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Cholesterol Metabolism by *Treponema hyodysenteriae*

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The sterol content of cellular lipids of *Treponema hyodysenteriae*, the agent of swine dysentery, was determined. When cultured in lipid-depleted brain heart infusion broth containing vesicles made from [4-^{14}C]cholesterol and phosphatidylcholine, *T. hyodysenteriae* cells incorporated radioactive label. Most (95%) of this radioactivity was associated with bacterial membrane preparations. Lipids were extracted from radiolabeled cells and fractionated by silicic acid column chromatography. Components of the neutral lipid fraction were separated by reversed-phase high-performance liquid chromatography and were detected by monitoring both radioactivity and UV absorption (210 nm) of the column effluent. Cholesterol represented only about 5% of the total radioactivity in the bacterial neutral lipids. The remaining radioactivity was associated with a compound that did not absorb light at 210 nm. This lipid was purified and, on the basis of results from thin-layer chromatography and mass spectrometry, was identified as cholestanol (5α-cholestan-3β-ol), a sterol lacking the unsaturated bond of cholesterol. Cholestanol was also present in cell-free culture broth, but only after growth of the spirochete. These results are evidence that cholesterol is used by *T. hyodysenteriae* for membrane synthesis. Cholesterol is converted to cholestanol in *T. hyodysenteriae* cultures and cholestanol is a major component (approximately 9% by weight) of *T. hyodysenteriae* cell lipids.

The agent of swine dysentery is a spirochete, *Treponema hyodysenteriae* (6). In studies described in the accompanying paper, erythrocytes were found to be a source of cholesterol and phospholipid for growth of this pathogen (27). The finding that cholesterol is a nutritional requirement for this spirochete confirmed earlier observations of Lemcke and Burrows (10). *T. hyodysenteriae* is one of only a few species of cell wall-containing bacteria reported to require cholesterol (5, 17, 24).

From an ecological and evolutionary viewpoint, the need of *T. hyodysenteriae* for cholesterol is intriguing. For the most part, procaryotes do not synthesize and do not require sterols (2). Consequently, bacteria metabolizing cholesterol must obtain the sterol from eucaryotic sources. It is likely that the need of *T. hyodysenteriae* for cholesterol both represents a physiological adaptation of this bacterium to existence within its animal host and indicates a nutritional dependence on that host.

Research described in this article was aimed at determining the metabolic fate of cholesterol in *T. hyodysenteriae* cultures. The sterol content of *T. hyodysenteriae* cellular lipids was analyzed, and the metabolism of [^{14}C]cholesterol by spirochete cells was investigated. Findings from these studies point to the conclusion that cholesterol is used for membrane synthesis by the spirochete. Additionally, an unexpected finding was that a major sterol in *T. hyodysenteriae* lipids is cholestanol (5α-cholestan-3β-ol) produced by chemical reduction of cholesterol in *T. hyodysenteriae* cultures.

MATERIALS AND METHODS

Culture conditions and cell harvest. *T. hyodysenteriae* B204, culture media, and conditions for culturing this spirochete have been described previously (27). For analysis of cellular lipids, *T. hyodysenteriae* cells were cultured in a 3-liter volume of either brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% (vol/vol) fetal calf serum (BHIS broth) or lipid-depleted BHI broth containing cholesterol-phosphatidylcholine vesicles (CPC vesicles) (DBC broth). DBC broth contained 10 to 15 μg of cholesterol per ml of medium. The same lot of BHI dehydrated broth was used throughout these and previous studies (27). The culture vessel was a 5-liter glass carboy sealed with a rubber stopper and vented with a stainless-steel one-way check valve (3SS-2C-1/3; Nupro Co., Willoughby, Ohio).

Bacterial cells in the late exponential phase of growth (3×10^8 to 5×10^8 cells per ml, direct counts) were harvested by centrifugation (10,000 × *g*, 25 min, 5°C). Cells were washed with 150 ml of cold buffer twice, suspended in 50 ml of distilled water, and freeze-dried. The buffer used to wash cells contained 4 g of NaCl and 0.51 g of $MgCl_2 \cdot 6H_2O$, in 500 ml of potassium phosphate buffer (0.01 M, pH 7.0). The average yield of cell material was 4.7 g (wet weight) or 1.1 g (dry weight).

