J. steroid Biochem. Vol. 27, No. 4–6, pp. 1154–1149, 1987
Printed in Great Britain. All rights reserved
0022-4731/87 $3.00 + 0.00
Copyright © 1987 Pergamon Journals Ltd

Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

BACTERIAL METABOLISM OF NATURAL AND SYNTHETIC SEX HORMONES UNDERGOING ENTEROHEPATIC CIRCULATION

JEANETTE WINTER and VICTOR D. BOKKENHEUSER

St Luke's-Roosevelt Institute for Health Sciences, New York, NY 10025, U.S.A.

Summary—Steroids undergoing enterohepatic circulation are exposed to bacterial metabolism particularly by obligate anaerobes which account for 99.99% of the fecal flora. The most common transformation is hydrolysis of conjugated steroids. The glucuronidases are synthesized by *Escherichia coli* and *Bacteroides* species. The bacterial catabolism of unconjugated steroids may be considered under several headings: 1. Reduction of ring-A due to clostridia species synthesizing specific enzymes; *C. paraputrificum*, $3\alpha,5\beta$-reductase; *C. innocuum*, $3\beta,5\beta$-reductase; and a new species *C.J*-1, $3\beta,5\alpha$-reductase. 2. Reduction of the Δ^5 bond by human fecal flora. The specific strain(s) synthesizing the enzyme have not yet been identified. 3. Reduction of 17-keto estrogens by the above mentioned ring-A reducing clostridia and by *Eubacterium lentum*. 4. Reduction of 17-keto androstenes by *Bacteroides fragilis*. 5. Desmolase mediated side chain cleavage at C17–C20 position of 17α-hydroxysteroids by two new species *Clostridium scindens* and *Eubacterium desmolans* isolated from human and cat fecal flora respectively and by *Clostridium cadavaris* isolated from New York City sewage. 6. And 16α- and 21-dehydroxylase by *E. lentum* a normal inhabitant of the human gut; it is the only organism known to synthesize 16α- or 21-dehydroxylases. Due to the high specificity of the enzymes and the simplicity of extracting the metabolites, biosynthesis of reference compounds and radioimmunoassay reagents is practical and inexpensive. The enzymes can also be used for titration of specific bacterial strains in fecal flora and as markers for bacterial identification in particular for the strains difficult to be defined by regular biochemical reactions.

The physiological role of the enterohepatic circulation (EHC) to which most steroids are subjected during their elimination is practically unknown. It consists of three major components: the liver, from which most of the steroids are excreted in the bile after conjugation with glucuronic or sulfuric acid; the intestinal tract, which contains approximately 1 kg of bacteria representing more than 400 bacterial species of which less than 1% are facultative anaerobes and more than 99% are obligate anaerobes; and the portal system, which returns the steroids whether altered or not to the liver for further enterohepatic circulation or renal excretion [1].

The first and most common alteration of the conjugated steroid in the gut is hydrolysis by intestinal wall (glucuronidases) or bacterial enzymes (glucuronidases and sulfatases). The glucuronidases are synthesized by both *Escherichia coli*, the most common of the facultative aerobes and *Bacteroides* sp., the most common of the obligate anaerobes [2]. The hydrolysis is very effective as over 98% of the steroids in feces are deconjugated and it is important from the standpoint of further bacterial catabolism as bacterial enzymes are incapable of dealing with the conjugates. Any changes in the intestinal flora such as antibiotic treatment or gastrointestinal disorders will have an impact on the EHC of steroids, i.e. decreased reabsorption of metabolites, decreased urinary excretion or increased fecal excretion resulting in a loss of circulating steroids [3]. The bacterial catabolism of steroids may be considered under several headings.

