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Erythrocytes as a Source of Essential Lipids for *Treponema hyodysenteriae*

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Mammalian erythrocytes were tested as a nutrient source for the spirochete *Treponema hyodysenteriae*, the etiologic agent of swine dysentery. Brain heart infusion (BHI) broth did not support growth of *T. hyodysenteriae* B204 or B78. However, BHI broth supplemented with washed bovine erythrocytes, erythrocyte membranes, or cholesterol was an excellent culture medium for these strains (4×10^8 to 10^9 cells per ml, final population densities). Small amounts of cholesterol (3 to 10 μg/ml of medium) were required for maximum cell yields of strain B204. Of various sterols and sterol-like compounds tested, cholestanol and sitosterol could substitute for cholesterol. BHI-dehydrated medium extracted with $CHCl_3$-CH_3OH (2:1, vol/vol) to remove lipids was used to prepare lipid-depleted culture broth. Lipid-depleted broth supplemented only with cholesterol did not support *T. hyodysenteriae* B204 growth. However, this same broth supplemented either with vesicles made from cholesterol and phosphatidylcholine or with washed erythrocytes was a good culture medium for the spirochete. Thus, both cholesterol and a phospholipid are needed for *T. hyodysenteriae* growth in lipid-depleted broth. Mammalian erythrocytes can be used by the spirochete as a source of these lipids.

Swine dysentery is a severe, mucohemorrhagic intestinal disease of major importance to the U.S. pork industry (10). The agent of swine dysentery is the spirochete *Treponema hyodysenteriae* (11, 26). A typical sign of the disease is profuse bleeding into the large bowel lumen through lesions induced by *T. hyodysenteriae* (1, 8, 13). Photomicrographs reveal that these lesions are populated by large numbers of *T. hyodysenteriae* cells, frequently in the midst of swine erythrocytes (1, 13). *T. hyodysenteriae* cells produce a hemolysin (24) which releases erythrocyte cytoplasmic contents into their environment.

Characteristics enabling *T. hyodysenteriae* to colonize and damage swine intestinal tissues have not been identified. Bacterial competition for limiting nutrients is considered a major determinant of the composition of the intestinal microflora (7). Intestinal colonization by *T. hyodysenteriae* undoubtedly depends on the ability of this pathogen to be equally or more efficient than indigenous bacteria in acquiring or using nutrients. An increased understanding of the success of *T. hyodysenteriae* as a pathogen would result from knowledge of the nutritional requirements, nutritional sources (in vivo), and metabolism of this spirochete.

There is evidence that host cells and cell products supply nutrients and growth substrates for intestinal bacteria (25, 27). The presence of erythrocytes in the vicinity of *T. hyodysenteriae* cells in the swine intestine and the hemolytic ability of the spirochete suggested to us that blood cells might serve as a source of nutrients for *T. hyodysenteriae*. In the investigations reported in this article, we tested this possibility. The results of these investigations indicate that *T. hyodysenteriae* can obtain essential lipids (cholesterol and phospholipid) from erythrocytes. We also report the results of preliminary investigations of cholesterol metabolism by this spirochete.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Stock cultures of *T. hyodysenteriae* strains used in these studies were supplied by I. M. Robinson, National Animal Disease Center, Ames, Iowa. Strain B78 has been designated the type strain of the species *T. hyodysenteriae* (11, 14). Strain B204 has frequently been used in experiments to infect animals (3, 12).

T. hyodysenteriae cells were routinely cultured in stirred BHIS broth under an atmosphere of N_2-O_2 (99:1, vol/vol). Cultures were incubated at 38 to 39°C for 20 to 36 h. BHIS broth was prepared anaerobically as follows. Dehydrated brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.), 3.7 g, was dissolved in 87 ml of distilled water containing resazurin (0.0001%, wt/vol). The pH was adjusted to 7.3, and the solution in a Florence flask was bubbled with oxygen-free N_2 and gently heated (<100°C). After the resazurin (resorufin) indicator became colorless, 3 ml of a 3.3% solution of L-cysteine hydrochloride-H_2O (adjusted to pH 7.0) was added. The flask was tightly stoppered and transferred into a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) inflated with N_2-H_2-CO_2 (85:10:5, vol/vol/vol). This basal medium, BHI broth, was dispensed (6.3 ml per tube) into anaerobic culture tubes (18 by 142 mm; Bellco Glass, Inc., Vineland, N.J.), each containing a Teflon-coated magnetic stirring bar (3 by 10 mm). Tubes were sealed with Neoprene rubber stoppers, removed from the anaerobic chamber, and autoclaved in a press. After the sterile medium had cooled to room temperature, 0.7 ml of heat-treated (56°C, 30 min) fetal calf serum (GIBCO Laboratories, Chagrin Falls, Ohio) was added to each tube while the tube was flushed with sterile, oxygen-free N_2. This serum-supplemented broth is BHIS broth. A tube of BHIS broth was inoculated under anaerobic conditions with 0.1 to 0.2 ml of spirochete culture. After the tube was stoppered and sealed with plastic tape, 0.2 ml of sterile O_2 was injected through the stopper into the headspace of the culture tube to

