation and identification of steroids were done as previously described [7, 8].

RESULTS

The steroid metabolizing activities of culture No. 116, of the neotype strain of *E. lentum*, and of mixed anaerobic cultures will be described separately.

(1) *Parameters of 21-dehydroxylation by culture No. 116*

pH. The pH of the media (PR) was monitored over a range of 5.5–8.6 by using 33% CH_3COOH and 30% NaOH or 0.1 M PO_4 buffer. The medium was supplemented with THDOC (16 μg/ml), seeded with culture No. 116 and incubated. Quantitative 21-dehydroxylation was obtained with an initial pH of the conversion medium between 6.5 and 7.3 (Fig. 1). Culture No. 116 grown in PR never lowered the pH with more than 0.2 units.

Arginine. The growth of culture No. 116, like that of *E. lentum*, was significantly stimulated by the presence of 0.5–1% arginine in the medium [10]. Table 1 shows that arginine neither increased the amount

Table 1. Effect of arginine on THDOC metabolism by culture No. 116

THDOC μg/ml media	PR-arginine*			PR (control)		
		Conversion			Conversion	
	Growth†	%	Days	Growth	%	Days
16	4	100	3–4	2	100	3–4
32	4	50	3–4	2	53	3–4
64	4	26	3–4	2	26	3–4

* Prereduced medium supplemented with 0.5% arginine. † Density of growth estimated on a scale 0–4.

Table 2. Promotion of 21-dehydroxylation in BHIC by aerobic organisms

Group	Bacterial species		Final Eh (mV)*	21-Dehydroxylation
	Culture No. 116		−80	0
A	Culture No. 116	+ *E. coli*	−280	+
		+ *Klebsiella sp.*	−260	+
		+ *Proteus mirabilis*	−200	+
B	Culture No. 116	+ *Staph. aureus*	−170	0
		+ *Staph. epidermidis*	−150	0
		+ *Enterococcus*	−100	0
		+ *Ps.*† *aeruginosa*	−160	0
		+ *Ps. stutzerii*	−180	0
		+ *Acinetobacter*	−130	0
	Culture No. 116	+ *E. coli* and strains of Group B	−270	+

* Measured at the end of the incubation (Eh of the uninoculated medium: −130 ± 20 mV). † *Pseudomonas.*

of THDOC that was dehydroxylated by culture No. 116 nor accelerated its conversion rate.

Effect of aerobic organisms in the cultures. Aerobic BHIC does not support growth of obligate anaerobes. However, simultaneous seeding of the medium with culture No. 116 and *E. coli* permits good growth of, and 21-dehydroxylation by, the former organisms [9]. To determine if the effect of *E. coli* is specific, BHIC with THDOC (16 μg/ml) was seeded with culture No. 116 and a number of aerobic organisms. Table 2 shows that *E. coli*, a *Klebsiella sp.*, and *Proteus mirabilis* were potent reducers of BHIC and provided a suitable environment for the synthesis and action of 21-dehydroxylase. On the other hand, 21-dehydroxylation was not observed when culture No. 116 was grown with gram negative non-fermenting rods and a number of gram positive cocci, all of which were weak reducers. The organisms in Group B (Table 2) did not inhibit culture No. 116 since *E. coli* mixed with any one of these and culture No. 116 resulted in 21-dehydroxylation. Control experiments showed that none of the aerobic organisms listed in Table 2 possessed 21-dehydroxylase.

Growth phase of E. coli consistent with 21-dehydroxylation in aerobic medium. 200 ml BHIC containing THDOC (16 μg/ml) was inoculated with 0.1 ml 24 h broth culture of *E. coli* and incubated at 37°C. Regardless of whether culture No. 116 was added early or after one week of incubation of the *E. coli* culture, THDOC was 21-dehydroxylated quantitatively within 7 days.

Growth phases of culture No. 116 consistent with 21-dehydroxylation of DOC. BHIC was seeded with culture No. 116 and incubated at 37°C. Whether added immediately or after one week of incubation of the culture, DOC was converted quantitatively to progesterone. Culture No. 116, incubated under similar conditions in PR, partially converted DOC to progesterone.

Concentration of substrate. 21-Dehydroxylation by dilute suspensions of fecal flora depends in part on the concentration of substrate [7]. To determine if this is true also for our isolate, culture No. 116 was seeded in media with various amounts of substrates. Figure 2 shows that in PR the culture 21-dehydroxylated a maximum of 2.4 μM THDOC/50 ml, whereas the conversion capacity was increased 8-fold in BHIC simultaneously seeded with *E. coli* (BHIC-Ec) and culture No. 116.

