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METABOLISM OF ANDROGENS AND ESTROGENS BY HUMAN FECAL MICROORGANISMS

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SUMMARY

Modifications of androgens and estrogens were observed in incubations with mixed human fecal cultures. Both oxidative and reductive reactions have been shown to occur. Moreover, the fecal concentration determines the types of metabolites formed. Analysis of reactions of high fecal concentration resulted in the identification of products formed by reduction. 4-ene-3-ketosteroids were reduced in ring A. 5β-3-ketosteroids were reduced to 5β-3-hydroxysteroids. 17-keto-androgens and estrogens were reduced to 17β-hydroxy products. Analysis of low fecal concentration incubations resulted in products formed by oxidation and by cleavage of conjugated steroids. 5β-androstane-3,17-dione was found to be converted to 4-androstene-3,17-dione. And 17β-hydroxysteroids were converted to 17-oxosteroids. Furthermore the estrogen conjugates estrone-3-sulfate and estradiol-3-glucuronide were hydrolyzed to form estrone and estradiol respectively.

A number of the reductive reactions occurred under aerobic conditions. This may reflect the ability of the fecal extract to lower the oxidation-reduction potential of the reaction mixture.

The androgen and estrogen metabolites varied depending on the concentration of feces and the availability of oxidizing agents, such as phenazine methosulfate or menadione

INTRODUCTION

Cancers of the large bowel and breast are considerably more frequent in Western Europe and North America than in Africa, Asia and South America. Several studies have found a strong correlation between these malignancies and a high dietary intake of fat and meat, and an inverse relationship with fiber[1–4]. On this basis, it has been suggested that the geographic distribution of these diseases could be related to variations in dietary practices.

Hill *et al.*[5, 6] have suggested that the intestinal flora is involved in the mechanism by which the diet influences incidence of bowel and breast cancer. These investigators have proposed that the intestinal flora can dehydroxylate and dehydrogenate bile acids and other steroids resulting in polycylic aromatic compounds[7, 8]. The dehydration of bile acids at the 7 position is a well-known bacterial reaction[9]. However, the aromatization of steroids had previously been shown only with the soil bacteria *Nocardia rubra* and *Pseudomonas testosteronii*[10, 11]. The aromatization of the A ring only occurred with substances such as nor-testosterone which lacks the C^{19} bridge methyl group or with 19 hydroxysteroids. Hill and his co-workers[7, 8, 12] showed that *Clostridium paraputrificum* isolated from human feces was capable of introducing double bonds into 3-oxo-5β-androgens. These reactions were run aerobically in the presence of oxidizing agents such as phenazine methosulfate or menadione.

Schubert *et al.*[13] had previously shown that *Clostridium paraputrificum* hydrogenates 3-ketosteroids at the 1 and 4 position under anaerobic conditions. They also found that the keto group was reduced to a hydroxy group. Eyssen and Paramentier[14] observed that bacteria in the large intestine and cecum were able to hydrogenate 5-ene-steroids. In another study by Eriksson and Gustafsson[15], 3-hydroxy,17-keto-androgens were incubated with human ileal contents, and the keto group was reduced to a hydroxy group. They also demonstrated dehydroxylation of pregnan derivatives at the 16 and 21 position. When 11-deoxycorticosterone was incubated with fecal contents, Bokkenhauser and coworkers[16, 17] found that dehydroxylation occurred at the 21 position and hydrogenation at the 4 position. In addition, the keto group was reduced to the hydroxy group at the 3 position.

These experiments indicate that the metabolic activity of human feces is predominantly reductive. The studies by Hill *et al.*[7, 8] were performed with pure cultures of fecal organism under aerobic conditions with the addition of oxidizing agents.

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Under such conditions, oxidation reactions were observed. However, the large bowel has a highly anaerobic milieu and contains a multitude of bacterial types—estimated at 500 different species of anaerobes. For these reasons, our studies of the transformation of estrogens and androgens used human fecal specimens which were incubated *in vitro* at various concentrations and under aerobic and anaerobic conditions.

METHODS

Fecal Extracts.

Fresh fecal samples were immediately placed in ice and were prepared within an hour of defecation. Extracts were made by mixing 1 g feces in 10 ml of 0.1 M phosphate buffer, pH 7.0. The mixture was centrifuged at low speed for 10 min, and was strained through gauze to remove large particles. Extracts were used immediately.

