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Biohydrogenation of Sterols by *Eubacterium* ATCC 21,408—*Nova* Species

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Biohydrogenation of Δ^5-steroids was studied *in vitro* by incubating various steroids with a pure culture of *Eubacterium* 21,408 in a brain-thioglycollate medium under anaerobic conditions.

1. *Eubacterium* 21,408, a strictly anaerobic bacterium, was isolated from rat cecal contents. It differed from any previously described *Eubacterium* species by its requirements for a Δ^5-3β-hydroxy steroid.

2. *Eubacterium* 21,408 reduced the 5,6-double bond of cholesterol, campesterol, β-sitosterol and stigmasterol, exclusively yielding the corresponding 5β-saturated derivatives. Similarly, the 4,5-double bond of allocholesterol was reduced to yield coprostanol. Neither the 7,8-double bond in 7-dehydrocholesterol or in lathosterol, nor the 22,23-double bond in the side chain of stigmasterol were reduced.

3. In the absence of a 3-hydroxyl function (*e.g.* in cholest-5-ene), when the 3-hydroxyl function was in the 3α-position (*e.g.* in epicholesterol) or was substituted (*e.g.* in 3β-chloro-cholest-5-ene and in cholesteryl esters), no saturation of the 5,6-double bond was observed.

4. When incubated with *Eubacterium* 21,408, the carbonyl group of 4-cholesten-3-one, was reduced to the corresponding 3β-hydroxyl group along with saturation of the 4,5-double bond. The bacterium also reduced the 3-oxo group of cholestanone and coprostanone to a 3β-hydroxyl group.

During their passage through the intestine, cholesterol and plant sterols such as β-sitosterol and campesterol are converted into the respective 5β-cholestan of 5β-stigmastan derivatives by microbial 5β-H reduction of the 5,6-double bond in the steroid nucleus. Coprostanol is not found in the feces of germ-free animals [1—3]. In conventional animals, coprostanol formation can be abolished by the administration of antibacterial agents [4—7]. In addition, hydrogenation of cholesterol has been conclusively demonstrated *in vitro* by anaerobic incubation of cholesterol with suspensions of feces, or with subcultures of feces containing undefined mixtures of several aerobic and anaerobic microorganisms [8—10]. However, all attempts to isolate a pure culture of a Δ^5-steroid-reducing microorganism were unsuccessful.

Biohydrogenation of Δ^5-sterols is of particular interest since this procedure yields the 5β-H derivatives. Conversely, catalytic hydrogenation almost exclusively yields the 5α-H derivatives. Moreover, coprostanol is less well absorbed than cholesterol and does not accumulate in the organism since it is preferentially eliminated, mainly *via* the bile salt pathway [11—13]. Although direct evidence was never presented, it has been assumed that microbial conversion of cholesterol into less efficiently absorbed coprostanol could affect the sterol balance of the host [14].

To investigate the implications to the host of 5β-H reduction of cholesterol and plant sterols in the intestine, we isolated a Δ^5-steroid-reducing microorganism in pure culture. The present paper describes its characteristics and its steroid-transforming capabilities.

A preliminary communication on this subject has been presented at the NATO Advanced Study Institute *The Germ-free Animals as a Tool in Research*, September 14—21, 1969, at Leuven, Belgium.

Trivial names. Cholesterol, 5-cholesten-3β-ol; coprostanol, 5β-cholestan-3β-ol; cholestanol, 5α-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; cholestanone, 5α-cholestan-3-one; cholesteryl chloride, 3β-chloro-cholest-5-ene; cholesteryl bromide, 3β-bromo-cholest-5-ene; epicholesterol, 5-cholesten-3α-ol; epicoprostanol, 5β-cholestan-3α-ol; allocholesterol, 4-cholesten-3β-ol; 7-dehydro-cholesterol, 5,7-cholestadien-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; cholesteryl stearate, 5-cholesten-3β-yl-stearate; cholesteryl acetate, 5-cholesten-3β-yl-acetate; cholesteryl heptadecanoate, 5-cholesten-3β-yl-heptadecanoate; campesterol, (24 *R*)-24-methyl-5-cholesten-3β-ol; 5β-campestanol, (24 *R*)-24-methyl-5β-cholestan-3β-ol; 5α-campestanol, (24 *R*)-24-methyl-5α-cholestan-3β-ol; β-sitosterol, (24 *R*)-24-ethyl-5-cholesten-3β-ol; 5β-stigmastanol, (24 *R*)-24-ethyl-5β-cholestan-3β-ol; 5α-stigmastanol, (24 *R*)-24-ethyl-5α-cholestan-3β-ol; stigmasterol, (24 *R*)-24-ethyl-5,22-cholestadien-3β-ol.

MATERIALS AND METHODS

Culture Media

The basal medium was a suspension of 300 mg of freeze-dried beef brain powder in 9 ml of thioglycollate medium without added dextrose (BBL-01-136C, Baltimore Biological Laboratories, Cockeysville, Maryland, U.S.A.) supplemented with 1% yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.) and 0.13% sodium thioglycollate. The freeze-dried beef brain powder contained about 120 mg cholesterol per g. This concentration of cholesterol supported adequate growth, and was in large excess of the amount required for checking transformation into coprostanol.

In studies on the biohydrogenation of β-sitosterol, campesterol, or stigmasterol, 15 g of brain powder were extracted by refluxing with 100 ml acetone in a Soxhlet apparatus for 12 h. This treatment yielded about 10 g of cholesterol-free extracted-brain powder. To 300 mg of this powder were added 8 ml of the basal medium and 20 mg of the plant sterol or steroid hormone suspended with 20 mg lecithin (E. Merck, Darmstadt, Germany) in 1 ml water.

Anaerobic Techniques

Dissolved oxygen was removed from the culture media by boiling for 30 min under a stream of purified nitrogen in a stainless-steel container fitted with nitrogen inlet and outlet valves. Thereafter the tubes were cooled to room temperature while under nitrogen and immediately inoculated with 1 ml of rat cecal content or with 0.5 ml of an active culture. After the excess cotton of the plugs of the tubes was burned off, the plugs were pushed into the tubes, soaked with 0.5 ml of a solution of 40% pyrogallic acid followed by 0.5 ml of 10% $NaHCO_3$ and sealed with rubber stoppers. All cultures were incubated at 37 °C for 48 h to 7 days. The oxidation-reduction potential of the culture medium at the time of inoculation was −170 mV at pH 7.