Kinetics of the metabolism of THDOC. Culture No. 116 was seeded in PR and BHIC-Ec (1600 ml) containing THDOC, 16 μg/ml. The cultures were incubated at 37°C; 50 ml was sampled every 24 h. Figure 3 shows that the yield of pregnanolone was 80% regardless of the medium employed; however, 21-dehydroxylation occurred 2–3 times faster in BHIC-Ec than in PR seeded only with culture No. 116.

Specificity of 21-dehydroxylase. To determine if the 4-ene-3 CO configuration interferes with 21-dehydroxylation, culture No. 116 was incubated in PR with DOC and THDOC (16 μg/ml), respectively. The culture converted DOC to progesterone and THDOC to pregnanolone. Identical results were obtained in BHIC-Ec.

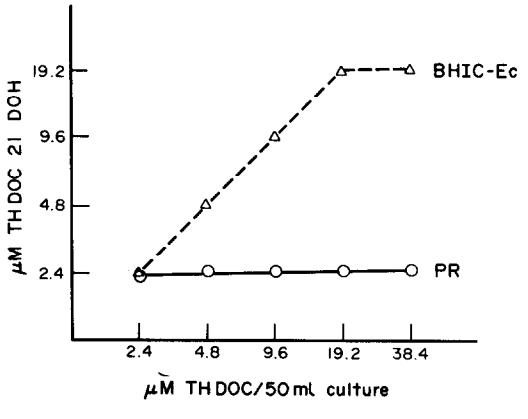

Fig. 2. 21-Dehydroxylation capacity by culture No. 116 at various concentrations of substrate.

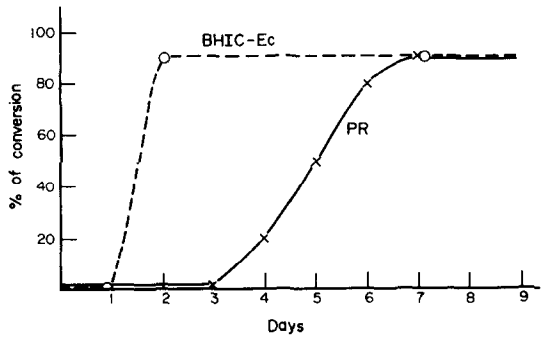

Fig. 3. 21-Dehydroxylation by culture No. 116 in PR and BHIC-Ec. Substrate: THDOC 16 μg/ml.

Metabolism of pregnanolone by culture No. 116. Culture No. 116 incubated with pregnanolone (16 μg/ml) in PR and BHIC-Ec failed to metabolize this steroid.

Product inhibition. To determine if pregnanolone inhibits 21-dehydroxylation of THDOC, culture No. 116 was incubated in both conversion systems containing THDOC (16 μg/ml) and unlabelled pregnanolone ranging from 16 to 128 μg per ml. Labelled THDOC was 21-dehydroxylated quantitatively regardless of the concentration of pregnanolone in the medium.

Constitutive enzyme. Seven passages of culture No. 116 in each of three different anaerobic substrate-free media (PR, SPB, and thioglycollate broth) did not reduce its 21-dehydroxylating capacity. Neither was the capacity affected by a year's propagation in substrate-free laboratory media.

(2) *Metabolism of THDOC by the neotype strain of Eubacterium lentum*

Because of the similarity between culture No. 116 and *E. lentum* [9], we decided to test the neotype strain of the latter for steroid metabolizing activity. Table 3 shows that the neotype strain of *E. lentum* indeed 21-dehydroxylated THDOC in BHIC-Ec. On occasions we noted a weak 3α-hydroxysteroid dehydrogenase activity (3α-DH). In PR, seeded with a pure culture of *E. lentum*, 3α-dehydrogenation of THDOC to dihydrodeoxycorticosterone (DHDOC) was the only metabolic alteration. The 21-dehydroxylation correlated with a low Eh whereas 3α-dehydrogenation was associated with a higher Eh (Table 3). Further studies showed that *E. lentum* (neotype) also 3α-dehydrogenated 3α-pregnanolone to pregnanedione; 3β-pregnanolone was formed in yields of 10–15%.

In contrast, culture No. 116 did not synthesize a 3α-DH.

