JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 323-330

Vol. 105, No. 1
Printed in U.S.A.

Sterol Requirements of T-Strain Mycoplasmas

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Received for publication 29 July 1970

T-strain mycoplasmas are very sensitive to digitonin, amphotericin B, and progesterone. This sensitivity and the relatively high content of cholesterol found in the cells indicated a possible requirement of T-strain mycoplasmas for sterols. This suspected requirement was demonstrated directly in a lipid-poor medium and can be met by cholesterol, as well as by β-sitosterol and to a lesser degree by 7-dehydrocholesterol, cholestanol, stigmasterol, and ergosterol but not by cholesterol laurate or cholestan-3-one. Coprostanol, epicoprostanol, and epicholestanol inhibited cell growth. This inhibition could be partially reversed by increasing the cholesterol concentration in the growth medium. Because of their sterol requirement and their unique requirement for urea, T-strain mycoplasmas might be classified as the third genus in the order *Mycoplasmatales*.

T-strain mycoplasmas (subsequently to be referred to as T-mycoplasmas), first isolated by Shepard (21), have been found to be part of the microbial flora of man and animals (22, 30). Because of their small colonial size and poor growth in liquid media (12), little is known about their chemical composition and biochemical activities. Two features investigated so far are their unique growth requirement for urea and their *p*H optimum in the acid range (23, 24). Nothing has been reported previously concerning the role of sterols in the growth of T-mycoplasmas although a requirement for serum has been shown. This was attributed mainly to the urea in the sera and not to the serum per se (24). The growth requirement for sterols is a unique feature of mycoplasmas (13) which is met by planar 3-hydroxy sterols (26). This requirement is a major consideration in the taxonomy of the class *Mollicutes*. Sterols are incorporated into the cell membrane (13), acting probably as stablizers of the bilayer membrane structure. This evidence concerning the role of sterols in the growth of large-type mycoplasmas prompted us to investigate their importance for the growth of T-mycoplasmas. Sterol specificity as well as the significance of our findings to the taxonomic position of T-mycoplasmas are discussed.

MATERIALS AND METHODS

Organisms and growth conditions. Three T-mycoplasma strains were used throughout this work. Strain Bowling was obtained from R. H. Purcell (National Institute of Health, Bethesda, Md.), strain T-960 was obtained from M. C. Shepard (Naval Medical Field Research Laboratory, Camp Lejeune, N.C.), and strain CH-12 was isolated in our laboratory from the urethra of a patient with nongonococcal urethritis. This strain was serologically related to the Henderson strain (A. Martinez-Lahoz and L. Hayflick, *unpublished data*). *Acholeplasma laidlawii* (oral strain) was obtained from S. Razin (The Hebrew University, Jerusalem, Israel), *M. gallisepticum* (A5969) was obtained from M. E. Tourtellotte (University of Connecticut, Storrs), and *M. hominis* (PG-21) was obtained from the American Type Culture Collection (Rockville, Md.).

For chemical analysis and electron microscopy, T-mycoplasmas were grown in 10-liter volumes of Hayflick's basal broth supplemented with 10% horse serum, 0.7% yeast extract (Difco), 0.5% K_2HPO_4, 0.2% urea, 0.002% phenol red, and 1,000 units of penicillin-G per ml. In some experiments, 1 μCi of [4-^{14}C]cholesterol (60.5 mCi/mmole, Calatomic, Los Angeles, Calif.) was added to each liter of the growth medium. The *p*H of the medium was adjusted to *p*H 6.0 with 1 N HCl. *A. laidlawii*, *M. gallisepticum*, and *M. hominis* were grown in the same medium except that, for *A. laidlawii* and *M. gallisepticum*, glucose (0.5%) was added to the medium and its *p*H was adjusted to 8.0. Arginine (0.15%) was added to the media used for the growth of *M. hominis*, and the *p*H was adjusted to 7.2. The organisms were grown for 24 to 48 hr at 37 C and harvested by centrifugation at 9,000 × *g* for 15 min. The cells were washed twice and suspended in 0.25 M NaCl.