Although this procedure could be successfully applied to transfer large inocula (0.5 to 1 ml) of mixed cultures, growth from small inocula (*e.g.* a loopful) was very irregular and unreliable. However, growth from diluted inocula could be obtained when the deaerated media were further prereduced for 48 h in a GasPak (BBL, Cockeysville, Maryland, U.S.A.) anaerobic system, and when transfers to the fresh medium were performed in an oxygen-free atmosphere. For this purpose the GasPak container with the prereduced media was brought into a Trexler plastic isolator (H. J. Kleinfeld, Hannover, Germany) as used in gnotobiotic experiments. The transfer lock of the isolator was exhausted and refilled three times with nitrogen (purified over copper wire at 400 °C, less than 1 vol./million oxygen), and once with 5% hydrogen in 95% nitrogen. The isolator chamber (110 × 60 × 60 cm) was kept under continuous anaerobiosis by recirculating a gas mixture of 5% hydrogen in 95% nitrogen over 400 g of palladium catalyst (Deoxo-D-catalyst, Englehard, Roma, Italy) placed inside the isolator. Under these conditions, the oxidation-reduction potential of the culture medium at the time of inoculation was −220 to −240 mV at pH 7. By use of this procedure all dilutions and subcultures could be made in the absence of oxygen and growth was obtained from diluted inocula.

When solid media were used, 1.5% agar was added to the liquid medium and the concentration of cholesterol was increased up to 5 mg/ml by addition of cholesterol in lecithin. The medium was dispensed into plastic petri dishes and prereduced for 72 h in a GasPak anaerobic jar before being introduced into the anaerobic chamber. After inoculation, the petri dishes were again placed in a GasPak jar with freshly activated carbon dioxide and hydrogen generator.

Production of acid and gas from carbohydrates was studied, using thioglycollate medium supplemented with 200 mg of whole-brain powder. The pH of the medium was determined after 3 and 7 days of incubation. The amount of acid produced was measured by titration of the culture with 0.1 N KOH to the pH of the control cultures incubated for the same period on the medium without carbohydrate. Production of gas was estimated by the appearance of gas bubbles in the sticky medium.

Extraction and Purification of the Reaction Products

When small amounts of sterols were extracted for analysis by thin-layer chromatography and gas-liquid chromatography, the contents of the tubes were transferred to 100-ml flasks, 30 ml of a solution of 5% KOH in ethanol were added and the suspension was refluxed for 2 h. After cooling, 30 ml water was added and the sterols were extracted three times with 30 ml petroleum ether (b.p. 40–60 °C). The petroleum ether layers were combined, washed with water until the wash-water was neutral, dried with sodium sulfate, filtered, and concentrated *in vacuo*.

When the reaction products were isolated for crystallization and determination of the physical characteristics, they were first purified by column chromatography on silica gel. To prepare coprostanol, for instance, 10 g crystalline cholesterol was suspended with 6 g lecithin in 700 ml brain-thioglycollate medium. After fermentation, the culture was extracted four times with an equal volume of petroleum ether. The extracts were pooled, washed with water, dried on Na_2SO_4, filtered, and evaporated under reduced pressure. The yellow oily residue was taken up in 400 ml 10% KOH in ethanol, and saponified by refluxing for 4 h. After cooling, 400 ml water was

added and the alkaline solution was extracted four times with an equal volume of petroleum ether. The organic layers were pooled, washed with water, dried, filtered and evaporated. The oily residue was taken up in 50 ml petroleum ether and filtered on a chromatography column (500 × 34 mm) prepared with 300 g silica gel (Merck "*unter* 0.08 mm"). Chromatography was carried out by stepwise elution with increasing concentrations of ethyl acetate in petroleum ether according to the following scheme: 200 ml petroleum ether, four portions of 500 ml petroleum ether—ethyl acetate (99:1, 98:2, 96:4, 94:6, v/v), followed by 2000 ml petroleum ether—ethyl acetate (92:8, v/v). Fractions of 25 ml were collected and examined by thin-layer chromatography. Coprostanol eluted from the column with the 8% ethyl acetate fraction and was well separated from cholesterol. Fractions containing the pure compound were pooled, evaporated, and dried *in vacuo* over P_2O_5. After recrystallization from dry acetone or ethanol, a white crystalline product was obtained. When less product was to be purified, the same procedure was followed but the amounts of the different solvents and reagents, as well as the dimensions of the silica gel column, were proportionally reduced.

Analytical Procedures

Qualitative and quantitative analysis of the sterol mixtures was carried out using different thin-layer and gas-liquid chromatography systems. The identity of the compounds was confirmed by mass spectrometry. The sterols were analyzed, either in the unsubstituted form, or as the trimethylsilyl ether derivatives. The latter were prepared by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine, for 30 min at room temperature [15].

Thin-Layer Chromatography. This was carried out on chromatoplates (20 × 20 cm) of silica gel G (Merck, Darmstadt), 0.25-cm thickness, using petroleum ether—ethyl acetate, or other suitable solvent systems for irrigation. The resolved steroids were visualized by phosphomolybdic acid reagent, or by a charr spray consisting of 50% sulfuric acid containing 0.6% potassium dichromate and heating at 180 °C. Spots to be scraped off for further analysis were visualized by iodine vapors.

Gas-Liquid Chromatography. The relative retention times (t_R) of unknowns were compared to those of reference products. Two types of apparatus were used. The first was a temperature-programmed instrument provided with an argon ionization detector. The straight column (120 cm × 4 mm i.d.) was packed with 1% JXR on Gas Chrom Q, 100—120 mesh. The second was a Pye series 104 instrument (Pye-Unicam, Cambridge, U.K.), provided with a flame ionization detector. Nitrogen was used as the carrier gas. The coiled column (140 cm × 4 mm i.d.) was packed with 3% QF-1 on Gas Chrom Q, 100 to 120 mesh. This column was kept at a constant temperature of 216 °C. The temperatures of flash heater and detector were 270—280 °C and 250 °C, respectively. The stationary phases were obtained as "pretested packing" from Applied Science Laboratories, Inc. (State College, Pennsylvania, U.S.A.).

Mass Spectrometry. Mass spectra of pure sterols were recorded by using the direct insertion system of an AEI-MS12 mass spectrometer (AEI, Manchester, U.K.) at an ion-source temperature of 120 °C to 140 °C. Unpurified samples and trimethylsilyl ethers were run *via* a gas-liquid chromatography mass spectrometry combination technique. The temperature of membrane separator (V-5620 molecular separator, Varian, Palo Alto, Calif., U.S.A.) and ion-source was maintained at 220—250 °C. Gas-liquid chromatography was carried out on a Pye series 104 apparatus with helium as the carrier gas.