(3) *Metabolism of DOC by the joint action of C. paraputrificum and culture No. 116*

Evidence from numerous experiments suggests that the metabolism of DOC to pregnanolone is a two-step conversion in which *C. paraputrificum* reduces DOC to THDOC, which, in turn, is 21-dehydroxylated by culture No. 116 or *E. lentum* [7, 8, 11]. Table 4 confirms this hypothesis. DOC was metabolized by culture No. 116 to progesterone and by *C. paraputrificum* to THDOC. When the two cultures were grown together in PR, both pregnanolone and THDOC

Table 3. Conversion of THDOC and pregnanolone by *E. lentum* (neotype) and culture No. 116

Bacterial strains	THDOC (16 μg/ml)		Pregnanolone (16 μg/ml)	
	PR	BHIC-Ec†	PR	BHIC-Ec
Culture No. 116	DOH‡	DOH	0	0
E. lentum (neotype)	DH§	DOH + DH*	DH	DH*
Final Eh (mV)‖	−160 ± 40	−280 ± 50	−160 ± 40	−280 ± 50

* Observed occasionally and in low yields. † BHIC reduced by growth of *E. coli*. ‡ 21-dehydroxylation. § 3α-dehydrogenation. ‖ Initial Eh: −130 ± 20 mV.

Table 4. Conversion of DOC by *C. paraputrificum* and culture No. 116

Media	Bacterial strains	Metabolites		
		THDOC	Progesterone	Pregnanolone
PR	No. 116	−	+	−
	C. paraputrificum	+	−	−
	No. 116 + *C. paraputrificum*	+	−	+
BHIC-Ec*	No. 116	−	+	−
	C. paraputrificum	+	−	−
	No. 116 + *C. paraputrificum*	−	−	+

Concentration of DOC = 16 μg/ml. * BHIC reduced by growth of *E. coli*.

Table 5. 21-Steroid metabolism by culture No. 116, *E. lentum* (neotype) and fecal flora

Parameters	Culture No. 116		*E. lentum* Media*		Fecal Flora
	PR	BHIC-Ec	PR	BHIC-Ec	BHIC
21-DOH (DOC, THDOC)	+	+	−	+	+
21-DOH capacity (μM)	2.4	19.2	ND†	5.6‡	19.2
Initial pH range	6.3–7.6	5.8–7.6	ND	5.7–7.9‡	5.8–7.6
Days for conversion	3–4	1–3	ND	1–2‡	1–2
Epimerization of pregnanolone	—	—	+	±	+
Final Eh (mV)	−160 ± 40	−280 ± 50	−160 ± 40	−280 ± 50	−280 ± 40‡

* Volume: 50 ml; substrate: 16 μg/ml except in capacity studies. † Not done. ‡ Unpublished observations. For other abbreviations: see Table 3.

were recovered. In the more efficient system, BHIC-Ec, DOC was quantitatively reduced to pregnanolone.

DISCUSSION

Metabolism of 21-hydroxy steroids by culture No. 116, E. lentum *and fecal flora*

21-Dehydroxylation of THDOC is accomplished rapidly and efficiently be fecal flora and by cultures No. 116 and *E. lentum* (neotype) provided they are grown in the presence of *E. coli* (Table 5). When culture No. 116 is grown in pure culture in PR, the 21-dehydroxylation is delayed and the conversion capacity of the culture reduced. The beneficial effect of *E. coli* upon conversion appears to be non-specific since it can be achieved with several other species of *Enterobacteriaceae*. These organisms, intrinsically unable to metabolize the steroids, appear to enhance the 21-dehydroxylating capacity of culture No. 116 and *E. lentum* by lowering the Eh of the medium. At the higher Eh in PR, culture No. 116 21-dehydroxylates THDOC at a slower pace and less efficiently. In PR *E. lentum* switches from 21-dehydroxylation to 3α-dehydrogenation. Thus it appears that the 21-dehydroxylase functions better at lower Eh (−230 to −300 mV) and that the 3α-DH requires a higher Eh (−120 to −200 mV). The absence of 3α-dehydrogenation in BHIC-Ec suggests that a highly reduced environment inhibits the production or the function of the enzyme. It is hard to understand why, at Eh ± −160 mV, the 21-dehydroxylase of culture No. 116 is active, albeit poorly, whereas the 21-dehydroxylase activity of *E. lentum* (neotype) is turned off.

Synthesis of 21-dehydroxylase by culture No. 116 in BHIC-Ec is unrelated to the density of the supporting *E. coli* at the time the organism is added. Moreover, the 21-dehydroxylase activity is independent of the growth phase of culture No. 116. Surprisingly, arginine, which stimulates the growth of culture No. 116 does not enhance the production of 21-dehydroxylase.

Whether or not the presence of an α-ketol group in the side chain is a necessity for the function of the 21-dehydroxylase is a moot point. We do know that the enzyme is unable to metabolize 20ξ, 21-dihydroxy-5ξ-pregnan-3-one [8]. On the other hand the enzyme is not specific to the ring A structure of the steroid. It appears that both atoms at C21 are involved in the dehydroxylation [12].