For testing the growth response to steroids, T-mycoplasmas were grown in 5-ml quantities of the T-mycoplasma medium described above in which 1% fatty acid-poor bovine serum albumin (Calbiochem, Los Angeles, Calif.) was substituted for the horse serum. The steroids were dissolved in 96% ethanol and were diluted in a 10% solution of the albumin; 0.5-ml amounts of the albumin solutions containing various concentrations of steroids were added to the tubes to yield a final concentration of 1% albumin. The maximum ethanol

concentration in the media did not exceed 0.6%. The tubes were inoculated with an 18-hr T-mycoplasma culture in a ratio of 1:100 or less. Growth was determined after 16 to 20 hr of incubation by the colony-counting technique (3) or by titrating the excess ammonia formed with a standard solution of 0.01 N HCl. For the colony counting, mycoplasma agar (Difco) supplemented with 0.7% yeast extract (Difco), 0.2% urea, and 10% agamma horse serum (Microbiological Associates, Inc., Bethesda, Md.) was used. The plates were dried for 2 hr at 37 C prior to inoculation and the inoculated plates were incubated in an atmosphere of 20% CO_2 in 80% N_2. Results were expressed as the number of colony-forming units (CFU) per ml. Titration of excess ammonia was performed only when the *p*H of the culture in the tube with the highest level of growth did not exceed 7.4. At *p*H range of 6.0 to 7.4, the titrimetric units corresponded with the CFU. Results were expressed as milliliters of 0.01 N HCl per 5 ml of media.

Inhibition of mycoplasma growth by amphotericin B (E. R. Squibb & Sons, New York, N.Y.), digitonin, and progesterone (Sigma Chemical Co., St. Louis, Mo.) was tested in 5 ml of the medium previously described containing 10% horse serum and various concentrations of the inhibitors added to the media as ethanolic solutions. After 24 hr of incubation at 37 C, the growth of T-mycoplasmas was determined by the colony-counting technique, the titrimetric technique as described above, or both. Growth of *A. laidlawii*, *M. gallisepticum*, and *M. hominis* was followed by measuring the absorbance at 640 nm with a Beckman DB spectrophotometer.

Analytical methods. Cell protein was determined by the method of Lowry et al. (10). Lipids were extracted from freeze-dried cells with chloroform-methanol (2:1) by the method of Folch et al. (7). Cholesterol and cholesterol esters in the lipid extracts were separated by thin-layer chromatography (2). Total cholesterol as well as free and esterified cholesterol were determined by the $FeCl_3$ method (32). When the cells were grown with ^{14}C-cholesterol, radioactivity in the free and esterified cholesterol fractions was determined, after extraction from the thin-layer plate, in a Packard Tri-Carb Liquid Scintillation Spectrometer with 10 ml of a scintillation fluid [5 g of permablend I (Packard Instrument Co., Inc., Downers Grove, Ill.) in 1 liter of toluene].

Density gradient centrifugation. Cell samples (0.2 ml, 1 to 2 mg of protein) suspended in 0.25 M NaCl were layered over 3.6 ml of a stepwise sucrose gradient (40 to 60% in six fractions). The gradients were centrifuged at 100,000 × *g* for 2 hr at 4 C in the SW39 rotor of a Spinco model L-2 ultracentrifuge. Fractions were collected by puncturing the bottom of the tube and were assayed for protein.

Electron microscopy. Pellets of sedimented cells were initially fixed in glutaraldehyde by the method of Sabatini et al. (20). These were then washed in 0.1 M cacodylate buffer (*p*H 7.4) and postfixed in 2% OsO_4 in the same buffer for 6 hr. The material was dehydrated and embedded in Epon by the method of Luft (11). Sections were stained with uranyl acetate and lead citrate (16) and examined in a Philips EM 200 electron microscope with an accelerating voltage of 100 kv, a 1- to 2-μm spot size, and a 50-μm objective aperture.

Chemicals. Coprostanol was obtained from K & K Laboratory (Hollywood, Calif.). Epicholestanol was kindly provided by C. Djerassi (Department of Chemistry, Stanford University, Stanford, Calif.). Cholesterol, 7-dehydrocholesterol, cholestanol, β-sitosterol, stigmasterol, ergosterol, epicholestanol, cholesterol laurate, 5-α-cholestane, and 5-α-cholestan-3-one were obtained from Sigma Chemical Co. All steroids were recrystallized several times from ethanol. The chemical formulas of most steroids used are shown in Fig. 1.