Vesicle preparation. Suspensions of CPC vesicles were prepared by sonicating cholesterol and phosphatidylcholine (1:1 molar ratio) in O_2-free phosphate buffer (0.11 M, pH 7.3) (9). The cholesterol content of each filter-sterilized (0.45-μm pore size) suspension was determined by enzymatic analysis (1). During preparation and storage, vesicles were kept cold (5°C) and under N_2.

Extraction and separation of lipids. Lipids in lyophilized *T. hyodysenteriae* cells (0.5 g) and in lyophilized culture media (approximately 5 g, equivalent to 200 ml of broth) were extracted with $CHCl_3$-CH_3OH (2:1, vol/vol) (22). Extracted lipids of culture media were directly analyzed for sterol content by thin-layer chromatography (TLC). Extracted lipids of bacteria were separated into neutral and polar lipid fractions by means of column chromatography with heat-activated silicic acid as the adsorbent (22, 23). Bacterial neutral lipids (including sterols) were recovered from column eluates and dissolved in CH_3OH for further analyses. All glassware used for lipid extractions and analyses had been acid washed and rinsed with distilled water.

[^{14}C]cholesterol metabolism. *T. hyodysenteriae* cells were cultured in DBC broth (300 ml) containing vesicles made from [4-^{14}C]cholesterol and phosphatidylcholine. The specific activity of cholesterol in the vesicles was 2×10^8 cpm/μmol. Cells in the late exponential growth phase were

harvested as described above. Portions of freeze-dried radiolabeled cells were combined with unlabeled cells before extraction and analysis of radioactive lipids.

In experiments to determine whether or not radioactivity from [^{14}C]cholesterol became associated with *T. hyodysenteriae* membranes, spirochete cells were harvested by centrifugation, washed twice with buffer, and lysed by sonication. Whole, unbroken cells were pelleted by centrifugation at 6,000 × *g* for 10 min and discarded. Cell membranes in the supernatant were pelleted by ultracentrifugation (144,000 × *g*, 2 h) and analyzed.

Radioactivity of various cell fractions and lipid extracts was determined with a Beckman LS-9800 liquid scintillation counter. The scintillation cocktail, Aquasol, and [4-^{14}C]cholesterol (NEC-018) were purchased from Du Pont New England Nuclear Corp. Research Products, Boston, Mass.

Lipid analysis by HPLC, enzymatic assay, and TLC. Extracted neutral lipids dissolved in CH_3OH were separated on a reversed phase high-performance liquid chromatography (HPLC) column (C_{18}, μBondapak, 0.78 by 30 cm; Waters Associates, Inc., Milford, Mass.). The mobile phase was CH_3OH-CH_3CN (75:25, vol/vol) flowing at a rate of 1 ml/min. Compounds eluting from the column were detected on the basis of their A_{210} (Spectroflow 757 absorbance detector; Kratos Analytical Instruments, Ramsey, N.J.). After calibration injections of cholesterol standard solutions, an SP4200 computing integrator (Spectra-Physics, San Jose, Calif.) was used to identify and measure cholesterol in unknown samples.

An enzymatic assay for cholesterol was used in place of or in addition to HPLC analysis (diagnostic kit no. 351, Sigma Chemical Co., St. Louis, Mo.). This assay is based on coupled reactions of three enzymes, including cholesterol esterase (EC 3.1.1.13), and measures both free and esterified cholesterol (1). In some experiments, enzymes of the assay were combined separately to distinguish between cholesterol esters and unesterified cholesterol. Cholesterol standards for calibrating the assay were prepared with the same solvent as unknown samples.