REDUCTION OF RING A

Ring A reduction is usually accompanied by a diminished hormonal activity of the molecule but there are some exceptions, especially when only the 4-ene bond is reduced, e.g. the dihydroderivative of testosterone is more potent than the parent molecule. The reduction of 4-ene-3-keto steroids begins with the hydrogenation of the double bond followed by the reduction of the keto function [4]. The reductases are synthesized by obligate anaerobic bacteria, among which *Clostridia* species are the most prominent. The enzymes have highly specific stereo-orientations leading to the formation of several isomers (Fig. 1). For example, we have shown that *Clostridium paraputrificum* reduces ring A to a $3\alpha,5\beta$ tetrahydro isomer, while *Clostridium innocuum* converts the substrate to the $3\beta,5\beta$ isomer and a new species, *Clostridium J*-1 to the $3\beta,5\alpha$ compound. Despite this extraordinary specificity of the bacterial enzyme, the substrate also plays a role in the outcome of the transformation [5]. A case in point is an aldehyde radical at C_{18} which will direct the hydroxy group at C_3 and the hydrogen at C_5 to the α position in the metabolic experiments with *C. innocuum* which with other steroids would produce the $3\beta,5\beta$ isomer. Conversion of progesterone and

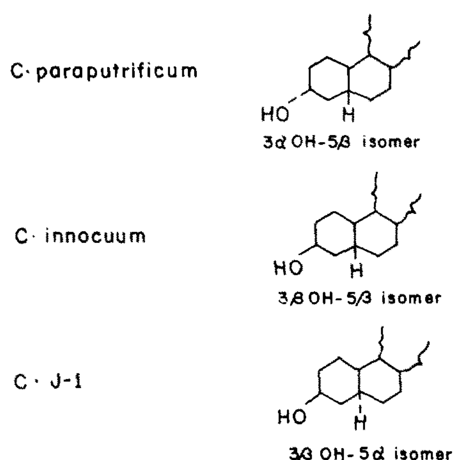

Fig. 1. Ring A reduction by *Clostridia* species.

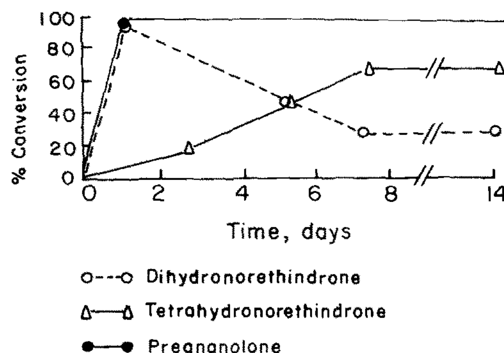

Fig. 2. Kinetics of the metabolism of noretheindrone vs progesterone. Concentration of substrate: 1 mg/50 ml of converting media. Inoculum: 0.25 ml of a 24 h culture of *C. paraputrificum*; converting medium: prereduced brain-heart infusion broth supplemented with 0.05% cysteine-hydrochloride. Incubation at 37°C.

other natural corticoids by *Clostridia* is complete within 16–18 h in cultures supplemented with up to 150 μg steroid/ml. Bacterial reduction of synthetic progestins is much slower (Fig. 2). For example, it takes *Clostridia* 3 days to reduce 20% and 14 days to reduce 70% of norethindrone or dimethisterone in culture supplemented with 20 μg steroid/ml. Norgestrel is even more resistant, with only 40% conversion after 14 days [4]. In kinetic experiments we demonstrated that the former compounds are ring A reduced by *C. paraputrificum* in a two-step fashion, as for corticoids, while the latter compound incubated with *C. innocuum* yielded the tetrahydro compound exclusively. This does not exclude the possibility that dihydroderivatives could be intermediary products, but in that case one may conclude that the conversion from dihydro to tetrahydro compound is very rapid.

At this point is may be appropriate to consider whether or not bacterial metabolism of synthetic progestins might be responsible for the rare failure of contraceptive medication of women receiving antibiotic treatment [4]. The synthetic progestins like natural progestins undergo EHC and are exposed to bacterial modification [3]. Conceivably, they could be metabolized at a higher than usual rate in the gut if an administered antibiotic kills a significant amount of bacteria incapable of synthesizing the specific enzyme and is without effect on those producing the reductase. The ring A reduced metabolites would then be absorbed, conjugated in the liver and excreted in the bile for further EHC. Since the deconjugated bacteria are destroyed by the antibiotics the conjugated progestins are excreted in the feces. The synthetic estrogens may follow the same fate, resulting in fecal excretion of conjugated estrogens.

Rifampin may exert its effect by another mechanism [6]. Rifampin stimulates the synthesis of conjugating enzyme and therefore increases the rate of steroid catabolism, resulting in an increase of fecal estrogenic excretion. In both cases, whether excessive ring A reduction or lack of deconjugation, there is a drain on the administered contraceptive and consequently a lower than expected blood level (Fig. 3).