Reference Sterols

Crystalline cholesterol and stigmasterol were obtained from E. Merck (Darmstadt). β-Sitosterol was also from E. Merck (Darmstadt) but contained about 10% campesterol. Campesterol (99% pure) was obtained from Applied Science Laboratories, Inc. (State College, Pennsylvania, U.S.A.). Epicholesterol, cholesteryl chloride, cholesteryl acetate and lathosterol were obtained from Mann Research Laboratories (New York, U.S.A.). Cholestanol, 4-cholesten-3-one, 5-cholesten-3-one, cholestanone, coprostanone, allocholesterol, epicoprostanol, and 7-dehydrocholesterol were obtained from Steraloids, Inc. (Pawling, New York, U.S.A.). Coprostanol was purchased from K & K Laboratories, Inc. (Plainview, New York, U.S.A.). 7-Dehydrocholesterol was impure and contained lathosterol and traces of cholesterol. The other products were at least 99% pure or were purified before being used. 5α-Stigmastanol and 5α-campestanol were prepared by hydrogenation of stigmasterol or campesterol, at room temperature, overnight in a Parr reaction apparatus (Parr Instrument Co., Moline, Illinois, U.S.A.) at a pressure of 50 lb/in² hydrogen and using 250 mg PtO_2 on charcoal for 1 g sterol dissolved in 500 ml absolute ethanol. After separation from the catalyst by filtration, the reduced sterol was crystallized from ethanol.

RESULTS

Isolation of Eubacterium *21,408*

One ml of rat cecal content was subcultured anaerobically in the basal brain-thioglycollate medium. Only small amounts of coprostanol were formed in the primary culture, but the reducing activity gradually increased during successive subculturing

until, at the 15th transfer, 90% of the substrate was hydrogenated in less than 72 h. From serial 10fold dilutions in the basal medium, coprostanol-producing subcultures were obtained which consisted of no more than 5 to 8 different species. These subcultures were plated out on the basal medium plus 1.5% of agar in petri dishes, but none of the pure strains which were started from single colonies produced coprostanol.

A pure strain was isolated from a mixed culture of *Clostridium perfringens* associated with a small gram-positive bacterium. A 1-to-5 dilution of this culture in prereduced diluent (1% yeast extract plus 0.2% sodium thioglycollate) was passed through a syringe fitted with a Millipore filter of average pore diameter of 0.8 μm to remove all aggregates and small particles. Subcultures were started from 2fold serial dilutions of the clarified filtrate. The whole procedure was carried out in the anaerobic isolator using media and diluents which were prereduced. Among the subcultures showing growth, those started from the highest dilutions of the filtrate contained a pure culture of the cholesterol-reducing microorganisms. When this strain was spread on the surface of brain-thioglycollate agar pinpoint watery colonies of 0.05-mm average diameter developed after 7 days of incubation under strictly anaerobic conditions.

Table 1. *Growth of* Eubacterium *ATCC 21,408 on various culture media*

All media were incubated anaerobically as described under Materials and Methods. Thioglycollate medium was used in culture media 1—11

Culture medium	Growth
1. Thioglycollate medium, without added dextrose	0
2. + 1% yeast extract	0
3. + 10% blood or serum	0
4. + 0.2% cholesterol in lecithin	++
5. + 3% whole brain powder[a]	+++++
6. + 3% sterol-free brain powder[b]	0
7. + 3% sterol-free brain powder and 0.2% cholesterol in lecithin	++++
8. + 3% whole-liver powder	++
9. + 3% whole-liver powder and 0.2% cholesterol in lecithin	++++
10. + 3% meat powder[c]	0
11. + 3% meat powder and 0.2% cholesterol in lecithin	++++
12. Cooked meat medium	±
13. As 12, + 0.2% cholesterol in lecithin	++++
14. Other media[d]	0

[a] Lyophilized beef brain powder.
[b] Acetone-extracted lyophilized beef brain powder.
[c] Lyophilized veal powder.
[d] Other media used were pleuropneumonialike organisms broth, trypticase soy broth, thioglycollate medium and dextrose, Todd-Hewitt broth, brain heart infusion, reinforced clostridial medium.

Sodium thioglycollate was added to a final concentration of 0.075%. Growth occurred in the media listed upon addition of 0.2% cholesterol in lecithin.

Characteristics of Eubacterium *21,408*

The cholesterol-reducing microorganism was a small anaerobic bacterium (0.3 to 0.5 μm by 1 μm). It was gram-positive in very young cultures and after mild alcohol treatment, becoming gram-negative after prolonged treatment with alcohol or in cultures more than 3-days old. In young cultures, many diploforms were observed. Branching or formation of chains or filaments were not observed.

As shown by the data from Table 1, the microorganism failed to grow on the common culture media such as: fluid thioglycollate medium, brain-heart infusion broth, trypticase soy broth, Todd-Hewitt broth, cooked-meat medium, thioglycollate medium supplemented with 3% freeze-dried beef meat powder. Growth on these media was not improved by addition of 10% serum or blood, or by yeast extract up to 1%. Conversely, all media supported growth when supplemented with cholesterol in lecithin, 1—2 mg/ml. Cholesterol could be replaced by Δ^5-3β-hydroxy plant sterols such as β-sitosterol, campesterol and stigmasterol. However, on further subculture on the aforementioned media supplemented with cholesterol in lecithin, growth became irregular and many cultures were lost before the 10th transfer.

The strain could be propagated without any restriction on thioglycollate medium plus 3% whole-brain powder. So far, more than 300 successive transfers have been made on this medium without loss of viability or steroid-transforming activity. No growth occurred on the basal medium in which the whole-brain powder was replaced by acetone-extracted cholesterol-free brain powder. Growth on this medium could be restored by addition of crystalline cholesterol, Δ^5-3β-hydroxy plant sterols, or steroid hormones such as pregnenolone or androstenolone. Good growth also occurred in thioglycollate medium supplemented with 3% liver powder or meat powder plus cholesterol in lecithin, 1—2 mg/ml. These results demonstrated that the bacterium had an absolute requirement for cholesterol or another unsaturated sterol. The data in Table 2 indicate that the growth stimulating activity of various Δ^5-sterols is limited to those compounds which can be hydrogenated by *Eubacterium* 21,408. This should be compatible with the hypothesis that these sterols are essential acceptors of metabolic hydrogen.

Growth was obtained over a broad range of temperatures (26—44 °C), the optimum being 37 to 40 °C. In all experiments, old as well as young cultures were killed by heating for 10 min at 60 °C, indicating that under our experimental conditions formation of spores did not occur.

Routine tests for production of indole, acetyl-methyl-carbinol, H_2S or catalase were negative.

Table 2. *Growth of* Eubacterium *21,408 on various substrates* 10 or 20 mg of substrate was suspended in lecithin, added to 9 ml thioglycollate medium with 3% extracted brain powder. Transformation of substrate was measured after a 7-day incubation

Substrate	Growth	Transformation of substrate
Cholesterol and its analogues:		
Cholesterol	++++	> 95% 5β-H reduction
4-Cholesten-3-one	+++	40% coprostanol
Cholesteryl acetate	0	no reduction
Cholesteryl chloride	0	no reduction
Plant sterols:		
β-Sitosterol	++++	> 95% 5β-H reduction
Campesterol	++++	> 95% 5β-H reduction
Stigmasterol	++++	> 95% 5β-H reduction
Hormones:		
5-Androsten-3β-ol-17-one	+++	> 80% 5β-H reduction
5β-Androstan-3β-ol-17-one	0	no reduction of 17-one
5α-Androstan-3β-ol-17-one	0	no reduction of 17-one
5-Pregnen-3,20β-diol	+++	> 80% 5β-H reduction

Reduction of NO_3^- to NO_2^- was not observed. Motility was never observed. The microorganism produced small amounts of acid (1—1.5 ml 0.1 N acid/10 ml) and gas from glucose, lactose, galactose and melibiose. Production of acid and gas was not observed in media supplemented with maltose, D-fructose, raffinose, mannose, trehalose, melezitose, xylose or rhamnose.