T. hyodysenteriae lipids, commercially obtained sterols, and culture broth extracts were analyzed by ascending TLC (11, 22), using either precoated Silica Gel G plates or Silica Gel 60 plates with concentrating zones (20 by 20 cm; 0.25-mm-layer thickness; Pierce Chemical Co., Rockford, Ill.). Plates were heat activated at 110°C for 30 min before use. The TLC solvent was benzene-ethyl acetate (5:1, vol/vol). Separated lipids were detected by exposing plates to iodine vapor at 37°C for 30 min (11), and their relative mobilities (R_f) were determined from the following equation: R_f = [distance sterol migrated (millimeters)/distance solvent front (150 mm)] × 100. In some experiments, cholesterol and cholestanol in different amounts were chromatographed on the same plate with unknown samples. After iodine staining, the concentrations of the sterols in the unknown were estimated by comparing the intensity of the cholesterol and cholestanol spots of the unknown sample to that of the standard spots. By this method, the minimum detectable amount of cholesterol in a spot was 0.2 to 0.5 μg and that of cholestanol was 1 to 2 μg.

Mass spectrometry. Mass spectrometry was used to identify a major cell lipid (see peak INV, Fig. 1) obtained by HPLC of *T. hyodysenteriae* neutral lipids. The mass spectrum of the unknown lipid was determined with a Finnigan 4000 mass spectrometer by Steve Veysey, Department of Chemistry, Iowa State University. Mass spectra of cholesterol and cholestanol were determined in parallel. The lipids were identified by a computer-based comparison of their mass spectra (ionization at 70 eV) to standard mass spectra in the computer reference library (National Bureau of Standards, Washington, D.C.).

TABLE 1. Cholesterol content of *T. hyodysenteriae* neutral lipids[a]

Culture broth	Material extracted	Assay method	Cholesterol content	
			mg/total extract	% Cell dry wt
BHIS	*T. hyodysenteriae* cells + cholesterol (25 mg) (control)	HPLC	24.3	
BHIS	*T. hyodysenteriae* cells	HPLC	ND[b] (<0.2)	<0.04
		Enzymatic	7.1	1.4
DBC	*T. hyodysenteriae* cells	HPLC	0.5	0.1
		Enzymatic	8.5	1.7

[a] Neutral lipids were obtained by silicic acid column chromatography of total lipids extracted from lyophilized *T. hyodysenteriae* cells (0.5 g).
[b] ND, Not detected (limit of detection).

Chemicals and reagents. Organic solvents were either HPLC grade (Burdick and Jackson Laboratories, Muskegon, Mich.) or were distilled before use. Cholestanol (5α-cholestan-3β-ol), epicholestanol (5α-cholestan-3α-ol) coprostanol (5β-cholestan-3β-ol) and epicoprostanol (5β-cholestan-3α-ol) were obtained from Steraloids, Inc. (Wilton, N.H.).

RESULTS

Analysis of sterol content of *T. hyodysenteriae* cellular lipids. Total extracted lipids of *T. hyodysenteriae* cells cultured either in BHIS broth or in DBC broth accounted for 18 to 19% of the cell dry weight. The neutral lipid fraction (eluted by $CHCl_3$ from a silicic acid column) was yellow-green in color, and lipids in this fraction were about one-third (by weight) of the total cell lipids.

By HPLC analysis (detection at 210 nm), there were two major and three to five minor peaks in chromatographs of *T. hyodysenteriae* neutral lipids (Fig. 1). Cholesterol was not a major lipid of *T. hyodysenteriae* cells. The sterol was not detected in extracts of cells cultured in BHIS broth except in control experiments when it was added to the cells before extraction (Table 1). From these control samples, 97% of the added cholesterol was recovered. Cholesterol was present only in small amounts (0.5 mg total, 0.1% of the cell dry weight) in the neutral lipids of bacteria cultured in DBC broth (Table 1).

In contrast to HPLC analyses, enzymatic analyses of the same extracts detected significant amounts of cholesterol-positive material, representing 1.4 and 1.7%, respectively, of the dry weight of cells cultured in either BHIS or DBC broth (Table 1). Together with the results obtained from HPLC analyses, these results indicated that a compound (unknown lipid) which was not cholesterol but which reacted with enzymes of the cholesterol assay was present in *T. hyodysenteriae* lipid extracts.