Steroids. All steroids were obtained from Steraloids Inc., except 5β-androstane-3,17-dione, 4-androstene-3,17-dione, and 1,4-androstadiene-3,17-dione which were obtained from Sigma. The purity of these compounds was found to be greater than 97% by thin layer chromatography (TLC) and gas liquid chromatography (GLC).

Media. BHI broth: 3.7 g dehydrated brain heart infusion (Difco) dissolved in 100 ml 0.05 m phosphate buffer, pH 6.8. This solution was sterilized by autoclaving for 20 min and then after cooling it was placed in an anaerobic chamber for several hours to remove dissolved oxygen. The broth was used immediately.

Lactate media was made as described by Marcus and Talalay[18].

Incubations. The fecal cultures were prepared by the addition of fresh fecal extracts to buffer or media. An appropriate amount of steroid was dissolved in 95% ethanol and added to the fecal extract. Reactions were run either aerobically or anaerobically at 37°C for 0 to 7 days. All reactions were stopped by freezing at −20°C except β-glucuronidase which was stopped by boiling 2 min. Table 1 summarizes the incubation conditions for the various substrates.

Extraction I—For reactions run with high fecal concentration: After the designated incubation periods, 3 ml methanol was added to thawed cultures. The mixture was centrifuged for 5 min at low speed. The supernatant was decanted and evaporated to near dryness. The aqueous residue was extracted between ethyl acetate and water, then chloroform–water, organic layers were combined, taken to dryness and purified by adsorption column chromatography. Extracts were chromatographed on a 5 g silica gel column in the appropriate solvent system. Fractions of 0.5 ml

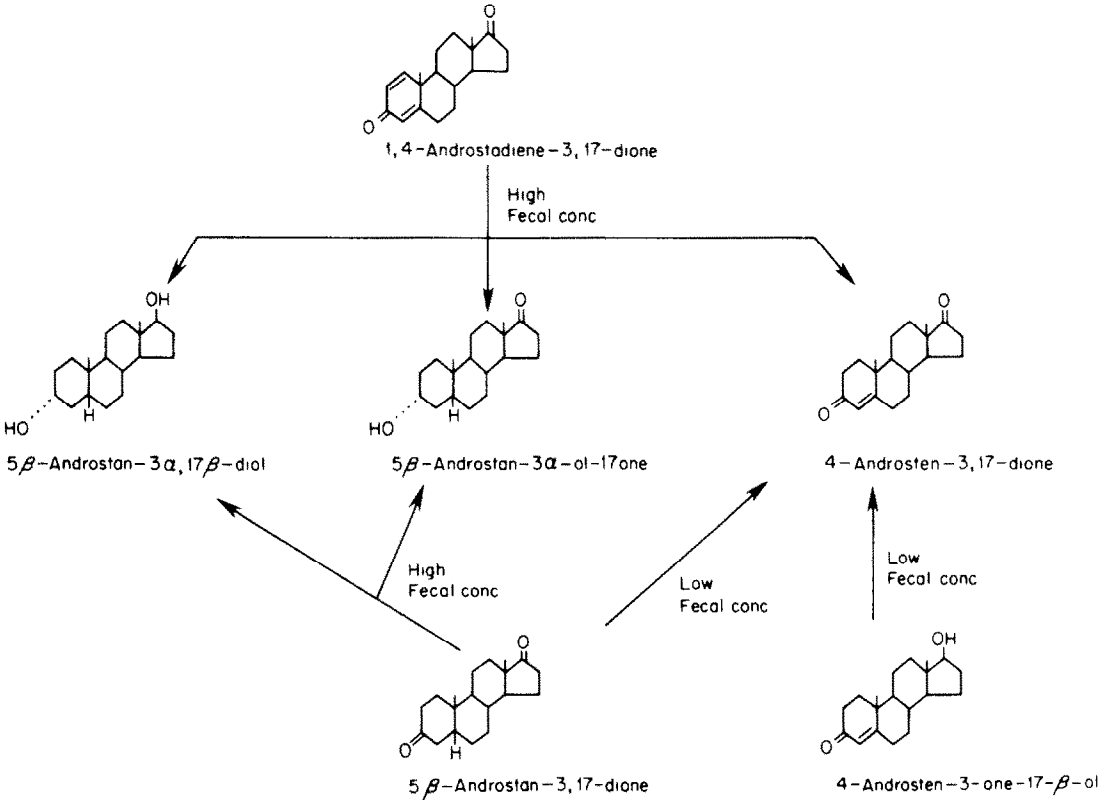

Fig. 1. Metabolic pathways for the fecal bacterial transformation of 5β-Androgens. All reactions were incubated with mixed fecal flora. The introduction of a double bond into the steroid nucleus required phenazine methosulfate or menadione.