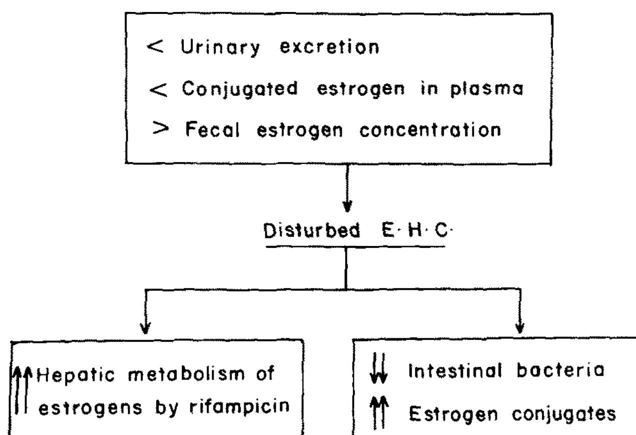

Fig. 3. Mechanism of contraceptive inactivation.

REDUCTION OF RING B

Reduction of the 5-ene bond was noticed in incubation of dehydroepiandrosterone with human fecal flora diluted up to 10^7 (Fig. 4). Three isomers were isolated: $3\beta,5\alpha$; $3\alpha,5\beta$; and $3\alpha,5\alpha$ tetrahydrocompounds. The organisms metabolizing dehydroepiandrosterone are not yet known. None of the three *Clostridia* reducing the 4-ene structure had any effect on the 5-ene bond (unpublished observation). In metabolic experiments we observed two additional metabolites which not only were hydrogenated at ring B as described above but also were reduced in the keto group at C_{17}.

REDUCTION OF 17-KETO STEROIDS

Phenolic steroids are reduced to 17-hydroxy-derivatives by *Clostridia* species synthesizing 4-ene-3-keto reductase and *E. lentum* [7]. The C_{19} steroids have received little attention but the reduction is performed by *Bacteroides fragilis*. Other *Bacteroides* species such as *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis* metabolize the C_{19} steroids to another two compounds that are not yet identified. Interestingly, *Clostridia* species are active on 17-keto phenolic steroids while *Bacteroides* species are active on 17-keto C_{19} steroids.

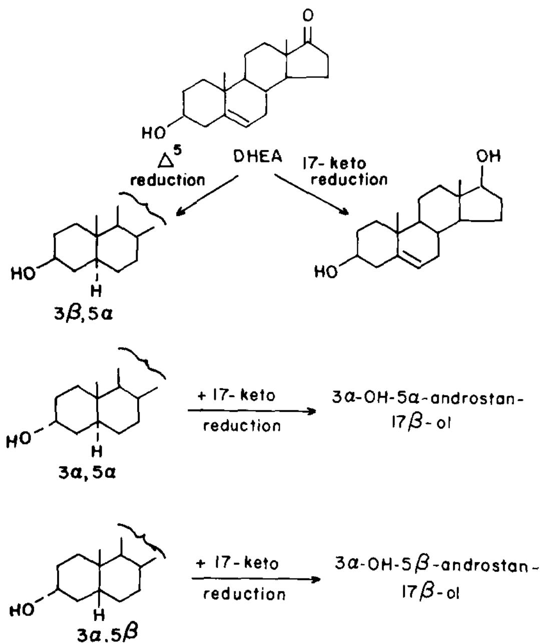

Fig. 4. Reduction of dehydroepiandrosterone by fecal flora.

SIDE-CHAIN CLEAVAGE AT C_{17}–C_{20}

Clostridium scindens, isolated from human feces [8, 9], *Eubacterium desmolans* recovered from cat feces and *Clostridium cadavaris* from New York City sewage [10, 11] synthesize the specific enzyme C_{17}–C_{20} steroid desmolase. All three organisms are obligate anaerobes and surprisingly perform the oxidative cleavage at -300 mV. The enzyme requires a 17-hydroxy group for function, making cortisol an excellent substrate. After 5–8 h incubation 10–20% of substrate is converted to 20-dihydrocortisol with the hydroxy group in the β-position when *E. desmolans* and *C. cadavaris* are used and in the α-position when *C. scindens* is employed. Both stereoisomers are further metabolized during the next 10 h to 11β-hydroxy-androstenedione (Fig. 5). At present we do not know whether or not the 20-hydroxy metabolite is an intermediary in the pathway for side-chain cleavage by anaerobes (Fig. 6).