Inasmuch as the enzymatic assay contains cholesterol esterase and detects both cholesterol and fatty acid esters of cholesterol (1), it seemed possible that the unknown enzyme-reactive compound in *T. hyodysenteriae* cells was esterified cholesterol. However, the results of assays of *T. hyodysenteriae* neutral lipids were the same when cholesterol esterase was omitted from the assay, indicating that the unknown lipid was not a cholesterol ester.

Metabolism of [4-^{14}C]cholesterol by *T. hyodysenteriae* cells. To investigate the metabolic fate of cholesterol in *T. hyodysenteriae* cultures, spirochete cells were cultured in DBC broth containing [^{14}C]cholesterol. On the basis of radioactivity measurements, about half the cholesterol initially present in culture broth was incorporated into spirochete cells. Essentially all (96%) of the radioactivity was associated with membranes pelleted by ultracentrifugation of cell lysates. Thus, cholesterol was ultimately a component of *T. hyodysenteriae* cell membranes.

In two experiments, *T. hyodysenteriae* cells incorporated radiolabel equivalent to 4.3 and 4.6 μmol of cholesterol per 100 mg of cell dry weight, or 1.7 and 1.8% of the cell dry weight, as determined from the specific activity of [^{14}C]cholesterol. These values were consistent with sterol estimates from enzymatic analysis (1.7% of cell dry weight [Table 1]) and contrasted with cholesterol measurements based on HPLC analysis (0.1% of cell dry weight [Table 1]). Thus, the unknown lipid reactive in the enzymatic assay was produced from cholesterol.

Detection and identification of cholestanol in *T. hyodysenteriae* lipid extracts. Lipids of *T. hyodysenteriae* cells cultured in broth containing [4-^{14}C]cholesterol were extracted and fractionated. Approximately 90% of the radiolabel in the cells was extracted by $CHCl_3$-CH_3OH. Of this radioactivity, 98% was in the neutral lipid fraction. Neutral lipids were separated on a reversed-phase HPLC column. Chromatographs based on UV absorbance contained two major peaks (peaks I and III, Fig. 1). On the basis of retention time, cholesterol was identified as the component of minor peak II and represented 5% of the total radioactivity. Most (94%) of the radiolabel in neutral lipids was eluted from the column immediately after cholesterol (peak INV, Fig. 1). The radioactive lipid in this peak did not absorb UV light at 210 nm, indicating that the compound lacked the unsaturated bond of cholesterol. This unknown lipid was recovered from HPLC column fractions and was analyzed by TLC and mass spectrometry. By TLC analysis, a single compound with a relative mobility, R_f, of 23 was detected. R_f values for cholesterol and various saturated sterols were: cholesterol, 26; cholestanol, 23; epicholestanol, 36; coprostanol, 32; and epicoprostanol, 33. The lipid from HPLC peak INV thus had a relative mobility identical to that of cholestanol.

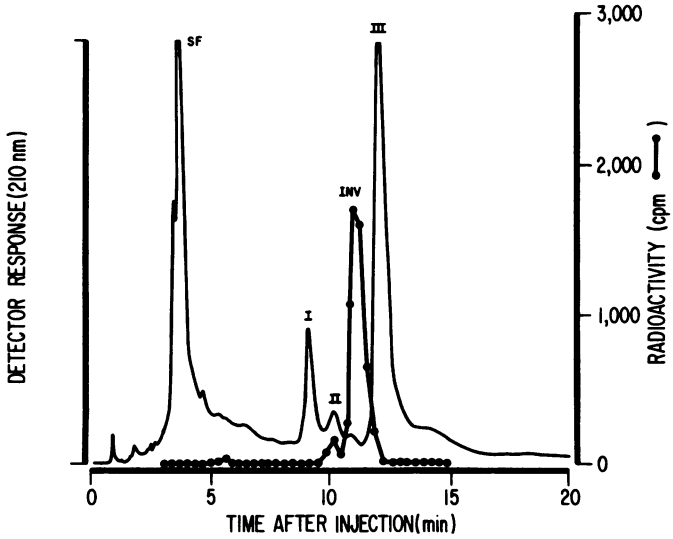

FIG. 1. Chromatograph (HPLC) of neutral lipids of *T. hyodysenteriae* B204 cells cultured in DBC broth containing [4-^{14}C]cholesterol. Separated compounds were monitored by UV absorbance and by radioactivity. Peak II contained cholesterol. SF, Solvent front.