Table 1. Conditions of incubation

Substrate	Fecal Extract Concentration mg/ml*	Aerobic/ Anaerobic	Buffer/Media	Time (h)	Temp (°C)	Substrate Concentration (μg/ml)
5β-Androstane-3,17-dione	High	Aerobic	0.1M PO_4, pH 7.0	72	37°	400
	Low	Aerobic Anaerobic	0.1M PO_4, pH 7.0	72	37°	
4-Androstene-3,17-dione	High	Aerobic	0.1M PO_4, pH 7.0	72	37°	400
Estrone	High	Aerobic Anaerobic	o.1M PO_4, pH 7.0	72	37°	100
16α-Hydroxyestrone	High	Aerobic	0.1M PO_4, pH 7.0	96	37°	85.7
	High	Anaerobic	3.7% BHI in PO_4, pH 6.8	72	37°	85.7
Estradiol-3-glucuronide	Low	Aerobic	PO_4, pH 6.8	20 min	37°	200
Estradiol	Low	Aerobic	PO_4, pH 6.8 and PO_4-Citrate, 6.4	24	37°	200
Estrone-3-sulfate	Low	Aerobic	PO_4, pH 6.8 and PO_4-Citrate, 6.4	24	37°	200
Testosterone	Low	Aerobic	PO_4-Lactate, pH 6.5	72	30°	100
1,4-Androstadiene-3,17-dione	High	Aerobic	0.1M PO_4, pH 7.0	72	37°	400

* High: 50–60 mg/ml Low: 6–10 mg/ml.

were taken. Generally 20–50 fractions were collected.

Extraction II—For incubations run with low fecal concentration: Thawed cultures were extracted 3 times with 3 ml of ethyl acetate or methylene chloride. Further purification was generally not necessary.

Thin layer chromatography. Silica gel precoated plates, either quantum Q5F of silica gel 60F-254 (EM Reagents), 250 μm thickness, were used for analytical purposes. Various solvent systems were used, depending of the steroid under investigation. Plates were inspected under a short wavelength U.V. lamp, and spots were visualized by spraying concentrated sulfuric acid, followed by heating for 10 min at 90°C.

Column chromatography. Incubations run with high fecal concentration required further purification before GLC or UV analysis could be undertaken. Purifications were achieved on silica gel 60, 70–230 mesh (EM Reagents). 5 g of adsorbant was mixed as a slurry with the appropriate solvent(s), and poured into glass columns 1 cm × 15 cm. Solvent systems which were the same as those used for TLC are listed as follows:

System 1. cyclohexane–ethyl acetate (2:1, v/v) 2. benzene–dioxane–acetic acid (100:10:1, by vol.) 3. benzene–methanol (80:20, v/v) 4. cyclohexane–ethyl acetate–methanol (45:45:10, By vol.)

Gas–liquid chromatography (GLC). Analysis by GLC was performed on a Packard Model 427 gas chromatograph using glass columns. Column packing of 3% OV-17 on Gas Chromosorb W (100/120 mesh) was obtained from Applied Science. Acetates were prepared by dissolving steroid in 0.1 ml pyridine to which was added 0.2 ml acetic anhydride; the reaction was run overnight at room temperature.

Identification of steroids. Extracted metabolites were purified if necessary. Identification was established by GLC analysis on 3% OV-17; relative retention times were compared with those obtained for authentic standards. In addition, R_f as were determined on TLC silica gel plates in several systems and compared with authentic standards. When appropriate, U.V. spectra of purified metabolites were recorded between 280 nm and 220 nm and were compared with authentic standards. Fluorometric analysis was used for the identification of estrogen metabolites and compared with authentic standards[19]. The metabolites and standards were dissolved in ethanol for the U.V. and fluorescence readings (Table 2). In addition, the Zimmerman reaction for 17 oxo steroids[20] was used on purified metabolites for further structural evidence.

