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Inactivation of Contraceptive Steroid Hormones by Human Intestinal Clostridia

V. D. BOKKENHEUSER,[1]* J. WINTER,[1] B. I. COHEN,[2] S. O'ROURKE,[1] AND E. H. MOSBACH[2]

Department of Microbiology, St. Luke's-Roosevelt Hospital Center, New York, New York 10025,[1] and Lipid Research Laboratory, Department of Surgery, Beth Israel Medical Center, New York, New York 10003[2]

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Steroid hormones reduced in ring-A are devoid of hormonal activity. In metabolic experiments we found that human fecal flora reduced the Δ^4-3-keto structure of natural progestins to 3α-hydroxy, 5β-steroid metabolites (3α,5β) and of synthetic progestins to a mixture of 3α,5β and 3β,5β compounds. 3α,5β-Reductase was synthesized by *Clostridium paraputrificum* and had a strong affinity for natural progestins such as progesterone. 3β,5β-Reductase was synthesized by *Clostridium innocuum* and had a stronger affinity for synthetic progestins. A third enzyme, 3β,5α-reductase, was synthesized by St. Luke's strain 209 (*Clostridium* species "J-1") but was only observed when pure cultures were used. Ring-A reduction of synthetic progestins was 3 to 10 times slower than that of natural progestins, thus explaining the pharmacological superiority of synthetic progestins over naturally occurring analogs.

The metabolism of synthetic progestins, which are components of oral contraceptives, was extensively studied in the late 1960s and early 1970s (5, 6, 9, 11, 12, 14, 16). The observed transformations, including oxidations and reductions, were attributed largely to hepatic enzymes. The fact that synthetic progestins, like other steroid hormones, undergo enterohepatic circulation (1) and therefore are exposed to bacterial enzymes in the intestinal stream was not considered. In this study we describe the role of the human intestinal flora in the transformation of synthetic progestins. Particular emphasis is placed on the bacterial species synthesizing the enzyme responsible for the metabolism.

MATERIALS AND METHODS

Abbreviations and trivial names. The abbreviations and trivial names used in the text are as follows: cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 21-deoxycortisol, 11β,17α-dihydroxy-4-pregnene-3,20-dione; dihydronorethindrone, 19-nor-17α-ethynyl-17β-hydroxy-5β-androstan-3-one; dimethisterone, 19-nor-17α-methylethynyl-17β-hydroxy-6α-methyl-4-androsten-3-one; 3α-HSDH, 3α-hydroxysteroid dehydrogenase; norethindrone, 19-nor-17α-ethynyl-17β-hydroxy-4-androsten-3-one; norgestrel, 13β-ethyl-17α-ethynyl-17β-hydroxy-gon-4-en-3-one; pregnanolone, 3α-hydroxy-5β-pregnan-20-one; progesterone, 4-pregnene-3,20-dione; tetrahydronorethindrone, 19-nor-17α-ethynyl-3α,17β-dihydroxy-5β-androstane; TLC, thin-layer chromatography.

Origin of bacteria. All three *Clostridium* species were isolated from human fecal flora: *Clostridium paraputrificum* by dilution technique (4) and *Clostridium innocuum* and St. Luke's strain 209 by the dilution and replicate-plating technique described by Macdonald et al. (13). The cultures were maintained lyophilized or frozen and were passaged twice in prereduced medium at 37°C before use. A 24-h culture was used for the conversion experiments.

Strain 209, a thin rod that forms terminal spores and is nonsaccharolytic and nonproteolytic, was identified as a member of *Clostridium* species "J-1" (Virginia Polytechnic Institute). It has not been frequently isolated from human feces (L. V. Holdeman, personal communication).

Steroids. Steroids were purchased from Steraloids, Inc., Wilton, N.H. Synthetic progestins, norgestrel, and 3-tetrahydronorgestrel isomers (3α,5β; 3β,5β; and 3β,5α) were kindly donated by Wyeth Lab, Inc., Philadelphia, Pa.; norethindrone and norethindrone acetate were donated by Parke, Davis & Co., Detroit, Mich., and Syntex Lab, Inc., Palo Alto, Calif.; and dimethisterone was donated by Mead-Johnson Co., Evansville, Ind.