The lipid of HPLC peak INV was analyzed by mass spectrometry along with cholesterol and cholestanol (Fig. 2). The molecular ion of the unknown lipid had a mass/charge (*m/z*) ratio of 388.5, indicating that its molecular weight was greater by two hydrogen atoms than that of cholesterol and was equal to the molecular weight of cholestanol. Identical fragment ions at *m/z* 373.5 (M − CH_3), *m/z* 355.4 (M − CH_3—H_2O), *m/z* 233.3 (M − side chain, ring D − H), and *m/z* 215.3 (*m/z* 233 − H_2O) in mass spectra of both cholestanol and the bacterial lipid confirmed identification of the unknown lipid as cholestanol (Fig. 2). Furthermore, in other studies, cholestanol reacted positively in the enzymatic test for cholesterol. This saturated sterol was also detected by TLC of the lipids of cells cultured in BHIS broth. These findings explained the discrepancy between HPLC and enzymatic determinations of cholesterol in *T. hyodysenteriae* neutral lipids (Table 1) and established that cholestanol was a major component of the cell lipids of the spirochete. Since lipids were about 18% and sterols (almost entirely cholestanol) were about 1.7% of the cell dry weight, cholestanol represented about 9% of the lipids of *T. hyodysenteriae* cells cultured in DBC broth.

Analysis of sterols in cell-free culture broth. The presence of radiolabeled cholestanol in lipids of *T. hyodysenteriae* cells cultured in medium containing [^{14}C]cholesterol indicated that the unsaturated cholesterol bond was reduced in bacterial cultures. In preliminary investigations of the mechanism of this reaction, sterile medium and the supernatant of spent culture medium were assayed for cholestanol and cholesterol. Appreciable amounts of cholestanol (5 μg/ml) were present in the culture supernatant (Table 2). Cholestanol was not found (<1 μg/ml, limit of detection) in incubated, uninoculated medium. This finding indicated that cholestanol was produced during *T. hyodysenteriae* growth.

DISCUSSION

Various bacteria chemically modify or metabolize cholesterol. A *Pseudomonas* strain degrades and uses cholesterol as a carbon-energy source (16). Certain other bacterial species convert cholesterol to coprostanol (3, 5, 24) or glucosylate the sterol (12). For still other species, cholesterol has been identified as a membrane component (18–20).

Of the bacteria that use cholesterol, a small subset require cholesterol for growth. *Eubacterium* sp., anaerobes isolated from the rat cecum, require substrate or millimolar amounts of cholesterol (1 to 2 mg/ml, 5 μmol/ml) (5). In cultures of these bacteria, the 5,6 double bond of cholesterol is reduced, yielding coprostanol (5β-cholestan-3β-ol), a common sterol in mammalian feces (13, 14). Cholesterol thus is a terminal electron-hydrogen acceptor in the metabolism of these *Eubacterium* strains and, for this function, is considered necessary for growth (5). *T. hyodysenteriae* (10, 27), *Treponema refringens* (previously designated "*Treponema pallidum*" Noguchi [17]), and cholesterol-requiring *Mycoplasma* spp. (21) require lower or nutrient amounts of cholesterol (1 to 20 μg/ml of medium). These mycoplasmas need sterol for membrane biosynthesis (19). At least one mycoplasma, *Mycoplasma capricolum*, can use cholestanol in place of cholesterol for growth (4).