RESULTS

Growth of T-mycoplasmas was obtained in media containing 0.5% Difco yeast extract and 2% mycoplasma serum fraction (Difco), which were substituted for the fresh yeast extract and horse serum in other T-mycoplasma media (8, 23). The addition of low concentrations of phosphate (up to 0.025 M) supported the growth of the celis, probably due to its buffering capacity. A toxic effect of the phosphate was noticed at concentrations higher than 0.025 M. When T-mycoplasmas were grown in media containing phosphate, white rhomboid crystals appeared at *p*H levels in excess of 8.1. These crystals were soluble in dilute acids but not in boiling water. The crystals contained ammonia and phosphate and are apparently ammonium triple-phosphate crystals. When the death rate of T-mycoplasma cells at *p*H 8.1 was analyzed, a higher death rate was observed in media containing phosphate (0.015 M) and ammonia (0.02 M) than in media containing ammonia alone (Fig. 2). In the absence of ammonia, the death rate of the cells was low even when phosphate was present in the medium.

Over 98% of T-mycoplasma cells could be har-

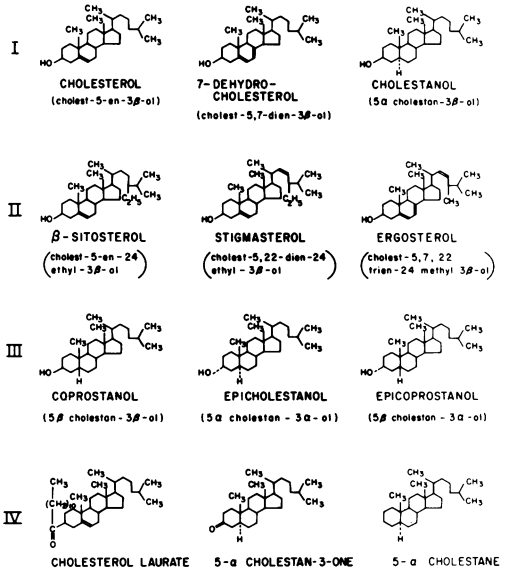

FIG. 1. *Chemical formulas of steroids used throughout this study.*

vested by centrifugation at 8,000 × *g* for 15 min. The yield of strain CH-12 cells grown in T-mycoplasma medium containing 0.015 M phosphate was about 1 mg of cell protein per liter. When thin sections of a pellet of T-mycoplasma strain CH-12 cells were viewed by electron microscopy, most of the cells appeared to be spherical, and bounded by a single membrane about 10 nm thick. The cell diameters ranged from 0.12 to 0.46 μm (Fig. 3); however, filaments more than 2 μm long and 0.15 to 0.20 μm in diameter were also observed. The cells with the small diameter were highly dense spherules (Fig. 3B). Among the larger cells, when the culture was harvested at *p*H 8.0, many "ghosts" or cells having empty-appearing areas (Fig. 3, A and B) were observed; in cultures harvested at *p*H 7.3, very few of such cells were found.

When the cells were grown in a medium containing 10% horse serum, the pellet obtained by harvesting the cells contained, in addition to T-mycoplasma cells, nonspecific precipitates, most of which could be separated from the cells by sucrose density gradient centrifugation for 2 hr. The band containing whole cells was in the interlayer between 52 and 56% sucrose. Cell membranes formed a band on top of the gradient whereas most of the nonspecific precipitates were sedimented to the bottom of the tube. In analyzing the total cholesterol content of T-mycoplasma cells obtained after sucrose density gradient centrifugation, T-mycoplasma cells were found to contain about fourfold more cholesterol than the nonsterol-requiring *A. laidlawii* (Table 1) but somewhat less than the sterol-requiring *M. hominis*, indicating a possible role of cholesterol in the growth of T-mycoplasmas. Due to the low yield of T-mycoplasmas, the results were expressed as micrograms of cholesterol per milligram of cell protein.