The comparative study on growth of the Δ^5-steroid-reducing bacterium in various culture media demonstrated that this microorganism did not develop unless the culture medium contained a Δ^5-sterol. Further experiments were designed to determine the minimal concentration of cholesterol required for optimal growth. Varying amounts of cholesterol were supplied either as a suspension in lecithin or by substituting whole-brain powder for part of the sterol-free brain powder. Growth was very sparse on media containing 0.125 or 0.25 mg cholesterol/ml, and all strains were lost on further subculture. With media containing cholesterol in amounts between 0.25—2.0 mg/ml, growth was directly proportional to the amount of sterol present. At least 1 mg sterol/ml was required for maintenance of the cultures, and maximal growth was not obtained unless the medium contained 1.5—2 mg cholesterol/ml.

Although the final taxonomy of this microorganism remains to be established, its general characteristics are compatible with those of the genus *Eubacterium*. However, the special growth requirements and the ability to produce coprostanol from cholesterol differentiate this strain from any species listed in the genus *Eubacterium* by Prevot *et al.* [16] or in Bergey's manual [17]. The microorganism has been registered by the American Type Culture Collection as *Eubacterium* ATCC 21,408.

Biohydrogenation of Cholesterol

Eubacterium 21,408 was incubated with 10 g cholesterol in brain-thioglycollate medium. Thin-layer chromatography of the crude extract of the culture showed one major spot with the R_F of coprostanol, and one minor spot with the R_F of cholesterol. Similar results were obtained by gas-liquid chromatography. At least 90% of the cholesterol was converted to coprostanol.

When the extract was chromatographed on a column of silica gel, the 8% ethyl acetate fraction yielded 7.95 g of a pure amorphous product. After recrystallization from hot dry acetone or hot absolute ethanol 7.25 g of white needles was obtained, the melting point of which was identical to that of reference coprostanol that had been crystallized from the same solvent (101—103 °C). The elemental analysis calculated for $C_{27}H_{48}O$ (coprostanol) was: C, 83.43%; H, 12.44%. The values found were: C, 83.23%; H, 12.29%. On thin-layer chromatography in petroleum ether—ethyl acetate (9:1, v/v), the substance showed only one spot, corresponding to that of authentic coprostanol (R_F 0.36). In this system the 5β-stanols (R_F 0.36) were well separated from the 5,6-unsaturated sterols (R_F 0.25) and the 5α-sterols (R_F 0.23), from coprostanone (R_F 0.77) and from 4-cholesten-3-one (R_F 0.48). By gas-liquid chromatography on 1% JXR or 3% QF-1 only one peak was detected, corresponding to that of reference coprostanol. On QF-1, the relative retention time (t_R 2.04) referred to 5α-cholestane, corresponded to that of reference coprostanol and differed from that of cholesterol (t_R 2.26), cholestanol (t_R 2.43), 4-cholesten-3-one (t_R 6.58), cholestanone (t_R 4.35) and coprostanone (t_R 4.00). The QF-1 phase was also able to separate coprostanol (t_R 2.04) from its epimer, epicoprostanol (t_R 2.26). No epicoprostanol could be detected in the crude extract of the culture or in the crystallized product. The mass spectrum of the compound (Fig. 1) was identical to that of authentic coprostanol. The molecular ion, found at *m/e* 388, corresponded to that of coprostanol (mol. wt of coprostanol is 388.66). Other fragment ions were found at *m/e* 373 (M-CH_3), *m/e* 370 (M-H_2O), *m/e* 355 (M-CH_3-H_2O), *m/e* 331 (M-C_3H_5O, a neutral fragment containing carbon atoms 1, 2 and 3 of ring A less one hydrogen atom), *m/e* 233 (M-side chain-ring D-hydrogen), *m/e* 215 (*m/e* 233-H_2O).

The 1% ethyl acetate fraction always contained a certain amount of coprostanone (± 2% of the total sterols), whereas trace amounts of 4-cholesten-3-one (less than 1% of the total sterols) eluted with the 8% ethyl acetate fraction. These substances were identi-

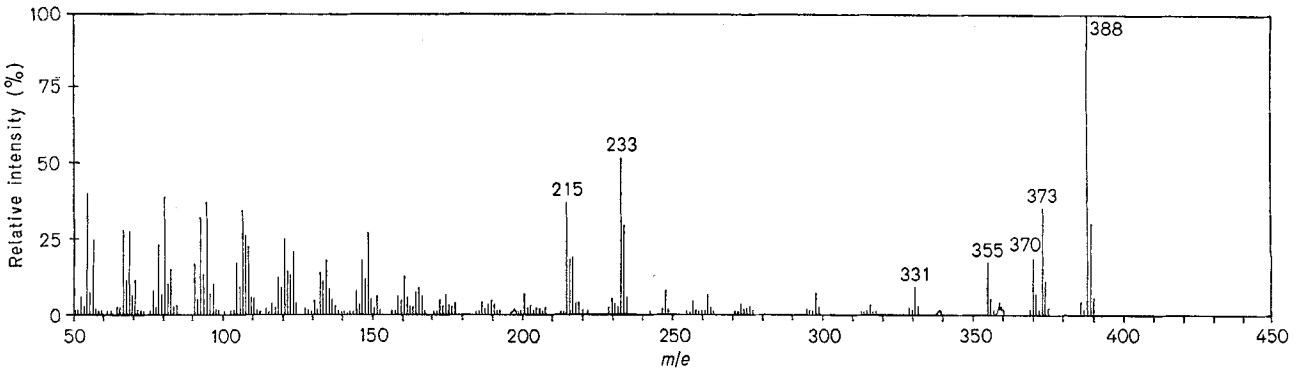

Fig. 1. *Mass spectrum of 5β-cholestan-3β-ol prepared by incubating* Eubacterium *21,408 with cholesterol*