An explanation for the need of *T. hyodysenteriae* for cholesterol may be inferred from the uses of cholesterol by

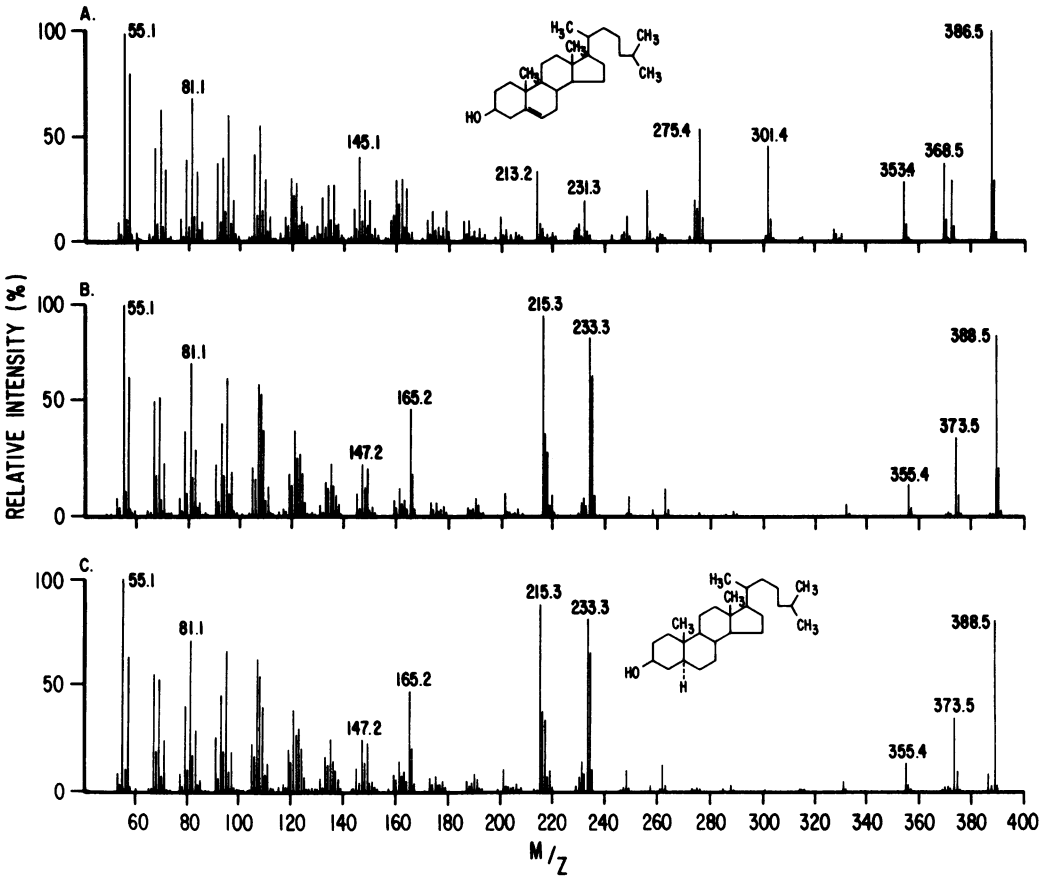

FIG. 2. Mass spectra of cholesterol (5 μg) (A), *T. hyodysenteriae* lipid (8 μg) from HPLC peak INV (Fig. 1) (B), and cholestanol (5 μg) (C).

other bacteria and from features of its own metabolism. Since *T. hyodysenteriae* requires nutrient amounts of the sterol and can use the saturated sterol cholestanol for growth (10, 27), cholesterol is not essential either as a growth substrate or as an electron-hydrogen acceptor. Quantities of cholesterol equivalent to what the spirochete needs for growth (27) were removed from the culture medium (Table 2) and incorporated into bacterial lipids as cholesterol or cholestanol (Table 1). Essentially all the radiolabel incorporated by cells cultured in medium containing [^{14}C] chol esterol was in bacterial membranes. The conclusion from these considerations is that *T. hyodysenteriae* requires sterol as a membrane component. It should also be mentioned that Lemcke and Burrows (10) first suggested that *T. hyodysenteriae* uses sterol as a membrane lipid based on their finding similar patterns of sterol use by the spirochete and by mycoplasmas.