An additional indication that T-mycoplasmas require sterol for growth was the inability of lipid-extracted serum to support the growth of T-mycoplasma strain CH-12. The gentle extraction of the serum (90% acetone containing 0.02% ammonia at −5 C) enabled the removal of over 96% of the total cholesterol from the serum. Likewise, digitonin, amphotericin B, and progesterone, known to affect sterol-containing membranes, were highly active in inhibiting the growth of T-mycoplasma strain CH-12 as well as of *M. hominis* in a serum-containing medium while growth of *A. laidlawii* was unaffected (Fig. 4).

The growth requirement of T-mycoplasma strain CH-12 for sterols was directly demonstrated by using T-mycoplasma medium in which horse serum or mycoplasma serum fraction was replaced by 1% fatty acid-poor bovine serum albumin (Table 2). A similar cholesterol requirement was found for strains T-960 and Bowling. Increasing the concentration of cholesterol in the growth medium (up to 6 × 10^{-5} M) resulted in an increase in both the growth rate and the maximum number of viable particles of T-mycoplasmas in the medium. At a concentration higher than 8 × 10^{-5} M, cholesterol was to some extent toxic. Cholesterol was added to the media as an ethanolic solution. Attempts to add cholesterol dispersed in Tween 80 were unsuccessful due to the sensitivity of T-mycoplasmas to Tween 80. At a concentration of 0.05%, Tween 80 almost completely inhibited the growth of T-mycoplasma strain CH-12 whereas the growth of *A. laidlawii* and *M. gallisepticum* was unaffected. The addition of saturated (myristic, palmitic, or stearic acid), unsaturated (oleic acid) fatty acids (1 to 10 μg/ml), or both to the growth medium containing 1% albumin and 2 × 10^{-5} M cholesterol had no effect on the growth of T-mycoplasmas, indicating that unlike some other large-colony mycoplasmas these cells do not require fatty acids for growth. Most of the cholesterol added to the growth medium was incorporated by the T-mycoplasmas without esterification or any other chemical change; 95.1% of the radio-

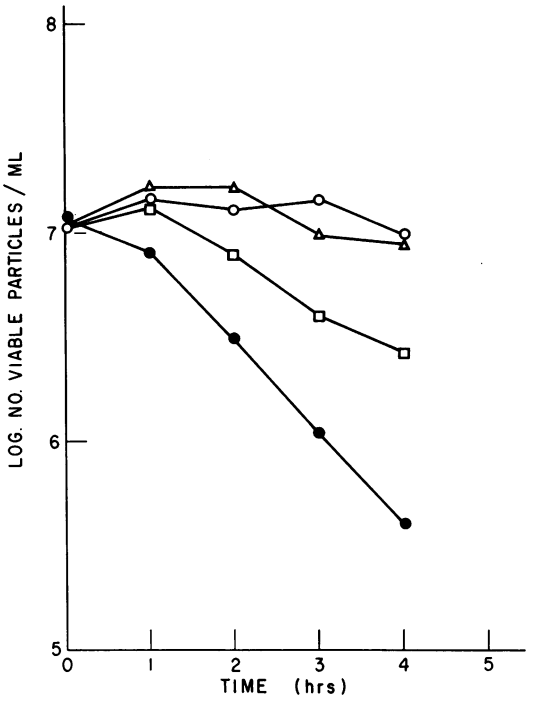

FIG. 2. *Effect of phosphate and ammonia on the death rate of T-mycoplasmas at pH 8.1 and 37 C. Symbols:* ○, *medium alone;* △, *medium plus phosphate;* □, *medium plus ammonia;* ●, *medium plus phosphate plus ammonia.*