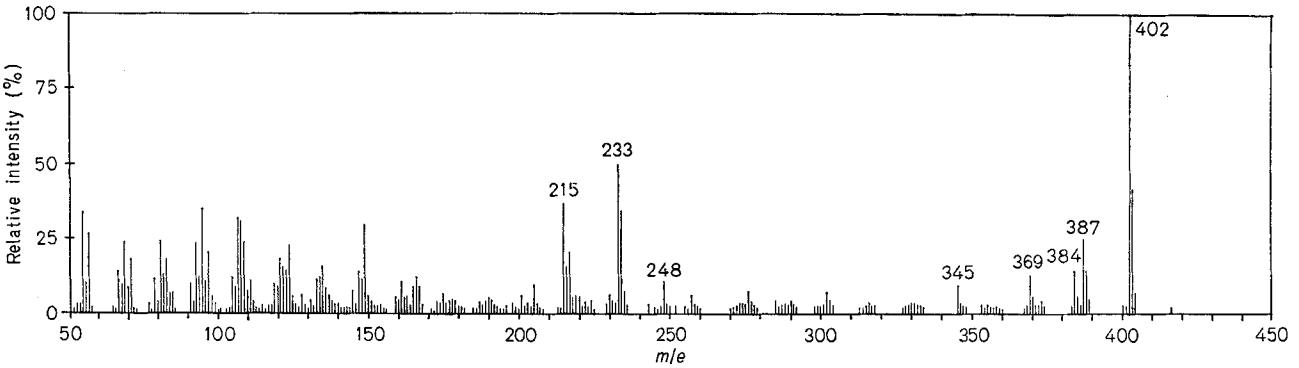

Fig. 2. *Mass spectrum of (24 R)-24-methyl-5β-cholestan-3β-ol prepared by incubating* Eubacterium *21,408 with campesterol*

fied by comparing the R_F values on thin-layer chromatography and the t_R values on gas-liquid chromatography with those of the reference substances.

BIOHYDROGENATION OF PHYTOSTEROLS

The possible influence of the structure of the side chain on biohydrogenation of the 5,6-double bond in the steroid nucleus was investigated by incubating *Eubacterium* 21,408 with several phytosterols.

Campesterol, (24 R)-24-Methyl-5-cholesten-3β-ol

When incubated anaerobically with *Eubacterium* 21,408, more than 90% of the campesterol was transformed into 5β-campestanol. The reaction product was isolated as described for coprostanol. The crystalline substance had a melting point of 121—122 °C. The compound showed only one peak in various thin-layer and gas-liquid chromatography systems. On thin-layer chromatography in petroleum ether—ethyl acetate (9:1, v/v) it was less polar (R_F 0.36) than either the starting compound (R_F 0.25) or 5α-campestanol (R_F 0.23). The hydroxyl group on C-3, being axial in the 5β- or A:B *cis* configuration, might be expected to decrease the polarity of the sterol. On gas-liquid chromatography on 3% QF-1 the transformed product (t_R 2.57) moved ahead of campesterol (2.94) and 5α-campestanol (t_R 3.17). This shift of the t_R was similar to that of coprostanol (t_R 2.04) compared to cholesterol (t_R 2.26) and cholestanol (t_R 2.43).

The mass spectrum, recorded as steroid alcohol, is shown in Fig. 2. The peak of the molecular ion was found at *m/e* 402 (mol.wt of 5β-campestanol = 402.676). Other fragment ions were found at *m/e* 387 (M-CH_3), *m/e* 384 (M-H_2O), *m/e* 369 (M-CH_3-H_2O), *m/e* 345 (M-C_3H_5O from C-1, C-2 and C-3 of ring A), *m/e* 248 (M-side chain-fragment containing carbon atoms 16 and 17 of ring D), *m/e* 233 (M-side chain-ring D-hydrogen), *m/e* 215 (*m/e* 233-H_2O). This, together with the thin-layer and gas-liquid chromatography data, led to the conclusion that *Eubacterium* 21,408 transformed campesterol into 5β-campestanol.

β-Sitosterol, (24 R)-24-Ethyl-5-cholesten-3β-ol

When incubated anaerobically with *Eubacterium* 21,408, more than 90% of the β-sitosterol was reduced to 5β-stigmastanol. The reduction product was isolated and purified as described for coprostanol;

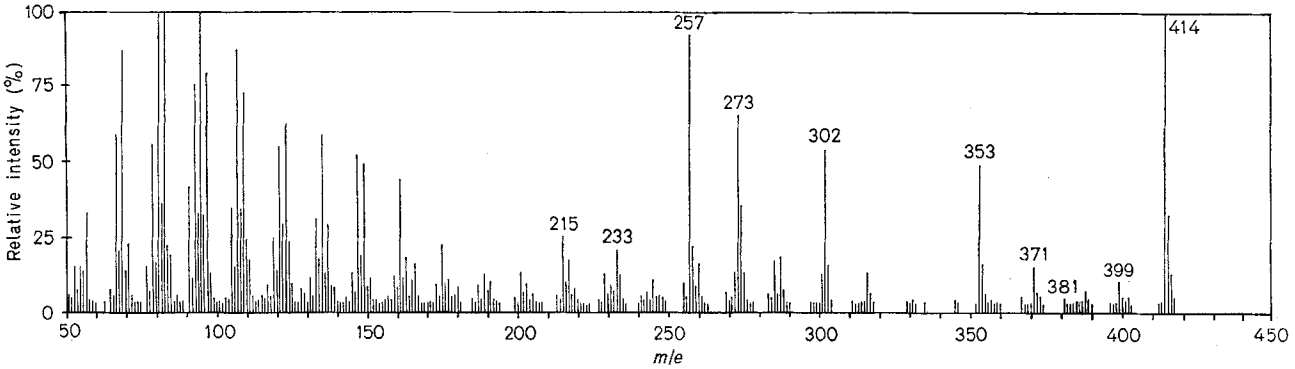

Fig.3. *Mass spectrum (24* R*)-24-ethyl-5β-cholest-22-en-3β-ol prepared by incubating* Eubacterium *21,408 with stigmasterol*

it yielded a crystalline compound with a melting point of 127—128 °C. This was in agreement with the data (m.p. 126—127 °C) reported by Neiderhiser [18]. However, on gas-liquid chromatography the compound showed two peaks. The first peak accounted for about 10% of the product, and corresponded to 5β-campestanol presumably derived from campesterol present in the starting compound. The second peak (t_R 1.64 on JXR; 2.97 on QF-1) was well separated from that of the starting compound (t_R 1.70 on JXR; 3.42 on QF-1) and from that of 5α-stigmastanol (t_R 1.70 on JXR; 3.72 on QF-1). The mass spectrum corresponded to that of a saturated derivative of β-sitosterol. The peak of the molecular ion was found at *m/e* 416 (mol.wt of 5β-stigmastanol = 416.706). Other important fragment ions were seen at *m/e* 401 (*M*-CH_3), *m/e* 398 (*M*-H_2O), *m/e* 383 (*M*-CH_3-H_2O), *m/e* 359 (*M*-C_3H_5O, loss of C-1, 2 and 3), *m/e* 248 (*M*-side chain-fragment containing carbon atoms 16 and 17 of ring D), *m/e* 233 (*M*-side chain-ring D-hydrogen), *m/e* 215 (*m/e* 233-H_2O). From these data it was concluded that the 5,6-double bond of β-sitosterol was hydrogenated yielding 5β-stigmastanol.