Reactions studied. Table 1 lists the various androgens and estrogens which were used as substrates for incubation with fecal specimens. Some reactions were run at a high fecal concentration, others at a low fecal concentration. Two blanks were run for each incubation. One contained everything except the fecal extract to determine if the reactions on the steroid were the result of anything other than the fecal extract. And the other blank contained everything except steroid to ensure that any metabolite formed was a product of the steroid. The blanks were worked up in the same way as incubation reactions and used for comparison on TLC and GLC. The anaerobic incubations were performed in tightly stoppered tubes with buffer or media that had been prereduced by placing them in an anaerobic chamber for 24 h. Additions were made under a flow of oxygen-free carbon dioxide. These conditions provided a highly anaerobic environment, as judged by the ability to maintain methyl viologen in a reduced state.

Table 2. Relative retention times of metabolites and reference compounds

Reaction	Metabolites/standards	Relative retention times
Rxn I-a	Metabolite I	0.39
(5β-cholestan-	3α-hydroxy-5β-androstan-17-one	0.39
3-one = 1.00)	Metabolite II	0.32
	5β-androstane-3α,17β-diol	0.32
Rxn I-b	Metabolite III	0.62
(4-androstene-	5β-androstane-3,17-dione	0.59
3,17-dione	Metabolite IV	0.51
= 1.00)	3α-hydroxy-5β-androstan-17-one	0.51
Rxn I-c	Metabolite V	0.48
(20-methyl	4-androstene-3,17-dione	0.50
cholanthrene	Metabolite VI	0.32
= 1.00)	3α-hydroxy-5β-androstan-17-one	0.32
Rxn I-d*	Metabolite VII	0.79
(5β-cholestan-	Estradiol	0.81
3-one = 1.00)		
Rxn I-e*	Metabolite VIII	1.83
(20-methyl chol-	Estriol	1.83
anthrene = 1.00)		
Rxn II-a*	Metabolite IX	0.42
(estriol = 1.00)	Estradiol	0.42
Rxn II-b*	Metabolite X	0.33
(estriol = 1.00)	Estrone	0.33
Rxn II-c*	Metabolite XI	0.78
(estradiol = 1.00)	Estrone	0.77
Rxn II-d*	Metabolite XII	0.81
(testosterone	4-androstene-3,17-dione	0.82
acetate = 1.00)		
Rxn IIe	Metabolite XIII	1.34
(5α-androstane-	4-androstene-3,17-dione	1.32
3,17-dione = 1.00)		

* Analysis done on acetates.

Steroids also were incubated with autoclaved fecal extracts.

RESULTS

Reaction mixtures were extracted and analyzed by TLC. Chromatograms were compared with those run on zero hour and blanks to ensure that only metabolites and not artifacts were being identified. Extracts from incubations with high fecal concentration were purified before GLC. Substrates and metabolites were separated from fecal impurities on silica gel column chromatography. Individual fractions were then analyzed by TLC and GLC to locate and identify substrates and products. Aliquots from fractions containing metabolites were mixed with reference standards, and derivatized if necessary. Relative retention times were determined by GLC (Table 2). Column purification was not necessary with extracts from incubations of low fecal concentration. After TLC analysis, the extracts were mixed with reference standards and derivatized if necessary. Relative retention times were determined by GLC (Table 2).

I. Incubations with high fecal concentration

(*a*) *5β-Androstane-3,17-dione.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 1:

5β-androstane-3,17-dione	0.30
3α hydroxy-5β-androstan-17-one	0.15
Metabolite I	0.14
5β-androstane-3α,17β-diol	0.08
Metabolite II	0.08

R_f values were also determined in system 2: In both systems, the chromatographic mobility of metabolite I and II were identical to standard 3α-hydroxy 5β-androstan-17-one and 5β-androstane-3α,17β-diol, respectively. System 1 was used for column purification.

(b) *4-Androstene-3,17-dione.* Extracted in-

cubation products were analyzed by TLC. R_f values obtained from system 1:

4-androstene-3,17-dione	0.25
3α-hydroxy-5β-androstan-17-one	0.15
Metabolite **III**	0.14
5β-androstane-3,17-dione	0.30
Metabolite **IV**	0.30

R_f values were also measured in system 2 and the chromatographic mobilities of metabolites were found to be identical to the respective standards. System 1 was used for column purification.

(c) 1,4-*Androstadiene*-3,17-*dione.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 1:

1,4-androstadiene-3,17-dione	0.23
4-androstene-3,17-dione	0.31
Metabolite **V**	0.30
3α-hydroxy-5β-androstan-17-one	0.20
Metabolite **VI**	0.21

R_f values were also measured in system 2 and in both systems the chromatographic mobilities of metabolites **V** and **VI** were identical to the respective standards. System 1 was used for column purification. U.V. analysis was done on fractions which contained metabolite **V**.