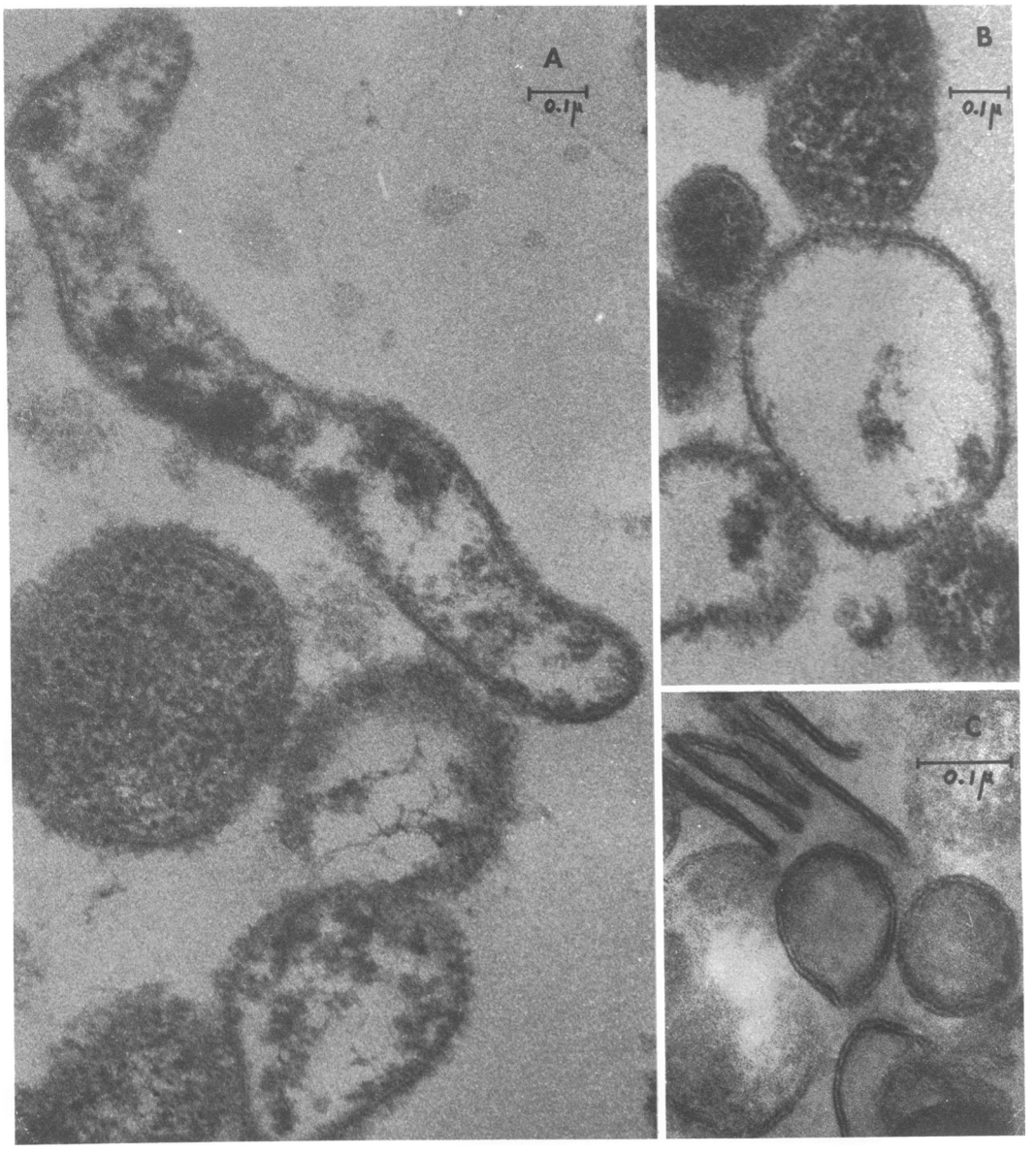

FIG. 3. *Thin sections through cell pellets of T-mycoplasma strain CH-12 harvested at pH 7.9. (A) Round and filamentous cells. (B) Highly dense and empty-appearing cells. (C) Cell membranes.*

activity derived from ^{14}C-cholesterol added to the growth medium could be recovered from the free cholesterol fraction of T-mycoplasma lipids.