yield a culture atmosphere containing approximately 1% (vol/vol) O_2. The inoculated culture was placed on a magnetic stirring platform within an incubator, and the stirring rate was adjusted so that a slight vortex was visible at the top of the broth. With our culture techniques, both the introduction of O_2 into the culture atmosphere and stirring of the culture have been necessary for optimal and consistent growth of *T. hyodysenteriae* (T. B. Stanton, manuscript in preparation).

Preparation of extracted (lipid-depleted) BHI culture broth. Lipids were extracted from dehydrated BHI culture medium (130 g) with $CHCl_3$-CH_3OH (2:1, vol/vol) (23). From the extracted BHI powder, lipid-depleted BHI culture broth was prepared like BHIS medium, except for the following modifications. The quantity of (extracted) BHI powder per 100 ml of culture broth was reduced to 2.6 to 2.8 g as an approximate compensation for lipid components removed during extraction. Additionally, to compensate for inorganic salts and glucose lost during extraction, the basal medium was prepared with sodium phosphate buffer (0.01 M, pH 7.3) instead of distilled water, and the final sterile medium (7 ml) was supplemented with 0.1 ml of a filter-sterilized glucose solution (14%, wt/vol).

Nutritional studies. Mammalian blood cells, blood cell fractions, artificial membranes, sterols, and sterol-like compounds were tested for the ability to support *T. hyodysenteriae* growth. Actively dividing bacteria (approximately 10^8 cells) cultured in BHIS broth were inoculated into 7 ml of BHI broth or lipid-depleted BHI broth containing one of these supplements in place of serum. Sterile phosphate buffer (0.7 ml; 0.1 M; pH 7.4) was added to control (unsupplemented) media. Additional culture conditions were similar to those given above. When cultures reached maximum absorbance, cells were transferred (1 to 2% [vol/vol] inoculum) into fresh medium containing the same supplements. After three or more transfers into the particular medium, bacterial growth was monitored spectrophotometrically at 660 nm and with a 18-mm pathlength. Bacterial cell densities at maximum culture absorbance were estimated by direct (Petroff-Hausser) cell counts.

Erythrocytes were aseptically harvested from defibrinated blood by centrifugation (1,000 × *g*, 10 min) and were washed three times in sterile, isotonic phosphate buffer (0.11 M, pH 7.4) (6). The buffy coat layer (leukocyte enriched) of the cell pellet was siphoned after each centrifugation. A membrane fraction was obtained from washed blood cells lysed in hypotonic buffer (6). A cytoplasmic fraction was obtained by freezing and thawing washed blood cells three times and centrifuging the lysate at 20,000 × *g* for 30 min. The dark red supernatant was filter sterilized (0.45-μm pore size) and used as a medium supplement.

Artificial membrane vesicles (cholesterol-phosphatidylcholine [CPC] vesicles) were made by the ether vaporization method of Deamer and Bangham (5). The final vesicle suspension was sonicated and then filter sterilized (0.45-μm pore size). Before a vesicle preparation was used as a medium supplement, its cholesterol content was determined (2).

Sterols and other compounds tested for their ability to support *T. hyodysenteriae* growth were dissolved in either ethanol or distilled water at 50 to 100 times their final concentration (0.1 μmol/ml) in culture broth. Vesicles and lipid solutions were made and stored at 5°C under oxygen-free N_2.