Analysis. Conversion experiments and extraction and purification of metabolites by TLC and identification by TLC, gas liquid chromatography, and gas liquid chromatography-mass spectrometry have been described previously (4, 19).

Enzymatic reaction for identification of 3α-hydroxy steroids was carried out in solution as described by Yamaguchi (20).

Identification of metabolites. A metabolite was considered identical to a given reference compound when (i) it had a molecular weight and mass spectrometry fragmentation pattern identical to those of the reference compound; (ii) its R_f value on TLC and relative retention time to 5α-cholestane on gas liquid chromatography agreed within 4% with those of the reference compound; and (iii) the reactivities of the unknown

TABLE 1. Characteristics of reference compounds for natural steroid metabolites[a]

Steroid metabolite	R_f[b]	RRT[c]	Reaction with 3α-HSDH
Series 1			
3α-Hydroxy-5β-pregnan-20-one	0.68	0.92	+
3β-Hydroxy-5β-pregnan-20-one	0.72	0.88	−
3β-Hydroxy-5α-pregnan-20-one	0.68	1.02	−
3α-Hydroxy-5α-pregnan-20-one	0.72	0.90	+
Series 2			
5β-Androstane-3α,11β,17β-triol	0.25	0.87	+
5β-Androstane-3β,11β,17β-triol	NA[d]	NA	NA
5α-Androstane-3α,11β,17β-triol	0.34	0.88	+
5α-Androstane-3β,11β,17β-triol	0.34	1.03	−
Series 3			
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one (tetrahydrocortisol)	0.28	1.44	+
3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-one (allo-tetrahydrocortisol)	0.36	1.58	+
Series 4			
3α,11β,17α-Trihydroxy-5β-pregnan-20-one (tetrahydro-21-deoxycortisol)	0.46	1.04	+
Series 5			
3α,21-Dihydroxy-5β-pregnan-20-one (tetrahydrodeoxycorticosterone)	0.53	0.83	+

[a] Mass spectrometry data for each series indicate M^+ (molecular ion) and B^+ (base peak). Series 1: $M^+ = 419$, $B^+ = 388$. Series 2: $M^+ = 524$, $B^+ = 434$. Series 3: $M^+ = 683$, $B^+ = 652$. Series 4: $M^+ = 595$, $B^+ = 564$. Series 5: $M^+ = 507$, $B^+ = 358$.

[b] TLC was done on Baker-flex silica gel 1B2-F, in the solvent system isooctane-ethyl acetate-acetic acid (15:75:0.6, vol/vol/vol).

[c] RRT, Retention time relative to 5α-cholestane on fused silica capillary column OV-101; oven temperature, 180 to 230°C; program rate, 3° per min. Steroids were analyzed as methoxime-trimethylsilyl ether derivatives.

[d] NA, Not available.

and the reference compounds with 3α-HSDH were identical (Tables 1 and 2).

Identification of metabolites for which reference compounds were not available was based on: (i) mass spectrometry, (ii) relative polarity as measured by TLC and gas liquid chromatography, (iii) reactivity with 3α-HSDH, and (iv) stereospecificity of enzymatic modification of a similar substrate for which reference compounds were available.

RESULTS

Mass spectra of metabolites of synthetic progestins. The extracted metabolites were converted to the volatile methoxime-trimethylsilyl ether derivatives. The mass spectrum of the tetrahydro metabolite of norgestrel is shown in Fig. 1. The molecular weight of the derivatized molecule is 460. Loss of $m/e = 31$ ($CH_3—O^-$) indicates a keto function in the molecule; successive losses of $m/e = 31$ indicate the total number of keto groups. Similarly, trimethylsilyl reagents reacted with hydroxy groups, and the number of fragments $M^+ - 90$, $M^+ - (2 \times 90)$, etc., indicates the number of hydroxy groups. Figure 1 shows that the norgestrel metabolite had two hydroxy groups but no keto groups. Moreover, the molecular weight was 4 mass units higher than the molecular weight of the substrate, indicating a reduction of both the keto group and the double bond. The base peak of $M^+ - 29$ reflected the loss of the ethyl group.