The growth requirement of T-mycoplasma cells for sterol could be fulfilled by other sterols as well as by cholesterol (Fig. 5). Complete saturation of the cyclopentanophenanthrene ring or unsaturation of the ring at different positions only slightly affect the growth-promoting activity of the sterol. Thus 7-dehydrocholesterol, having an unsaturated ring at C5 and C7, and cholestanol, having a saturated ring (*see* Fig. 1), support the growth of the cells. Various degrees of growth-promoting activity were noticed with sterols having an unsaturated ring at C5 and different hydrocarbon side chains (*see* Fig. 1, II). β-Sitosterol, having an additional ethyl group on the hydrocarbon side chain, was even more effective at low concentrations than cholesterol (Fig. 5). Other changes in the hydrocarbon side chain, such as the introduction of a double bond into the hydrocarbon side chain (stigmasterol and ergosterol),

TABLE 1. *Cholesterol content of T-mycoplasmas*

Organism	Cell yield[a]	Total cholesterol[b]
T-mycoplasma strain CH-12	1.24	46.4
T-mycoplasma strain T-960	1.08	61.1
Acholeplasma laidlawii	96.00	14.2
Mycoplasma hominis	26.80	113.6

[a] Cells were grown in media containing 10% horse serum. Values are expressed as milligram of cell protein per liter of culture.

[b] Values are expressed as micrograms of cholesterol per milligram of cell protein.

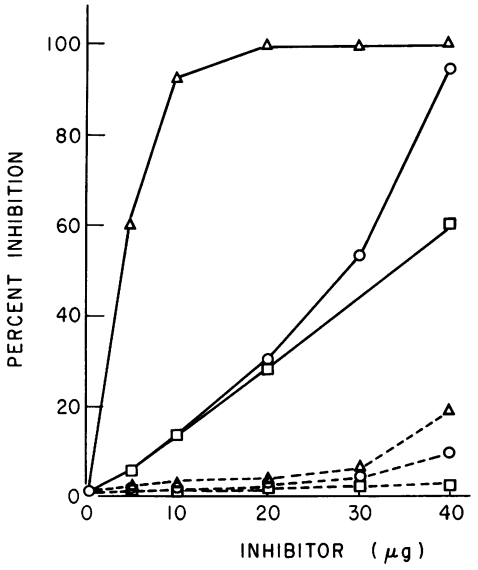

FIG. 4. *Growth inhibition of T-mycoplasmas and A. laidlawii by digitonin, amphotericin B, and progesterone. Symbols:* O, *digitonin;* Δ, *amphotericin B;* □, *progesterone. Solid lines are T-mycoplasma strain CH-12; broken lines are A. laidlawii.*

TABLE 2. *Effect of cholesterol on the growth of T-mycoplasmas*[a]

Cholesterol (M)	CFU/ml[b]	Ammonia production (μmoles/ml)
0	1.1×10^4	1.2
10^{-5}	1.7×10^6	8.3
2×10^{-5}	8.0×10^6	20.0
4×10^{-5}	1.2×10^7	27.4

[a] T-mycoplasma strain CH-12 was grown in a medium containing 1% albumin. Cholesterol was added to the media as ethanolic solutions as described in Materials and Methods.

[b] Colony-forming units.

resulted in less effective compounds. Compounds differing from cholesterol in their stereo-configuration, such as epicoprostanol, coprostanol, epicholestanol, and coprostan-3-one, were unable

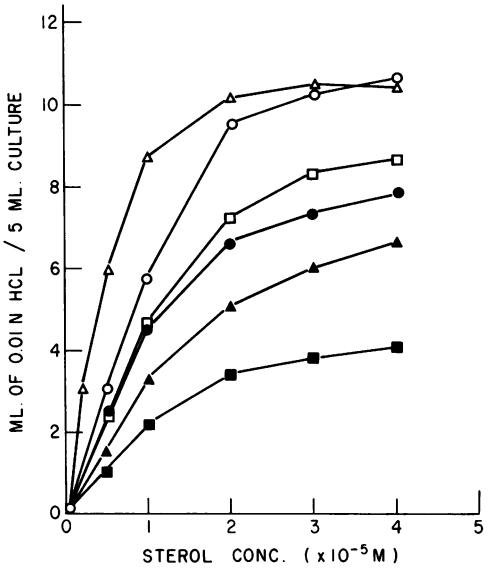

FIG. 5. *Growth response of T-mycoplasmas to various sterols. Symbols:* O, *cholesterol;* Δ, *β-sitosterol;* □, *7-dehydrocholesterol;* ●, *cholestanol;* ▲, *stigmasterol;* ■, *ergosterol.*