Chemicals and reagents. Organic solvents were either high-performance liquid chromatography (HPLC) grade or were distilled before use. Cholesterol (stock code CH-S), phosphatidylcholine (P-2772), and phosphatidylglycerol (P-0514) were from Sigma Chemical Co. (St. Louis, Mo.). Other sterols or sterol-like compounds were the purest forms available from Sigma, Aldrich Chemical Co., Inc. (Milwaukee, Wis.), or Steraloids, Inc. (Wilton, N.H.). Most of these sterols and all sterols that supported *T. hyodysenteriae* growth were analyzed by reversed-phase HPLC or by enzymatic assay (Sigma kit no. 351) to detect any contaminating cholesterol.

TABLE 1. Utilization of bovine erythrocytes, erythrocyte fractions, and cholesterol by *T. hyodysenteriae* for growth in BHI broth

Medium supplement[a]	Strain	No. of population doublings[b]	Final population densities (10^8 cells/ml)[c]
Phosphate buffer (control)	B78	3–4	
	B204	3–10	
Fetal calf serum	B78	32+	8
	B204	32+	11
Washed erythrocytes	B78	30+	4.5
	B204	30+	4
Erythrocyte membranes	B78	30+	4
	B204	30+	4.5
Erythrocyte cytoplasmic contents	B204	5–7	
CPC vesicles	B78	32+	10
	B204	32+	8
Phosphatidylcholine vesicles	B78	2–3	
	B204	3–4	
Cholesterol (in ethanol)	B204	32+	7

[a] Final concentration of serum and whole blood was 10% (vol/vol). Media contained erythrocytes, membranes, and cytoplasmic contents in amounts equivalent to 10% whole blood.

[b] Estimated from inoculum volume, number of consecutive culture transfers, population density at transfer. +, Continuous growth throughout experiment.

[c] Final population densities were not determined for cultures in media that did not support growth.

RESULTS

Growth in BHI broth. In unsupplemented BHI broth (phosphate buffer added), growth of *T. hyodysenteriae* B78 and B204 ceased after a limited number of population doublings (Table 1). Apparently, cell division in unsupplemented broth was possible until the bacteria had depleted the medium of nutrients carried over with the inoculum. In BHI broth supplemented with either washed bovine blood cells or fetal calf serum, *T. hyodysenteriae* cells reached high population levels (4×10^8 to 1.1×10^9 cells per ml of broth [Table 1]) at each of five successive transfers into the particular medium. There was no sign of growth limitation, and spirochete cells presumably could have been subcultured indefinitely in these broths. Although bovine blood cells were routinely used because they were easy to obtain, *T. hyodysenteriae* B78 cells also grew well in BHI broth containing swine, human, horse, guinea pig, or rabbit blood cells, reaching population densities of 6.5×10^8, 4.3×10^8, 6.5×10^8, 9.0×10^8, and 5.0×10^8 cells per ml, respectively, after four successive culture transfers.

Although washed erythrocytes supplied one or more nutrients essential for *T. hyodysenteriae* growth and missing from BHI broth, erythrocytes by themselves were insufficient for growth of the spirochete. That is, strain B204 cells did not grow in a medium consisting of anaerobic phosphate

buffer, glucose (0.2% [wt/vol], final concentration), and washed erythrocytes at a final concentration equivalent to 20% whole blood.

To identify the location in the blood cell of the nutrient(s) essential for growth, we inoculated *T. hyodysenteriae* cells into BHI broth supplemented with either membrane preparations or cytoplasmic contents from erythrocytes. Strain B204 cells exhibited only limited carry-over growth in BHI broth containing cytoplasmic contents (Table 1). Both strains grew well in broth supplemented with blood cell membranes (Table 1). In separate experiments, membrane preparations that had been heated for 10 min in a boiling water bath supported spirochete growth.

Cholesterol and various phospholipids make up a large portion (40 to 50% by weight) of the mammalian erythrocyte membrane (20, 22). Furthermore, cholesterol has been reported to be an essential growth nutrient for *T. hyodysenteriae* (16). On the basis of this information, we tested whether or not cholesterol would support *T. hyodysenteriae* growth in BHI broth. To prevent cholesterol precipitation and to provide spirochete cells with a more accessible source of the sterol, cholesterol and phosphatidylcholine (1:1 molar ratio) were made into membrane vesicles, and these vesicles were added to the culture medium. The particular lot of BHI broth used throughout these investigations did not contain detectable amounts of cholesterol (<1 μg/ml of prepared broth, HPLC analysis).