Stigmasterol, (24 R*)-24-Ethyl-5,22,cholestadien-3β-ol*

When stigmasterol was incubated with *Eubacterium 21,408*, more than 90% of the starting product was converted into a compound that, after purification as described for coprostanol, could be crystallized from dry ethanol. The melting point was 151—152 °C, this corresponded to that reported [18] for 5β-stigmast-22-en-3β-ol (m.p. 153—155 °C).

The mass spectrometric data of the product of biotransformation of stigmasterol (Fig.3), indicated a 5β-stigmast-22-en-3β-ol structure with a peak for the molecular ion at *m/e* 414 (mol.wt of 5β-stigmast-22-en-3β-ol = 414.72). Other characteristic peaks were found at *m/e* 399 (*M*-CH_3), *m/e* 381 (*M*-CH_3-H_2O), *m/e* 371 (*M*-$(CH_3)_2CH$ radical from the side chain), *m/e* 353 (*m/e* 371-H_2O), *m/e* 302 (cleavage of the side chain between C-20 and C-22 with loss of one hydrogen atom), *m/e* 273 (*M*-side chain-2H, with rearrangement of a hydrogen atom to the neutral fragment), *m/e* 257 (*M*-side chain-H_2O), *m/e* 233 (*M*-side chain-ring D-hydrogen), *m/e* 215 (*m/e* 233-H_2O). The fragmentation pattern of the steroid nucleus corresponded to that of the other 5β-saturated-3β-hydroxy sterols. These results indicated that *Eubacterium* 21,408 reduced the 5,6-double bond, whereas the 22,23-double bond was unaffected.

Reference 5β-stigmast-22-en-3β-ol or 5α-stigmast-22-en-3β-ol were not available. However, on thin-layer chromatography in petroleum ether—ethyl acetate (9:1, v/v) the transformed product (R_F 0.36) was clearly separated from the 3β-hydroxy-Δ^5-steroids (cholesterol, campesterol, β-sitosterol and stigmasterol, R_F 0.25), and from their respective 5α-saturated derivatives (R_F 0.23). The R_F-value was identical to that of the 5β-saturated derivatives of these substances (R_F 0.36). The thin-layer chromatography data also indicated that the compound was less polar than stigmasterol, and this was compatible with an axial hydroxyl group on C-3 and a *cis*-fusion of the A/B rings. More indications that the transformed compound was the 5β-saturated derivative of stigmasterol, not the 5α-saturated analogue, were obtained from the gas-liquid chromatography data. On the JXR stationary phase, cholesterol, β-sitosterol and campesterol could not be separated from their respective 5α-saturated derivatives, and one would expect a similar behavior for stigmasterol and 5α-stigmast-22-en-3β-ol. However, the transformed product (t_R 1.55 on JXR; 2.64 on QF-1) eluted in front of stigmasterol (t_R 1.60 on JXR; 2.95 on QF-1), as did the other 5β-saturated compounds with respect to the corresponding 5,6-unsaturated-analogues.

Further evidence for the selective 5β-hydrogenation of the 5,6-double bond was obtained by catalytic hydrogenation of the 22,23-double bond of the product isolated from the fermentation medium. Therefore 2.011 g of the transformed product were acetylated with dry pyridine—acetic anhydride

(1:1, v/v) under reflux at 65 °C during 4 h and recrystallized from ether (yield 2.066 g; m.p. 139—141 °C). One gram of the acetylated derivative was dissolved in 100 ml acetic acid and hydrogenated under a pressure of 0.5 lb/in^2 hydrogen with 125 mg PtO_2 catalyst, during 2 h. The hydrogenated product was recrystallized from ethanol—water (yield 0.616 g; m.p. 87—88 °C), and saponified by refluxing with 5% KOH in ethanol during 4 h. The free sterol was extracted with ether and recrystallized from dry methanol, yielding 433 mg of a white crystalline product (m.p. 127—129 °C). On thin-layer and gas-liquid chromatography this product was identical to 5β-stigmastanol, the compound obtained by biohydrogenation of β-sitosterol, and was clearly separated from 5α-stigmastanol. From these experiments it was concluded that biohydrogenation of stigmasterol by *Eubacterium* 21,408 yielded 5β-stigmast-22-en-3β-ol.

BIOHYDROGENATION OF ALLO-CHOLESTEROL AND 7-DEHYDROCHOLESTEROL

Allocholesterol (4-Cholesten-3β-ol)

When incubated anaerobically with *Eubacterium* 21,408, allocholesterol was almost quantitatively transformed. On thin-layer and gas-liquid chromatography, the reaction product was indistinguishable from reference coprostanol. After purification by column chromatography on silica gel, the transformed compound was crystallized from ethanol. The melting point (101—103 °C) was identical to that of coprostanol. Mass spectral analysis showed the peak of the molecular ion at *m/e* 388, with a distribution of *m/e* signals corresponding to those of a sample of authentic coprostanol.

7-Dehydrocholesterol (5,7-Cholestadien-3β-ol) and Lathosterol (5α-Cholest-7-en-3β-ol)

Commercial 7-dehydrocholesterol (provitamin D_3) was not pure: thin-layer chromatography revealed other spots than 7-dehydrocholesterol, *e.g.* lathosterol; by gas-liquid chromatography a small amount of cholesterol could also be detected. No attempts were made to purify commercial 7-dehydrocholesterol to eliminate the trace amounts of cholesterol present. It is also known that 7-dehydrocholesterol is unstable. However, no differences were observed between freshly prepared culture media before and after sterilization. Moreover, after 7 days incubation at 37 °C in the dark, the amount of 7-dehydrocholesterol recovered from the uninoculated blanks was comparable to that in freshly prepared media. The conditions of incubation were the same as for the other sterols, but the cultures were extracted with diethyl ether instead of petroleum ether because 7-dehydrocholesterol was incompletely extracted with the latter solvent. Since the product obtained after incubation of 7-dehydrocholesterol with *Eubacterium* 21,408 was not separated from coprostanol on thin-layer chromatography with a single development in petroleum ether—ethyl acetate (9:1, v/v), the "multiple development" method was used. This procedure consisted of two successive developments in petroleum ether—ethyl acetate (9:1, v/v) and allowed partial separation of coprostanol from the compound assumed to be 5β-cholest-7-en-3β-ol.

On thin-layer chromatography with multiple development, the extracts of the cultures with *Eubacterium* 21,408 showed two major spots. The R_F value of the first spot (R_F 0.22) corresponded to that of unmodified 7-dehydrocholesterol. The second spot (R_F 0.34) migrated somewhat more slowly than coprostanol (R_F 0.36). The size of this spot increased with the number of subcultures made on the 7-dehydrocholesterol medium. Concomitantly, the size of the spot of 7-dehydrocholesterol decreased, indicating that the microorganism became adapted to the new substrate. The minor spots, which were found in the extracts of the cultures, corresponded to trace amounts of lathosterol and coprostanol. They were assumed to be derived from traces of cholesterol and lathosterol present in the starting product.