to support growth of T-mycoplasmas. Furthermore, these compounds were found to inhibit the growth of the cells. Of the compounds listed, epicoprostanol was the most effective inhibitor. In the presence of 2×10^{-5} M epicoprostanol, growth of cells in media containing 10^{-5} M cholesterol was inhibited by about 70%, whereas, under the same conditions, coprostan-3-one (2×10^{-5} M) only inhibited growth by 10%. Epicoprostanol very effectively inhibited the growth of T-mycoplasma as well as of *M. gallisepticum* in media containing mycoplasma serum fraction; the non-sterol-requiring *A. laidlawii* was only slightly affected (Table 3). The growth inhibition of T-mycoplasma by epicoprostanol, coprostanol, epi-

TABLE 3. *Growth inhibition of mycoplasmas by epicoprostanol*[a]

Epicoprostanol (M)	Per cent inhibition[b]		
	Acholeplasma laidlawii	*Mycoplasma gallisepticum*	T-mycoplasmas[c]
2×10^{-5}	5.9	67.4	54.1
4×10^{-5}	9.8	85.2	92.0
8×10^{-5}	16.2	98.0	99.4

[a] Cells were grown in media containing 2% mycoplasma serum fraction as described in Materials and Methods. Growth was tested after 18 to 24 hr of incubation at 37 C.

[b] Compared to growth with no inhibitor.

[c] Same inhibition was found for strain CH-12 and Bowling.

cholestanol, and coprostan-3-one in media containing 1% albumin and cholesterol could be partially reversed by increasing the cholesterol concentration in the media (Fig. 6) for epicoprostanol and epicholestanol. Steroids lacking a free 3-hydroxyl group such as 5-α cholestane, 5-α coprostane, 5-α cholestan-3-one, and cholesterol-laurate when tested in concentrations of up to 8 $\times$ 10^{-5} M neither support nor inhibit the growth of T-mycoplasma strains CH-12, Bowling, and T-960. For inhibition experiments, these compounds were added to the growth medium simultaneously with cholesterol (10^{-5} M).

DISCUSSION

The high toxicity of ammonia for T-mycoplasmas was previously reported (8, 24). Our observations suggest that this toxicity was much more pronounced in the presence of phosphate, but it is not yet clear to what extent the crystals which appeared in the presence of phosphate and ammonia affected the death rate of the cells. The possibility that these crystals reduce toxicity by neutralizing excess ammonia can not be excluded.

The electron microscope studies revealed that, in common with the large-colony mycoplasmas (1), T-mycoplasmas are spherical cells bounded by a single triple-layered membrane. The empty areas in the cells might be attributed, at least in part, to degeneration resulting from the toxic effect of ammonia accumulation. Such areas appeared even in filamentous cells that are considered, according to the filamentous growth concept, as reproductive units (9).

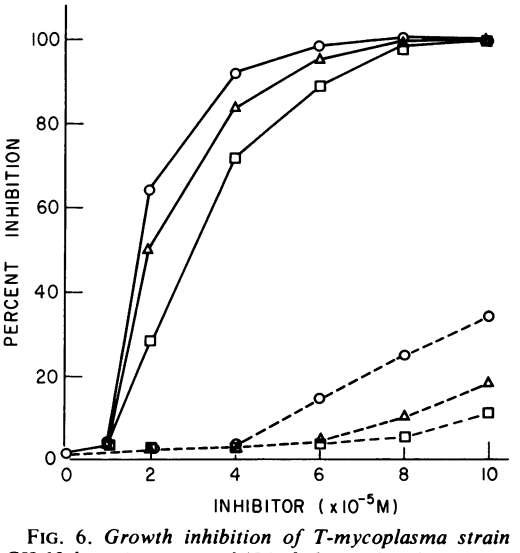

FIG. 6. *Growth inhibition of T-mycoplasma strain CH-12 by epicoprostanol (5β-cholestan-3α-ol) and epicholestanol (5α-cholestan-3α-ol) and its partial reversion by cholesterol. Symbols:* O, *10^{-5} M cholesterol;* Δ, *2 $\times$ 10^{-5} M cholesterol;* $\square$, *4 $\times$ 10^{-5} M cholesterol. Solid lines are epicoprostanol; broken lines are epicholestanol.*