Both *T. hyodysenteriae* strains grew to high population densities (8×10^8 and 10^9 cells per ml of broth, respectively) through five successive subcultures in BHI broth supplemented with CPC vesicles (Table 1). Only limited carry-over growth of spirochete cells occurred in broth containing vesicles prepared from phosphatidylcholine alone. Strain B204 cells grew to high population densities (7×10^8 cells per ml) when cholesterol was the only supplement (Table 1). Because cholesterol by itself could not be made into vesicles, it was added as a solution in ethanol. In additional studies, strain B204 cells have undergone more than 50 population doublings in BHI broth supplemented either with CPC vesicles or with cholesterol. These results indicated that *T. hyodysenteriae* required cholesterol for growth in BHI broth.

All further investigations involved the use of only strain B204, for the sake of convenience and because we have used this strain frequently in other areas of swine dysentery research.

***T. hyodysenteriae* growth at various cholesterol concentrations.** To determine the cholesterol concentration supporting maximum growth yields of *T. hyodysenteriae*, strain B204 cells were inoculated into BHI broth containing various amounts of cholesterol, supplied either as CPC vesicles or as a solution in ethanol (Fig. 1). The cholesterol concentration necessary for maximum population densities was 3 to 10 μg (0.008 to 0.026 μmol)/ml of broth (Fig. 1). Direct microscope counts indicated that final cell densities ranged from 4×10^8 to 10^9 cells per ml. Although the nature of the cholesterol supplement had no significant effect on final cell densities of the spirochete, the time required to reach maximum cell density for cultures supplemented with ethanol solutions of the sterol was usually longer (36 to 72 h) than that of vesicle-supplemented cultures (20 to 36 h). Additionally, a cholesterol precipitate formed in BHI broth supplemented with the ethanol solution if the final cholesterol concentration was 10 μg/ml or higher. Inasmuch as this precipitate interfered with culture absorbance measurements, CPC vesicles were a more appropriate supplement. On the basis of the above results, culture broth for the spirochete in subsequent experiments contained sterol (cholesterol) concentrations of 0.05 to 0.1 μmol/ml of medium, or approximately twice the concentration needed for maximum growth yields.

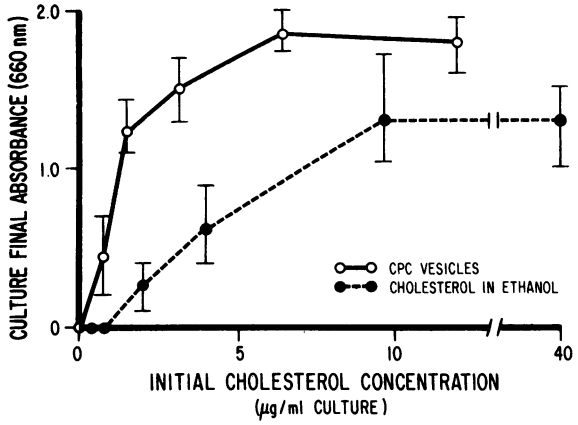

FIG. 1. Growth yields (final culture absorbance) of *T. hyodysenteriae* B204 in BHI broth containing various cholesterol concentrations. Graphed values are the average final absorbance values for three to four cultures with the range of absorbance measurements indicated by bars.

Growth on various sterols and sterol derivatives. Various sterols and compounds chemically derived from cholesterol were tested for the ability to support *T. hyodysenteriae* growth in BHI broth. Because many of these compounds could not be made into vesicles with phosphatidylcholine, all were dissolved in ethanol or water and added to BHI broth (0.1 μmol/ml, final concentration).