It is noteworthy that the ring-A isomers of a given tetrahydro compound showed the same fragmentation pattern.

Conversion of natural steroids. Progesterone (Fig. 2), 11β,17β-dihydroxy-4-androsten-3-one, cortisol, 21-deoxycortisol, and deoxycorticosterone were all reduced in ring-A by mixed human

TABLE 2. Characteristics of reference compounds for synthetic progestins[a]

Standard[b]	R_f[c]	RRT[d]	Reaction with 3α-HSDH
3α,5β	0.14	0.88	+
3β,5β	0.32	0.89	−
3β,5α	0.21	0.95	−
3α,5α	NA[e]	NA	NA

[a] Mass spectrometry data indicate M^+ and B^+ (see Table 1, footnote *a*). $M^+ = 460$; $B^+ = 431$.

[b] Ring-A reduced norgestrel: 3,17β-dihydroxy-13β-ethyl-17α-ethynylgonane.

[c] TLC was done on Analtech alumina G-F (fluorescent indicator) plates in the solvent system benzene-acetone (80:20, vol/vol).

[d] See Table 1, footnote *c*.

[e] NA, Not available.

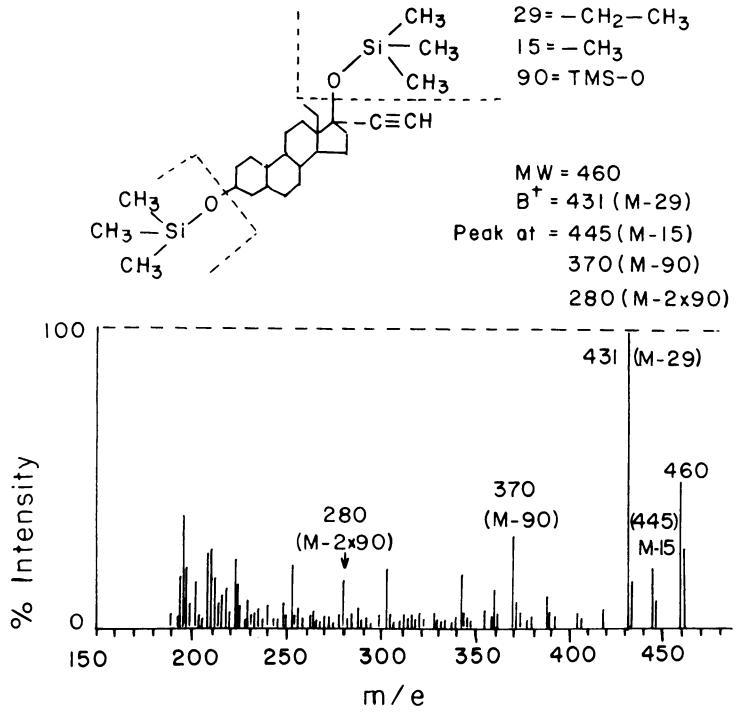

FIG. 1. Mass spectra of tetrahydronorgestrel (trimethylsilyl derivative).

fecal flora and by pure cultures of *C. paraputrificum* to the 3α,5β compounds. In contrast, pure cultures of *C. innocuum* and *Clostridium* species J-1 converted the substrates to 3β,5β and 3β,5α compounds, respectively.

Conversion of synthetic progestins. Norgestrel and dimethisterone (Fig. 2) were reduced in ring-A by fecal flora to a mixture of 3α,5β and 3β,5β compounds. As suggested by the observations with natural steroids, *C. paraputrificum* synthesized the 3α,5β-reductase and *C. innocuum* the 3β,5β-reductase. The 3β,5α-reductase synthesized by *Clostridium* species J-1 metabolized synthetic progestins, although no trace of the metabolite was observed in experiments with mixed fecal flora.

Norethindrone acetate incubated with fecal flora was quickly hydrolyzed and concomitantly reduced to the mixture of 3α,5β and 3β,5β metabolites. It is noteworthy that fecal flora could be diluted to 10^9 and still perform the hydrolysis, whereas reduction of ring-A was not obtained with fecal dilutions above 10^7, suggesting that different organisms are responsible for the two reactions. The transformation of norethindrone by fecal flora was adequately explained by the presence of *C. paraputrificum* and *C. innocuum*. *Clostridium* species J-1 did not appear to play an important role in this conversion.