λ_{max} metabolite **V** = 239. λ_{max} 4-androstene-3,17-dione = 239. λ_{max} 1 (5α)-androsten-17-β-ol-3-one = 228.

The finding of 4-androstene-3,17-dione differed from the results reported by Schubert with *Clostridium paraputrificum.*

(d) *Estrone.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 1:

estrone	0.45
estradiol	0.24
Metabolite **VII**	0.23
17α estradiol	0.29

R_f values were also measured in system 2 and 3. In all systems, the chromatographic mobility of metabolite **VII** was identical to 17β-estradiol. System 1 was used for column purification. Acetylated derivatives of estrone and metabolite **VII** were prepared for GLC analysis.

(e) 16α-*Hydroxyestrone.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 4:

16α-hydroxyestrone	0.46
estriol	0.24
Metabolite **VIII**	0.24
16 epiestriol	0.29
17 epiestriol	0.41

R_f values were also measured in system 3. In both systems the chromatographic mobility of metabolite **VIII** was identical to estriol. Purification of the extracts was achieved on an adsorption column using system 4. Acetylated derivatives of metabolite **VIII**, 16α-hydroxyestrone and estriol were prepared for GLC analysis.

II. Incubations with low fecal concentration

(a) *Estradiol-3-glucuronide.* The free steroids were separated from conjugated substrate by partitioning between ethyl acetate and water. The chromatographic mobility of metabolite **IX** proved to be identical with estradiol on TLC. Acetylated derivatives of incubation extracts and estradiol were prepared for GLC analysis.

(b) *Estradiol.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 3:

Estradiol	0.33
Estrone	0.52
Metabolite **X**	0.51

Acetylated derivatives of the reaction mixtures, estradiol and estrone were prepared for GLC analysis.

(c) *Estrone sulfate.* The free steroids were separated from sulfates by partitioning between ethyl acetate and water. R_f values of free metabolite was determined by TLC, system 3:

Estrone sulfate	0.00
Estrone	0.34
Metabolite **XI**	0.33

Acetylated derivatives of metabolite **XIII**, and estrone were prepared for GLC analysis.

(d) *Testosterone.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 3:

Testosterone	0.37
4-androstene-3,17-dione	0.43
Metabolite **XII**	0.43

R_f values were also determined in system 4. In both systems, the chromatographic mobility of metabolite **IX** was identical to standard 4-androstene-3,17-dione.

Acetylated derivatives of the extraction mixtures, and testosterone were prepared for GLC analysis.

(e) 5β-*Androstane*-3,17-*dione.* Extracted incubation products were analyzed by TLC. R_f values obtained from system 1:

5β-androstane-3,17-dione	0.23
4-androstene-3,17-dione	0.13
Metabolite **XIII**	0.13

R_f values were also obtained from system 3. In both systems the chromatographic mobility of metabolite **X** was identical to standard 4-androstene-3,17-dione. System 1 was used for column purification. U.V. analysis was done on fractions which contained metabolite **XIII**.

λ_{max} metabolite **XIII** = 239. λ_{max} 4-androstene-3,17-dione = 239.

Fractions containing metabolite **XIII** were combined. An aliquot was taken and mixed with reference standard and taken for GLC analysis.

Controls

Fecal extracts incubated in media without steroids produced background metabolites which did not interfere with the assay of steroid substrates or their products.

Steroid substrates incubated in buffer or media, in the absence of fecal extract, aerobically or anaerobically, did not undergo any measurable transformation. This observation indicates that low oxidation-reduction potential alone is not responsible for reductive reactions.

As an additional control, fecal extracts were prepared as usual, then autoclaved at 120°C for 20 min, before being added to the incubation medium. The following substrates were incubated in this manner: estrone and 4-androstene-3,17-dione using high fecal concentration, and estradiol using low fecal concentration. None of these incubations resulted in the formation of metabolites which could be detected by TLC and GLC. 5β-androstane-3,17-dione was incubated with a low fecal concentration and menadione with no metabolites being formed. Incubations with a high autoclaved fecal concentration under reductive conditions with this compound also failed to produce metabolites.