The unidentified spot was eluted from preparative thin-layer chromatography plates and was subjected to mass spectrometry and gas-liquid chromatography. Reference 5β-cholest-7-en-3β-ol was not available. However, mass spectral analysis showed a fragmentation pattern compatible with that of 5β-cholest-7-en-3β-ol (Fig.4). The peak of the molecular ion was found at *m/e* 386. As the molecular weight of 7-dehydrocholesterol is 384.62, this indicated that one of the original double bonds was left unreduced. Other fragments ions were seen at *m/e* 371 (M-CH_3), *m/e* 353 (M-CH_3-H_2O), *m/e* 273 (M-side chain), *m/e* 231 (M-side chain-ring D-hydrogen), *m/e* 213 (*m/e* 231-H_2O).

On gas-liquid chromatography, the compound assumed to be 5β-cholest-7-en-3β-ol (t_R 1.31 on JXR, 2.04 on QF-1) was clearly separated from the starting compound (t_R 1.45 on JXR and 2.56 on QF-1), from 5α-cholest-7-en-3β-ol (t_R 1.45 on JXR, 2.56 on QF-1), from cholesterol (t_R 1.35 on JXR, 2.26 on QF-1), from allocholesterol (t_R 0.99 on JXR; 1.03 on QF-1) and from coprostanol (t_R 1.27 on JXR, 2.04 on QF-1). As reduction of the 7,8-double bond would have given rise to the formation of cholesterol and hence of coprostanol, it must be assumed that only the 5,6-double bond was reduced with formation of 5β-cholest-7-en-3β-ol.

Further evidence that *Eubacterium* 21,408 did not reduce the 7,8-double bond of 3β-hydroxy steroids was provided by the observation that lathosterol was not reduced to cholestanol. After 7-days incubation the unmodified lathosterol was quantitatively recov-

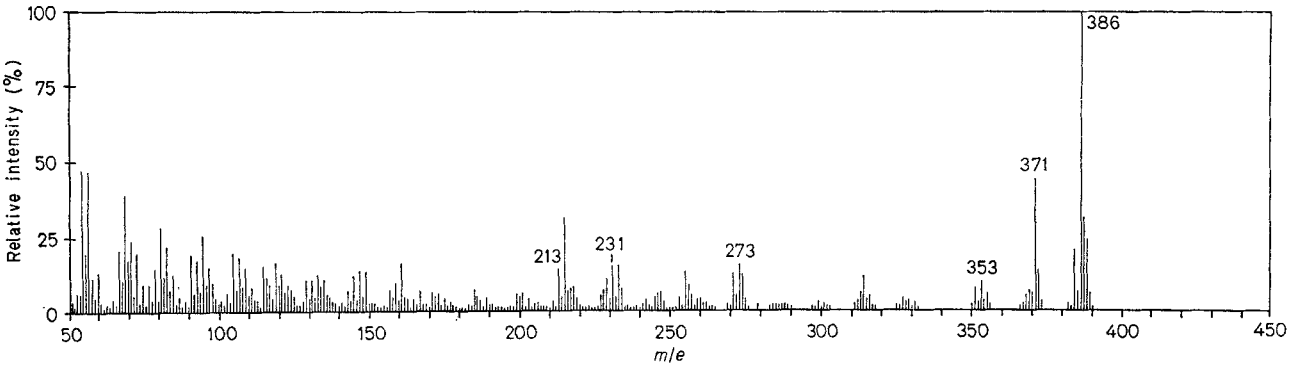

Fig. 4. *Mass spectrum of 5β-cholest-7-en-3β-ol prepared by incubating* Eubacterium *21,408 with 7-dehydrocholesterol.* The product was isolated by preparative thin-layer chromatography with "multiple" developments. Peaks at *m/e* 215 and *m/e* 233 are due to contamination of the sample by traces of coprostanol

ered from the culture medium. Under the same conditions, β-sitosterol was almost quantitatively reduced to 5β-stigmastanol.

LACK OF BIOHYDROGENATION OF CHOLESTERYL ESTERS, CHOLESTERYL HALIDES, 5-CHOLESTEN, AND EPICHOLESTEROL

As *Eubacterium* 21,408 failed to grow when incubated with cholesteryl esters, cholesteryl halides, 5-cholesten or epicholesterol in a cholesterol-free medium with acetone-extracted beef brain powder, the incubations were carried out in a medium containing half the optimal amount of campesterol (1 mg/ml) to assure good growth of *Eubacterium* 21,408.

Cholesteryl Esters

The following esters were examined: cholesteryl stearate, cholesteryl acetate and cholesteryl heptadecanoate. After extraction of the culture with petroleum ether, the ester fraction was separated from the free sterols by gradient elution chromatography on a silica gel column (11 × 1.5 cm). A gradient of polarity was obtained by pumping 150 ml of a solution of 20% (v/v) of ethyl acetate in petroleum ether (b.p. 40–60 °C) into a mixing chamber containing 100 ml petroleum ether. The free-sterol fraction contained 5β-campestanol almost exclusively, thus proving that the reductive processes were operating. The ester fraction was evaporated and saponified by refluxing in a solution of 3% potassium hydroxide in a mixture of equal volumes of toluene, isopropanol and methanol. The free sterols were extracted with petroleum ether after addition of an equal volume of water. They were quantitated and identified by gas-liquid and thin-layer chromatography. In all experiments, only cholesterol and no coprostanol could be detected in the ester fraction. The amount of cholesterol recovered from the ester fraction corresponded to the amount added as cholesteryl ester to the culture medium. These results were not influenced by repeated subculturing *Eubacterium* 21,408 on the medium with the esters, thus demonstrating that the microorganism did not become adapted to the esterified substrate.

Cholesteryl Chloride and Cholesteryl Bromide

When incubated with *Eubacterium* 21,408 3β-chloro-cholest-5-ene was not reduced, either in the presence or in the absence of campesterol or β-sitosterol. The substrate was recovered unmodified in amounts comparable to these in the blanks. Quantitative determinations were performed on a 3% QF-1 column which separated cholesteryl chloride (t_R 2.09) from the other sterols. On mass spectrometry, the fragmentation pattern of the substance assumed to be unmodified cholesteryl chloride was identical to that of the starting compound. Similar results were obtained with cholesteryl bromide.

Cholest-5-ene

Eubacterium 21,408 did not transform cholest-5-ene. The unmodified starting compound was quantitatively recovered from the cultures, whereas added campesterol was quantitatively transformed into 5β-campestanol. These results demonstrated that the lack of a hydroxyl group in the C-3 position prevented the 5,6-double bond to be hydrogenated by *Eubacterium* 21,408.