Kinetics of ring-A reduction. The intestinal flora produced similar alterations in ring-A of both natural and synthetic progestins. However, orally administered synthetic progestins, in contrast to natural progestins, are potent contraceptives. This difference in biological activity prompted us to compare the kinetics of ring-A

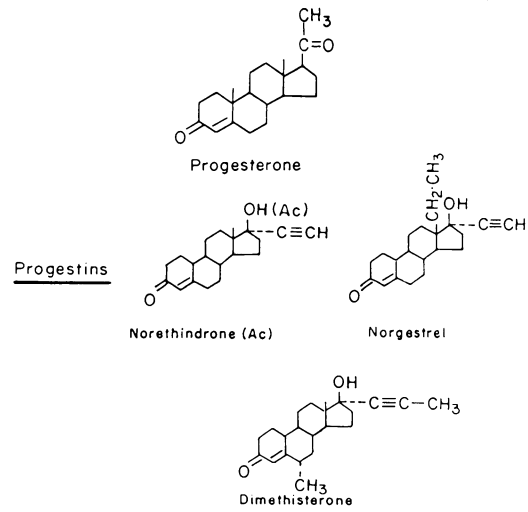

FIG. 2. Structure of progesterone and synthetic progestins.

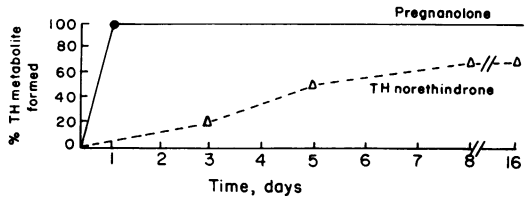

FIG. 3. Comparative kinetics of the metabolism of progesterone and norethindrone. Concentration of substrate: 1 mg per 50 ml of converting medium. Inoculum: 0.25 ml of a 24-h culture of *C. paraputrificum*. Converting medium: prereduced brain heart infusion broth supplemented with 0.05% cysteine-hydrochloride obtained from Scott Laboratories, Inc., Fiskeville, R.I. Incubation: 37°C. TH, Tetrahydro derivative.

reduction by pure cultures of steroid-converting organisms.

C. paraputrificum, *C. innocuum*, and *Clostridium* species J-1 all quantitatively reduced ring-A of natural progestins. In contrast, ring-A reduction of synthetic progestins was slow, taking 6 to 7 days to reduce about 70% of the molecules (Fig. 3).

C. paraputrificum reduced the synthetic progestins in a two-step reaction, best observed with the substrates norethindrone and dimethisterone (Fig. 4). Within 18 to 24 h, the substrate was quantitatively reduced to dihydronorethindrone, which over the next 6 to 7 days was partially reduced to tetrahydronorethindrone. Dihydro intermediates were not observed in metabolic experiments with fecal flora or with *C. innocuum* and *Clostridium* species J-1.

Under the conditions we used, the maximum amount of natural progestins converted in 24 h by the three *Clostridium* species was 9 mg per 50 ml of medium. These species had a 3 to 10 times lower capacity to reduce synthetic progestins, as compared with the natural compounds. The reduction rate, however, varied with the chemical structure of the synthetic progestins.

Substrate competition among *C. paraputrificum*, *C. innocuum*, and *Clostridium* species J-1. As observed above, ring-A reduction of progestins by fecal flora reflected the enzymatic activity of *C. paraputrificum* and, to a lesser degree, of *C. innocuum*. *Clostridium* species J-1 activity was not expressed. In experiments with mixed cultures of *Clostridium* species (Table 3), *C. paraputrificum* had a greater affinity for natural progestins, whereas *C. innocuum* and *Clostridium* species J-1 were more active upon synthetic progestins. In mixtures of *C. innocuum* and *Clostridium* species J-1, the enzymatic expression of *C. innocuum*, i.e., the formation of the 3β,5β isomer, prevailed.