Each compound could be placed into one of four categories based on its effect on *T. hyodysenteriae* growth. Ergosterol, stigmasterol, cholesteryl sulfate, cholesteryl chloride, cholesteryl propionate, cholesteryl oleate, cholesteryl palmitate, and cholesteryl laurate neither supported nor inhibited growth. *T. hyodysenteriae* growth with these compounds was equivalent to carry-over growth detected for control cultures, BHI broth plus phosphate buffer. Spirochete cells could not be subcultured in these media. In a second category, cholesteryl hemisuccinate supported limited growth. That is, bacteria could be subcultured into BHI broth containing this sterol, but not past the second subculture. *T. hyodysenteriae* growth was inhibited by compounds in a third category, cholestenone (4-cholesten-3-one) and 7-ketocholesterol (5-cholesten-3β-ol-7-one), oxidation products of cholesterol. Spirochete cells did not exhibit even limited carry-over growth in BHI broth containing these compounds. In separate experiments, *T. hyodysenteriae* growth in BHIS broth containing 7-ketocholesterol was inhibited, and growth in BHIS broth containing cholestenone required an incubation period that was 60 to 84 h or three to four times longer than that of control cultures without this compound. Finally, BHI broth containing cholesterol (5-cholesten-3β-ol), cholestanol (dihydrocholesterol, 5α-cholestan-3β-ol), or sitosterol (5-cholesten-24β-ethyl-3β-ol) supported spirochete growth through five successive cell transfers into the particular medium with average final cell densities of 5×10^8, 4.5×10^8, and 3×10^8 cells per ml, respectively. Cultures in BHI broth containing cholesterol or cholestanol reached maximum cell densities after a 36- to 48-h incubation period. Cells in BHI broth containing

TABLE 2. *T. hyodysenteriae* B204 growth in lipid-depleted BHI broth

Medium supplement	No. of population doublings	Incubation period[a] (h)	Final population densities (10^8 cells/ml)
None (phosphate buffer)	NG[b]		
Fetal calf serum (control)	30+[c]	18–24	12
Washed erythrocytes	28+	24–48	5
CPC vesicles	30+	24–36	7.5
Cholesterol[d]	Variable[e]	72–120	0.5
Cholesterol + phosphatidylcholine[d]	30+	36–48	6.5
Cholesterol + phosphatidylglycerol[d]	30+	36–72	4.7

[a] Time for an inoculated culture to reach maximum absorbance (660 nm).
[b] NG, No detectable growth.
[c] +, Continuous growth throughout experiment.
[d] Dissolved in ethanol and added to medium.
[e] Variable, Eight cultures ceased growing after 8 to 12 population doublings. Values in the table are for two other cultures.

sitosterol grew slowly, reaching maximum densities after 72 to 120 h.

***T. hyodysenteriae* B204 growth in lipid-depleted BHI broth.** Cells of strain B204 did not grow in unsupplemented lipid-depleted broth (Table 2). In lipid-depleted broth supplemented with cholesterol, the spirochete grew poorly, if at all. That is, in four experiments, a total of eight cultures could not be transferred beyond two subcultures in this medium. In one other experiment, cells in two cultures continued dividing through four subcultures. However, final population densities in these cultures were low (5×10^7 cells per ml), and incubation times were long (72 to 120 h [Table 2]). Thus, solvent extraction of BHI removed a medium component, other than cholesterol, that was necessary for optimum growth of the spirochete.

Washed erythrocytes and fetal calf serum supported good growth in lipid-depleted BHI broth (Table 2). *T. hyodysenteriae* cells also attained high final cell densities in broth supplemented with both cholesterol and phospholipid, either phosphatidylcholine or phosphatidylglycerol. These results indicated that the extracted BHI component essential for *T. hyodysenteriae* was either phospholipid or another lipid-like compound that substituted for phospholipid. Blood cells were a source of both cholesterol and this compound.

DISCUSSION

In our investigations, mammalian (bovine) erythrocytes supported *T. hyodysenteriae* growth in BHI broth (Table 1). The essential nutrient supplied by blood cells was cholesterol, based on the following observations. (i) Cholesterol is a major lipid of the erythrocyte membrane (20, 22), and the essential nutrient was a heat-stable component of the blood cell membrane (Table 1). Although cholesteryl esters, such as cholesteryl palmitate and cholesteryl oleate, are commonly found in mammalian serum (15) and are possibly present at low levels in erythrocyte membranes, these compounds did not support *T. hyodysenteriae* growth. (ii) *T. hyodysenteriae* cells could be cultured in BHI broth with cholesterol as the only supplement (Table 1). Unsupplemented BHI broth used in these studies did not support growth of the spirochete and did not contain cholesterol (<1 μg/ml, HPLC analysis).