It is noteworthy that while a 17-hydroxy group is an absolute requirement for the side-chain cleavage by anaerobic bacteria, the C_{17}–C_{20} bond can be cleaved by certain aerobic bacteria and fungi without a hydroxy group at C_{17}.

In experiments with cell-free extracts of *C. scindens* we demonstrated that the desmolase requires NAD and manganese and benefits from thiamine pyrophosphate and Co-enzyme A [12]. Oxygen is not required. This contrasts with the requirements of the desmolase produced by *Cylindrocarpon radicicola*, *Pseudomonas chlororaphus*, *Arthrobacter simplex* and *Nocardia* species [13], which require NAD(P)H and oxygen as cofactors [14]. It is conceivable that NAD(P)H and O_2 are required for the 17-hydroxylation step in the 17-deoxysteroids while

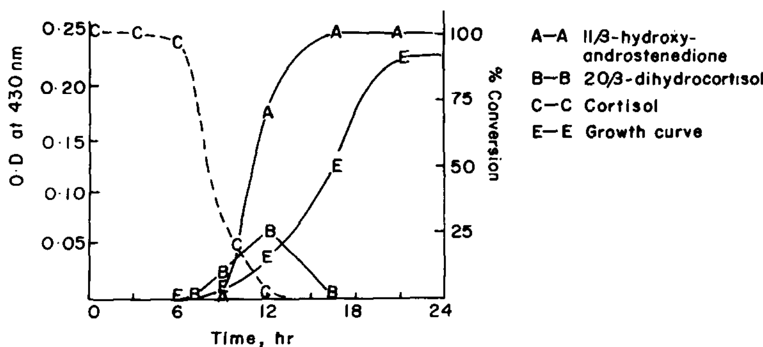

Fig. 5. Side-chain cleavage of cortisol.

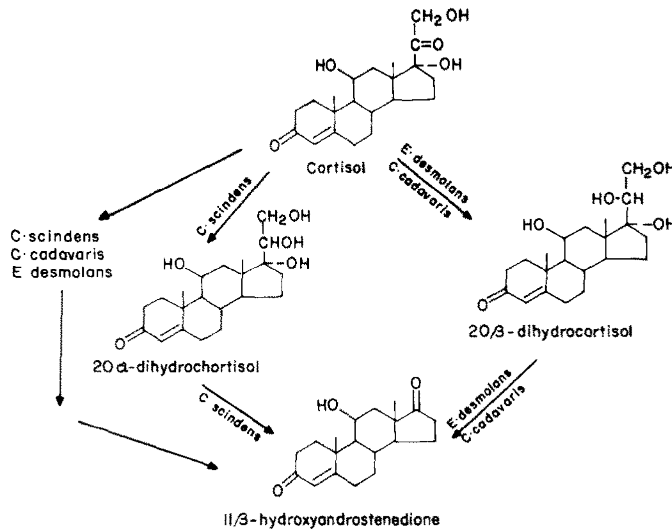

Fig. 6. Desmolase activity as a function of bacterial multiplication. O–O, 11β Hydroxyandrostenedione. ●–●, Growth curve. Inoculum: 0.25 ml of a 24-h culture of *C. scindens*. For details see legend of Fig. 2.

cleavage of 17-hydroxysteroids is accomplished in the presence of the oxidated form of coenzyme NAD.

The types of desmolase also differ in their substrate specificity at C_{11} and C_{20}. While *C. radicicola* has no activity on cortisol molecule [11] and prefers substrates with a 20-carbonyl group *C. scindens* prefers cortisol as substrate and metabolizes 20-carbonyl steroids and their corresponding 20-hydroxy metabolites equally well [8].