DISCUSSION

Origin of bacterially synthesized steroid-inactivating enzymes. The predominant and perhaps the sole effect of the intestinal flora on the synthetic progestins studied in the present investigation is the reduction of the Δ^4-keto structure of ring-A. For almost 10 years it has been known that the human intestinal flora contains bacteria capable of reducing ring-A and thereby inactivating endogenous progestins (4). Our observations show that at least three *Clostridium* species synthesize ring-A active enzymes with different affinities and different stereochemical effects. The enzyme produced by *C. paraputrificum* is particularly active upon natural progestins, whereas those produced by *C. innocuum* and *Clostridium* species J-1 are relatively more active upon the synthetic progestins used for contraceptive purposes. Whether the observed differences in their abilities to reduce ring-A of the substrates are due to differences between synthesis of the enzyme in mixed flora or differences in enzymatic affinity for the substrates is unknown. Presently, it is difficult to offer a convincing hypothesis for the existence of three different Δ^4-3-keto reductases, namely, 3α,5β-, 3β,5β-, and 3β,5α-reductases, all acting upon ring-A.

Preservation of contraceptive properties of synthetic progestins. Our experiments show that the minimal loss of biological activity of synthetic progestins in the intestinal environment is attributable to the ethynyl group which enhances the resistance of ring-A to reducing enzymes. It is more difficult to explain the rare failure of these contraceptive hormones in women receiving antibiotic therapy (3, 8, 17). Perhaps the antibiotics reduce the intestinal population of deconjugating bacteria and thereby promote a substantial loss of hepatically conjugated hormones through fecal excretion. Alternatively, it is possible that alteration of the flora results in a relative increase in the population of ring-A

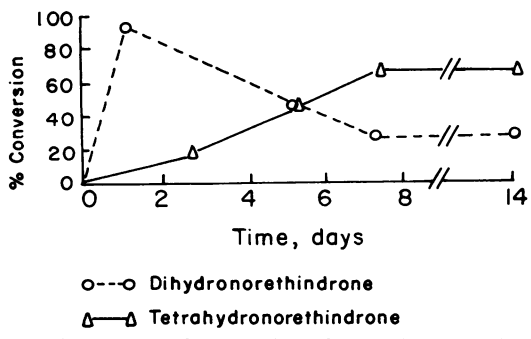

FIG. 4. Rate of conversion of norethindrone with *C. paraputrificum*. For details, see legend to Fig. 3.

TABLE 3. Substrate competition between *Clostridium* species

Substrate	Conversion in culture mixture with *C. paraputrificum* (%)			
	C. innocuum:		*Clostridium* species J-1:	
	3α,5β	3β,5β	3α,5β	3β,5α
Progesterone	80	20	80	20
Norethindrone	50	50	50	50
Dimethisterone	20	80	10	90
Norgestrel[a]	10	40	15	35

[a] 50% of this substrate was unconverted.

reducing bacteria, i.e., the reducing organism might be resistant to the administered antibiotics and multiply at the expense of sensitive intestinal strains.

Sites of inactivation of progestins. Natural progestins are inactivated mainly in the liver through reduction of the double bonds (10, 15). However, the small proportion of Δ^4-3-keto steroids which enters the enterohepatic circulation undergoes hydrogenation by the intestinal flora to form ring-A saturated hydroxy steroids. In contrast, the hepatic reduction of the Δ^4-3-keto structure of synthetic progestins is slow and incomplete, allowing the majority of molecules to enter the general circulation and perform their hormonal functions. The synthetic progestins undergoing enterohepatic circulation (1, 2) are slowly reduced by bacterial enzymes as described above.

For patients treated with norgestrel, Sisenwine et al. (16) noted that all four variants of the hydrogenated Δ^4-3-keto function (3α,5α; 3α,5β; 3β,5α; and 3β,5β) appeared in urine and feces. Evidence suggests that the human liver is capable of performing the 3α,5β reduction, but whether it synthesizes the other reductases is unknown. Our results show that intestinal *Clostridium* species synthesize at least three of the reductases active upon ring-A. If it is permissible to apply the results from animal experiments (7, 18) to the human situation, it seems likely that ring-A reduction of synthetic progestins is carried out both by the liver and by certain intestinal *Clostridium* species.

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