Epicholesterol (5-Cholesten-3α-ol)

In subsequent studies we investigated whether the orientation of the 3-hydroxy group would influence the biohydrogenation of the 5,6-double bond. Therefore, *Eubacterium* 21,408 was incubated with epicholesterol or with epicholesterol plus campesterol. When chromatographed as the free sterols, epicoprostanol and coprostanol were not separated by

gas-liquid chromatography on 1% JXR. On 3% QF-1, epicoprostanol (t_R 2.26) was separated from coprostanol (t_R 2.04) and from epicholesterol (t_R 2.19), but eluted together with cholesterol (t_R 2.26). The order of elution of the trimethylsilyl ethers from a QF-1 column was: epicholesterol plus coprostanol, epicoprostanol, cholesterol. By combination of the data obtained by chromatography of the free sterols and the trimethylsilylethers on QF-1, it was proven that no epicoprostanol was produced from epicholesterol. The starting compound was quantitatively recovered. It was concluded that a 3α-hydroxyl group, as found in epicholesterol, was not inverted, and interfered with biohydrogenation of the 5,6-double bond.

REDUCTION OF 3-OXO-STEROIDS

According to Schoenheimer *et al.* [19], 4-cholesten-3-one and coprostanone might be intermediates in the conversion of cholesterol into coprostanol. We investigated, therefore, whether *Eubacterium* 21,408 was able to grow on 3-oxo steroids and to produce the respective 3-hydroxyl derivatives.

Cholestanone (5α-Cholestan-3-one) and Coprostanone (5β-Cholestan-3-one)

On gas-liquid chromatography, the only reduction product detected in the cultures after incubation of *Eubacterium* 21,408 with cholestanone could not be separated from cholestanol. The identification was confirmed by thin-layer chromatography with petroleum ether—ethyl acetate (9:1, v/v) to which 0.5% bromine was added to permit the separation of cholesterol and cholestanol. Furthermore, oxidation with chromic acid yielded a ketone which on thin-layer and gas-liquid chromatography was identified as 5α-cholestan-3-one. Similarly, *Eubacterium* 21,408 reduced coprostanone into coprostanol. This demonstrated that *Eubacterium* 21,408 was able to reduce 5β- or 5α-saturated-3-oxo steroids into the respective 3β-ol derivatives. However, yields of cholestanol and coprostanol were poor, usually less than 25%, and most cultures were lost after a few transfers on the medium with either cholestanone or coprostanone, because *Eubacterium* 21,408 failed to develop adequately on this substrate.

4-Cholesten-3-one

Incubation of 4-cholesten-3-one with *Eubacterium* 21,408 yielded two reduction products: coprostanone and coprostanol. On thin-layer and gas-liquid chromatography the crude extracts of the cultures were shown to contain three neutral sterols. The first compound (R_F 0.48; t_R 1.54 on JXR and 6.58 on QF-1) accounted for 40 to 45% of the neutral sterol fraction and could not be separated from 4-cholesten-3-one, the starting compound. The second compound accounted for 5 to 10% of the total neutral sterols and corresponded to coprostanone (R_F 0.77; t_R 1.35 on JXR and 4.00 on QF-1). The third spot (R_F 0.36; t_R 1.27 on JXR and 2.04 on QF-1) corresponded to authentic coprostanol and accounted for 45 to 55% of the sterol fraction. It would have been of interest to determine whether 5-cholesten-3-one was reduced as well but this compound was unstable and under the conditions of the experiment it was spontaneously transformed into 4-cholesten-3-one even in the uninoculated blanks.

DISCUSSION

Recently it has been demonstrated in several laboratories that many strict anaerobic intestinal microorganisms require culture media with a very low oxidation-reduction potential and are so sensitive to oxygen that the whole isolation procedure has to be carried out in complete absence of air, using prereduced culture media [20—22]. In the present studies a similar system was applied to isolate a Δ^5-steroid-reducing microorganism from mixed cultures derived from rat cecal contents.

The Δ^5-steroid-reducing microorganism isolated from the intestine of the rat is a small gram-positve strictly anaerobic bacterium which does not conform to any of the species listed in Bergey's [17] or Prévot's manuals [16]. On the basis of its morphological and physiological characteristics it has been tentatively classified into the genus *Eubacterium*. However, it differs from any previously described *Eubacterium* species by its requirements for a Δ^5-3β-hydroxy steroid. In the range of 0.2—2 mg cholesterol/ml, growth of *Eubacterium* 21,408 was directly proportional to the amount of Δ^5-sterol available. The large amount of steroid required for maximal growth would indicate that cholesterol is not acting as a true growth factor. It seems more logical to assume that the 5,6-double bond of cholesterol, plant sterols and steroid hormones is acting as a hydrogen acceptor in the microorganism's metabolism.

Pure cultures of *Eubacterium* 21,408, reduced cholesterol to coprostanol with an efficiency exceeding 90%. Similarly, campesterol, β-sitosterol and stigmasterol were reduced to the respective 5β-stanols, thus indicating that the structure of the side chain was not of paramount importance for hydrogenation of the 5,6-double bond in the nucleus. The 22,23-double bond in the side chain of stigmasterol was left unreduced. This demonstrated that the biohydrogenating activities of *Eubacterium* 21,408 were primarily restricted to double bonds in the steroid nucleus. The 5,6-double bond in 7-dehydrocholesterol and the 4,5-double bond in allocholesterol was efficiently hydrogenated. However, the 7,8-double bond in 7-dehydrocholesterol and in lathosterol was not reduced.

According to Schoenheimer, microbiological conversion of cholesterol to coprostanol would involve the initial oxidation of cholesterol to 4-cholesten-3-one, followed by successive reduction to coprostanone and coprostanol [19]. On the other hand, Rosenfeld *et al.* [23] and Rosenfeld and Gallagher [24] eliminated the ketones from the pathway for coprostanol formation. In studies on the conversion of [3-^{2}H]- and [3-^{3}H]cholesterol to the corresponding labeled coprostanol, these investigators found that 60—80% of the label was retained after hydrogenation, and postulated a direct stereospecific reduction of the 5,6-double bond. These conclusions were invalidated in a recent study by Björkhem and Gustafsson [25] who demonstrated that conversion of cholesterol into coprostanol by cecal content of rats proceeds to at least 50% by means of the intermediate formation of 4-cholesten-3-one. Although the pathway for conversion of cholesterol into coprostanol by *Eubacterium* 21,408 remains to be established, the results of the present investigations lend support to Schoenheimer's hypothesis. We first demonstrated that the same bacterium which is capable of converting cholesterol into coprostanol, also converts 4-cholesten-3-one and coprostanone into coprostanol. However, the bacterium did not reduce cholesterol esters, cholesteryl halides, epicholesterol or 5-cholesten. From these data it would appear that the substituent at carbon-three is important for hydrogenation of the 5,6-double bond. Experiments in progress have also shown that about 70% of the label of [3β-^{3}H]cholesterol is lost during conversion to coprostanol by *Eubacterium* 21,408. This, together with the fact that production of coprostanol on preparative scale always yielded small amounts of 4-cholesten-3-one and coprostanone, tends to favor the theory of the indirect pathway for formation of coprostanol.

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