Besides enzymatic conversion of sex hormones, a series of reactions occur due to the action of bacterial enzymes on corticosteroids. Many of these enzymes are synthesized by *E. lentum* and phenotypically related organisms. They are the only organisms known to produce 21-dehydroxylase (Fig. 7) [15], 16α-dehydroxylase (Fig. 8) [16, 17], reductases for the double bond of the lactone ring of digoxin (Fig. 9) [18] and for the conjugated double bond in the fatty acids (Fig. 10) [19]. The practical application of

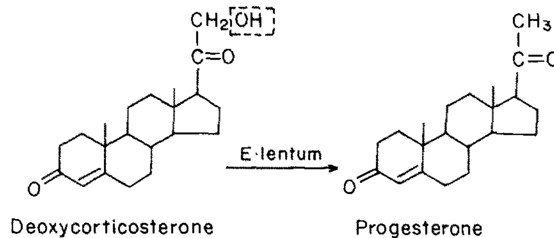

Fig. 7. 21-Dehydroxylation of corticoids. Concentration of deoxycorticosterone: 1–5 mg/50 ml media. Inoculum: 0.25 ml of a 48-h culture of *E. lentum*; converting media: prereduced brain–heart infusion broth supplemented with 0.05% cysteine–hydrochloride. Incubation: 37°C for 7 days.

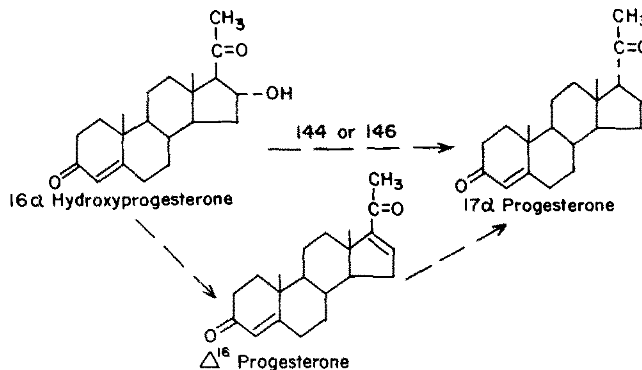

Fig. 8. Steroid 16α-dehydroxylation by *E. lentum*. For details see legend of Fig. 7.

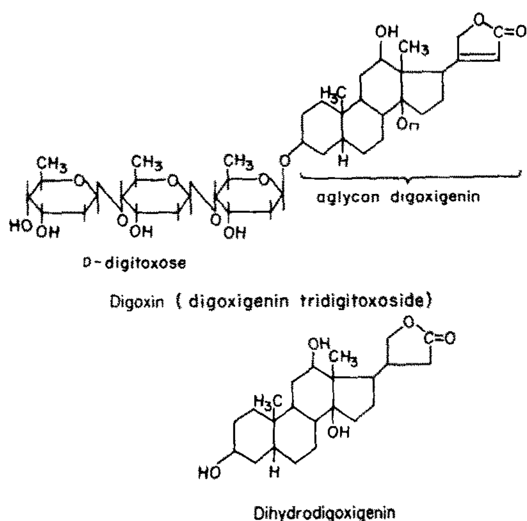

Fig. 9. Catabolism of digoxin by *E. lentum*.

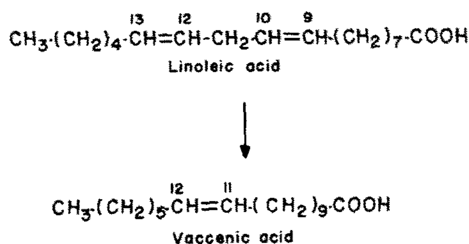

Fig. 10. Reduction of fatty acid by *E. lentum*.

these enzymatic conversions include: biosynthesis of unavailable reference compounds; biosynthesis of reagents for radioimmunoassay; titration of specific bacterial strains in fecal flora; markers for bacterial identification; isolation and identification of new enzymes; and establishment of metabolic pathways.