TABLE 2. Sterol content of *T. hyodysenteriae* culture broth[a]

Sterol	Concn (μg/ml of medium) in:			
	Uninoculated medium		Spent culture supernatant	
	A	B	A	B
Cholesterol	11.3	12.5	0.75–1.0	0.6–1.25
Cholestanol	ND[b] (<1.0)	ND (<1.0)	3.75–5.0	5–6.3

[a] In two experiments (A and B), lipids were extracted from DBC broth (200 ml) and analyzed for sterol content. The cholesterol content of uninoculated medium was estimated by enzymatic assay after TLC analysis revealed that there was no detectable cholestanol. The cholesterol and cholestanol contents of spent medium were estimated by a TLC-iodine staining method.

[b] ND, Not detected (limit of detection).

As determined from enzymatic analysis of lipid extracts (Table 1) and measurements of [^{14}C]cholesterol uptake by *T. hyodysenteriae* cells, cholesterol plus cholestanol made up 1.7 to 1.8% of the dry weight of bacteria cultured in DBC medium. It can be calculated from published data (19) that mycoplasma cells contain comparable amounts of total sterol. However, unlike *T. hyodysenteriae*, mycoplasmas incorporate cholesterol and esterified cholesterol from their culture medium with no chemical modifications to the sterols (19). Also, unlike mycoplasmas *T. hyodysenteriae* cells have two membranes, a cytoplasmic membrane and an outer membrane or sheath. At this time, it is not known whether sterol is present in one or both of these membranes. Neither can the significance of sterol in *T. hyodysenteriae* membranes be evaluated. Sterol undoubtedly provides this pathogen with membrane properties not possessed by other bacteria.

An unexpected finding in these investigations was the reduction of cholesterol to cholestanol in *T. hyodysenteriae* cultures. Cholestanol is present as a minor component (2 to 5%) of the neutral sterols of rat and human feces (14, 15). Rat fecal cholestanol is made from cholesterol (14). The cell source for cholestanol production has not been established and could be intestinal bacteria, intestinal tissue, or both (7, 13). As far as I am aware, this is the first report of

cholestanol production in cultures of a specific intestinal bacterium.

From the data of Table 2, the difference in total sterol content between uninoculated medium (12 μg of cholesterol per ml) and spent culture supernatant (5 μg of cholestanol plus 1 μg of cholesterol per ml) was 6 μg/ml, representing sterol taken up by bacteria. It can be calculated that the cholestanol content of bacteria in 1 ml of medium was 5.6 μg (94% of 6 μg/ml). Thus, the cholestanol contents of 1 ml of spent medium and of bacterial cells from 1 ml of medium were essentially the same. Interestingly, the ratio of cholestanol to cholesterol was 4 to 5:1 in spent medium (Table 2) and 19:1 in *T. hyodysenteriae* cell lipids, as determined from radioactivity measurements (Fig. 1). Therefore, relative to cholesterol, there was more cholestanol associated with bacterial cells than with spent medium. A possible explanation for this result is that the site of cholesterol reduction was the spirochete cell.

The mechanism of cholesterol reduction in *T. hyodysenteriae* cultures and the significance of this reaction to spirochete physiology remain to be determined. In mammalian tissues, multienzyme pathways have been considered to convert cholesterol to cholestanol (25, 26). However, cholestanol can also be formed from cholesterol by nonenzymatic means (8). Thus, it is conceivable that the catalyst for cholesterol reduction in *T. hyodysenteriae* cultures is not an enzyme. Inasmuch as the unsaturated bond of cholesterol and, therefore, cholesterol hydrogenation is not needed for growth in vitro (27), identification of a specific enzyme(s) for cholesterol reduction would be an important step toward concluding that this reaction has physiological relevance for *T. hyodysenteriae* in vivo.

The possibility that cholesterol metabolism is involved in pathogenesis deserves consideration. *T. hyodysenteriae* cells can obtain cholesterol from eucaryotic (erythrocyte) membranes (27) and can generate exogenous cholestanol during growth (Table 2). Damage to membranes of intestinal epithelium in the vicinity of *T. hyodysenteriae* cells could result from cholesterol depletion or cholestanol incorporation.

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