It is unlikely that swine erythrocytes are the sole source of cholesterol (and phospholipid) for *T. hyodysenteriae* cells in vivo. The spirochete requires only low or nutrient amounts of cholesterol for growth (Fig. 1). Before disease is clinically apparent, when *T. hyodysenteriae* cells populate colonic crypts and colonize intestinal mucosa, sterol in quantities adequate for growth would likely be supplied by materials in the diet of the host, by membranes of mucosal epithelial cells (4), and by intestinal mucus (19). During the severe hemorrhagic stages of the disease, blood serum as well as erythrocytes would enter the environment of the spirochete and could serve as a cholesterol source. In view of these alternative sources, swine erythrocytes could be regarded as an accessory source of cholesterol and phospholipid for *T. hyodysenteriae*, serving perhaps to enhance or facilitate growth in vivo, especially at peak severity of the disease. In view of the finding that erythrocyte lipids can support growth of *T. hyodysenteriae* cells in vitro, it is plausible that other blood cell components may benefit the growth and survival of the spirochete in vivo. Blood cell lysates contain low-molecular-weight substances conferring serum resistance on *Neisseria gonorrhoeae* (21). Heme or hemoglobin can be used as an iron source by a number of bacterial pathogens (9).

The demonstration of a cholesterol requirement for *T. hyodysenteriae* and the results of experiments dealing with cholesterol metabolism confirmed and extended the observations of Lemcke and Burrows (16). They reported maximum growth yields (10^7 CFU/ml) of *T. hyodysenteriae* P18A in Trypticase soy broth supplemented with 1.2 μg of cholesterol per ml of medium. We found that cholesterol concentrations of 3 to 10 μg/ml support maximum cell yields of *T. hyodysenteriae* B204 in BHI broth (Fig. 1). Thus, both studies indicate that *T. hyodysenteriae* requires only nutrient levels of cholesterol for growth.

It is noteworthy that the final cell yields of *T. hyodysenteriae* in BHI broth supplemented with CPC vesicles (if expressed as CFU) are 20- to 50-fold greater than those in supplemented Trypticase soy broth (16). We have found BHI broth to be more favorable than Trypticase soy broth for *T. hyodysenteriae* growth (T. B. Stanton and D. F. Lebo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D25, p. 70).

T. hyodysenteriae cells could not use for sustained growth any of the tested cholesteryl esters. This result indicated that the free 3-OH group of cholesterol was essential for growth and that *T. hyodysenteriae* cells did not possess a cholesterol ester hydrolase (EC 2.1.1.13). Both cholestanol, a sterol formed by the chemical reduction of the unsaturated bond of cholesterol, and sitosterol, a plant sterol with an ethyl group bonded to the cholesterol side chain, supported spirochete growth, as Lemcke and Burrows have reported (16). The bacterium either can make cholesterol from these compounds or, more feasibly, requires neither the double bond nor the specific side chain of cholesterol. It was reported (16) that *T. hyodysenteriae* growth did not increase when cholestenone was added in place of cholesterol to the culture medium. Our findings indicated that cholestenone and 7-ketocholesterol inhibited growth of *T. hyodysenteriae* in basal BHI broth and in BHIS broth. Eucaryotic membrane-associated functions and membrane morphology are affected by 7-ketocholesterol and other oxygenated sterols (28). Possibly, these compounds have similar effects on the spirochete. Clearly, agents that interfere with the cholesterol metabolism of *T. hyodysenteriae* by complexing with cholesterol, chemically modifying cholesterol, or displacing cholesterol are potential inhibitors of the pathogen.

T. hyodysenteriae cells required, in addition to choles-

terol, phospholipid for growth in lipid-depleted BHI broth (Table 2). One explanation for this requirement is that *T. hyodysenteriae* resembles certain other spirochetes and is incapable of making long-chain fatty acids for cellular phospholipids (17). Another explanation is that phospholipids form micelles or lipid vesicles with cholesterol, solubilizing the sterol and making it more accessible for the spirochete. Regardless of its role, phospholipid was essential for growth, and erythrocytes satisfied this requirement. Although *T. hyodysenteriae* cellular phospholipids and glycolipids have been quantitated and identified (18), lipid metabolism of the pathogen has not been studied. Lipid-depleted broth or BHI broth supplemented with specific lipids or lipid precursors should be a useful medium for such investigations. In the accompanying article, we describe the use of lipid-depleted broth to investigate cholesterol metabolism by *T. hyodysenteriae*.

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