REFERENCES

1. Macdonald I. A., Bokkenheuser V. D., Winter J., McLernon A. M. and Mosbach E. H.: Degradation of steroids in the human gut. *J. Lipid Res.* **24** (1983) 675–700.
2. Lombarde P., Goldin B., Britin E. and Gorbach S. L.: Metabolism of androgens and estrogens by human fecal microorganisms. *J. steroid Biochem.* **9** (1978) 975–801.
3. Adlercreutz H., Martin F. Järvenpaä P. and Fotsis T.: Steroid absorption and enterohepatic recycling. *Contraception* **20** (1979) 201–223.
4. Bokkenheuser V. D., Winter J., Cohen B. I., O'Rourke S. and Mosbach E. H.: Inactivation of contraceptive steroid hormones by human intestinal clostridia. *J. clin. Microbiol.* **18** (1983) 500–504.
5. Winter J., Shackleton C. H. L., O'Rourke S. and Bokkenheuser V. D.: Bacterial formation of aldosterone metabolites. *J. steroid Biochem.* **21** (1984) 563–569.
6. Skolnick J. L., Stoler B. S., Katz D. B. and Anderson W. H.: Rifampin, oral contraceptive and pregnancy. *J. Am. med. Ass.* **236** (1976) 1382.
7. Winter J., O'Rourke-Locascio S., Bokkenheuser V. D., Mosbach E. H. and Cohen B. I.: Reduction of 17-keto steroids by anaerobic microorganisms isolated from human fecal flora. *Biochem. biophys. Acta* **795** (1984) 208–211.
8. Winter J., Morris G.N., O'Rourke-Locascio S., Bokkenheuser V.D., Mosbach E. H., Cohen B. I. and Hylemon P. B.: Mode of action of steroid desmolase and reductases synthesized by *Clostridium "scindens"* (formerly *Clostridium* strain 19). *J. Lipid Res.* **25** (1984) 1124–1131.
9. Morris G. N., Winter J., Cato E. P., Ritchie A. E. and Bokkenheuser V. D.: *Clostridium scindens* sp. nov., a human intestinal bacterium with desmolitic activity on corticoids. *Int. J. syst. Bact.* **35** (1985) 478–481.
10. Morris G. N., Winter J., Cato E. P., Ritchie A. E. and Bokkenheuser V. D.: *Eubacterium desmolans* sp. nov., a steroid desmolase-producing species from cat fecal flora. *Int. J. syst. Bact.* **36** (1986) 183–186.
11. Bokkenheuser V. D., Winter J., Morris G. N. and Locascio S.: Steroid desmolase synthesis by *Eubacterium desmolans* and *Clostridium cadavaris*. *J. appl. environ. Microbiol.* **52** (1986) 1153–1156.
12. Krafft A. E., Winter J., Bokkenheuser V. D. and Hylemon P. B.: Co-factor requirement of steroid 17–20 desmolase and 20α-hydroxy steroid dehydrogenase activities in cell extracts of clostridium scindens. *J. steroid Biochem.* **26** (1987) in press.
13. Vezima C. and Rakhit S.: Microbial transformation of steroids. In *Handbook of Microbiology* (Edited by A. I. Laskin and M. A. Lechevalier). CRC Press, Cleveland, Ohio. Vol. IV (1974) pp. 333–341.
14. Rahim M. A. and Sih C. J.: Mechanism of steroid oxidation by microorganisms. Enzymatic cleavage of the pregnane side-chain. *J. biol. Chem.* **241** (1966) 3615–3623.
15. Winter J., Bokkenheuser V. D. and Ponticorvo L.: Bacterial metabolism of corticoids with particular reference to the 21-dehydroxylation. *J. biol. Chem.* **254** (1979) 2626–2629.
16. Glass T. L., Winter J., Bokkenheuser V. D. and Hylemon P. B.: Biotransformation by *Eubacterium* sp. 144: non-enzymatic addition of L-cysteine to Δ^{16}-progesterone. *J. Lipid Res.* **23** (1982) 352–356.
17. Winter J., O'Rourke S., Bokkenheuser V. D., Hylemon P. B. and Glass T. L.: 16α-Dehydration of corticoids by bacteria isolated from rat fecal flora. *J. steroid Biochem.* **16** (1982) 231–237.
18. Lindenbaum J., Rund D. G., Butler V. P. Jr., Tse-Eng D. and Saha J. R.: Inactivation of digoxin by the gut flora: reversal by antibiotic therapy. *New Engl. J. Med.* **305** (1981) 789–794.
19. Eyssen H., Verhulst A., DePauw G. and Asselberghs S.: Biotransformation of C-18 fatty acids and bile acids by *Eubacterium lentum*. *Antonie van Leeuwenhoek* **48** (1982) 499–500.