DISCUSSION

Earlier studies[15–17] have shown that the human intestinal microflora can metabolize C_{21} steroids, primarily by reductive reactions. Our experiments demonstrate that the fecal microflora can perform reductive and oxidative reactions on the androgens and estrogens. The only previous work in this field was the report by Erikson and Gustafsson[15] on the conversion of 3β-hydroxy-5-androstene-17-one to 5β-androstane-3α,17-diol by human ileal contents.

We have shown that incubates with fecal concentrations above 50 mg/ml produce reduction of the carbonyl group to a hydroxy group at the 3 and 17 position of androgens and at the 17 position of estrogens. In addition, double bonds at the 1 and 4 position of androgens are saturated. These reactions occur under aerobic or anaerobic conditions. That reduction occurs under aerobic growth conditions may be explained by a decrease in the oxidation–reduction potential of the buffer solution brought about by the high concentration of feces which contains many anaerobic bacteria. These organisms grow in the depth of the liquid medium and maintain a highly anaerobic, reductive environment. A low oxidation–reduction potential alone, however, does not result in steroid conversions since autoclaved feces cannot perform any of the reductive or oxidative reactions described in this report. Therefore, these metabolic conversions appear to be dependent on viable bacterial organisms and their enzymes.

Oxidation of the hydroxyl group at 17 position of testosterone and estradiol occurs when the fecal

Table 3. Major metabolites formed upon incubation of androgens and estrogens with human fecal extracts

Substrate	Fecal concentration	Major metabolites	Yield* (%)
5β-Androstane-3,17-dione	high	3α-hydroxy-5β-Androstan-17-one &	40
		5β-Androstane-3α,17β-diol	10
5β-Androstane-3,17-dione	low	4-Androstene-3,17-dione &	5
		3α-hydroxy-5β-Androstan-17-one	30
4-Androstene-3,17-dione	high	5β-Androstane-3,17-dione &	25
		3α-hydroxy-5β-Androstan-17-one	25
1,4-Androstadiene-3,17-dione	high	4-Androstene-3,17-dione &	45
		3α-hydroxy-5β-Androstan-17-one &	45
		5β-Androstane-3α,17β-diol	
Estrone	high	Estradiol	5–10†
			10–20§
Estradiol	low	Estrone	64
16α-Hydroxyesterone	high	Estriol	2–3
Estradiol-3-glucuronide	low	Estradiol	ND
Estrone-3-sulfate	low	Estrone	ND
Testosterone	low	4-Androstene-3,17-dione	5

* Measured by GLC.
† Aerobic.
§ Anaerobic.
ND not determined.

concentration is below 10 mg/ml. Addition of oxidizing agents is not necessary for this reaction. The introduction of double bonds at the 4 position of androstane-3,17-dione requires a low concentration of feces in the incubation mixture and oxidizing agents such as menadione or phenazine methosulfate.

Menadione is a precursor of vitamin K_2 and is synthesized by the bowel flora. It does not appear, however, that the concentration of endogenous menadione or vitamin K_2 in the feces is sufficient to permit the introduction of double bonds into the steroid nucleus. This does not exclude the possibility that this reaction occurs naturally in some area of the large bowel, since the concentration of vitamin K_2 or menadione may be higher in colonic contents than in fecal contents. (Absorption of the vitamin from the bowel may produce lower amounts in the feces.)

This study clearly demonstrates that the intestinal flora of humans can carry out a variety of reductive, oxidative and hydrolytic reactions on androgens and estrogens. Reductive reactions are observed at a high fecal concentration while the oxidative reactions occur at a low concentration, and in the case of double bond formation there is a requirement for the addition of an oxidizing agent. The fecal flora also is capable of hydrolyzing estrogen sulfate and glucuronide conjugates. A previous study[21] has shown that the intestinal flora of the rat can hydrolyze androgen and pregnane substituted sulfates and glucuronides. The rate of the hydrolytic reactions is directly proportional to the concentration of feces in the culture medium. These findings suggest that multiple androgen and estrogen conversions can occur in the large bowel.

The introduction of a double bond into the steroid nucleus confirms the earlier studies of Aries *et al.*[7] indicating that *Clostridium paraputrificum* can convert androstane-3,17-dione to 4-androstene-3,17-dione. It was not possible, however, to demonstrate in our experiments further aromatization of the steroid nucleus by the fecal flora. The 3,17 oxidoreduction of androgens and estrogens by the bacterial flora can produce shifts in the relative concentrations of estrone, estradiol, testosterone and androstendione in the bowel. The subsequent reabsorption of these compounds would result in changes in the circulating concentrations of estrogens and androgens.

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