The 3α-DH of *E. lentum* (neotype) offers an explanation for the formation of pregnanedione and

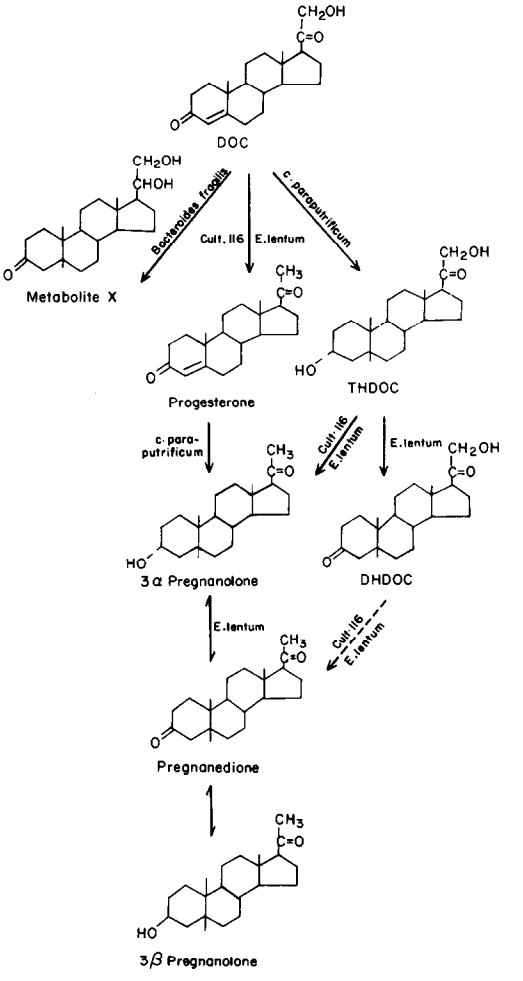

Fig. 4. Bacterially mediated metabolic pathways of DOC and allied steroids.

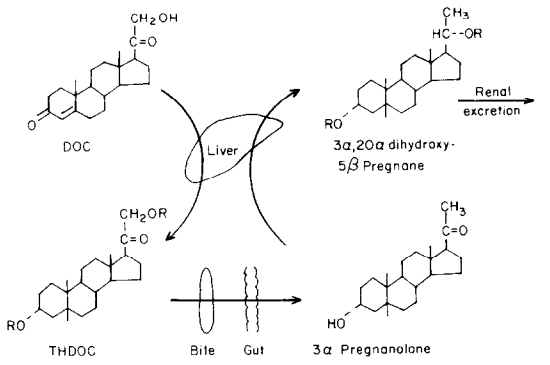

Fig. 5. Proposed *in vivo* metabolic pathway of DOC undergoing enterohepatic circulation. R = glucuronic acid.

3β-pregnanolone observed in cultures of mixed fecal flora [8]. Strains similar to *E. lentum* are normal inhabitants of the intestinal tract [13, 14]. The 3α-DH of these organisms, an oxido-reductive enzyme seems to epimerize 3α-pregnanolone to 3β-pregnanolone via pregnanedione (Fig. 4).

Bacterially mediated metabolic pathways of DOC and THDOC

Based on present and earlier experiments, it is now possible to present diagrammatically the bacterially mediated metabolic pathways of DOC (Fig. 4). Most of the DOC is rapidly metabolized to THDOC by *C. paraputrificum* [8, 15, 16] and, to a much lesser degree by other intestinal bacteria [8]. About 10% of the DOC is metabolized by *Bacteroides fragilis*, *Bifidobacteria* species and other common intestinal anaerobes to a 20,21-diol which is resistant to any further enzymatic alterations by fecal bacteria [8]. Small quantities of the substrate may also be 21-dehydroxylated by culture No. 116 or *E. lentum* to progesterone. This compound has not been observed in fecal cultures where it probably is reduced almost instantly by the fast acting *C. paraputrificum* to 3α-pregnanolone. The principal metabolite of DOC, THDOC, is somewhat more slowly 21-dehydroxylated to 3α-pregnanolone by culture No. 116 or *E. lentum*. 10–15% of the 3α-pregnanolone is epimerized to 3β-pregnanolone probably via pregnanedione by the 3α-DH of *E. lentum* (neotype).

Proposed catabolism of DOC in vivo

In man urinary 3α, 20α-dihydroxy-5β-pregnane is a 21-dehydroxylated derivative of DOC [17, 18], for which we propose the *in-vivo* metabolic pathway depicted in Fig. 5. DOC is reduced to THDOC in the liver, conjugated, excreted in the bile, transported into the intestine, probably deconjugated, and dehydroxylated by culture No. 116, *E. lentum* or allied bacteria to pregnanolone. Although the fate of pregnanolone is unclear, it is probably absorbed, reduced in the liver to 3α, 20α-dihydroxy-5β-pregnane, conjugated with glucuronic acid and returned to the blood for renal excretion.

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