The lysis of mycoplasmas by digitonin and amphotericin B, as well as the growth inhibition of mycoplasmas by digitonin, have been previously described (15, 31). The action of these substances was attributed mainly to their interaction with sterols known to be located in mycoplasmas exclusively in the cell membrane. The fact that digitonin and amphotericin strongly inhibited the growth of T-mycoplasmas as well as *M. hominis*, but had little effect on the growth of the non-sterol-requiring *A. laidlawii*, underscores the important role of sterols in the growth of T-mycoplasmas.

The poor growth of T-mycoplasmas in liquid media did not enable the absolute determination of the percentage of cholesterol in the cells. However, assuming that the protein content of T-mycoplasmas is close to that reported for other mycoplasmas (15), and based on the cholesterol to protein ratio in the cells, T-mycoplasmas contain much more cholesterol than the saprophytic *A. laidlawii* but still less than *M. hominis*. The separation of the cells on sucrose density gradient allows relatively clean T-mycoplasma preparations to be obtained as judged by the small amount of amorphous material seen in material examined in the electron microscope.

A cholesterol requirement for the growth of mycoplasmas was first described by Edward and Fitzgerald (5). This requirement could be fulfilled by certain other sterols, each having various degrees of efficiency (19, 27). Although some inconsistencies in the results were reported, the conformation and configuration of the sterol necessary to support the growth of mycoplasmas have been discussed by Smith and his coworkers (25–29). Our data indicate that saturation or unsaturation of the cyclopentanophenanthrene ring is of minor importance for T-mycoplasmas. Although the unsaturated ring at C5 was the most effective compound tested, a complete saturation (cholestanol) or unsaturation at C5 and C7 (7-dehydrocholesterol) resulted in an active compound. In other large-colony mycoplasmas, 7-dehydrocholesterol did not replace cholesterol (25) and even inhibited growth (18). Changes in the growth-promoting activity of the sterols were noticed as a result of changing the hydrocarbon side chain. β-Sitosterol having a 10-carbon side chain was more effective than cholesterol. However, introduction of a double bond at C22 (stigmasterol) resulted in a much less active compound. Coprostane-type steroids having A and B rings of the cyclopentanophenanthrene molecule in a *cis* position did not replace cholesterol in the

medium and even inhibited growth in its presence. This inhibition could be reversed by increasing the cholesterol concentration in the medium. Coprostane-type steroids also inhibited the growth of *M. gallisepticum* but not of the non-sterol-requiring *A. laidlawii* even when tested in a cholesterol-containing medium.

Similar to the inhibitory effect of coprostanol, steroids having the C3 hydroxyl group in an α configuration (epicholestanol) were also highly inhibitory, but steroids lacking the free hydroxyl group at C3 could neither support nor inhibit growth of T-mycoplasmas. The inability of cholesterol esters to support growth of mycoplasmas was described previously (5, 17). However, cholestan-3-one actively inhibited the growth of *A. laidlawii* (4) as well as of sterol-requiring mycoplasmas but only slightly inhibited T-mycoplasmas.

These results which indicate for T-mycoplasmas a sterol requirement similar to that for large-colony mycoplasmas have important implications for their taxonomic position. The T-mycoplasmas, although possessing most of the general properties of large-colony mycoplasmas, have several distinctive characteristics. On the basis of their major properties, most important of which is the lack of a cell wall, T-mycoplasmas should be included in the new class *Mollicutes* along with the large-colony mycoplasmas. Recently the single genus within this class has been expanded to two genera in appreciation of the distinction that can be made between sterol-requiring and non-sterol-requiring species (6). Thus, sterol-requiring organisms are classified in the genus *Mycoplasma* and non-sterol-requiring organisms in the genus *Acholeplasma*. It is our view that demonstration here of a sterol requirement for the T-mycoplasmas along with a prior description of their requirement for urea (24) now allows for classification of these organisms. We would conclude that a requirement for urea is as significant as a sterol requirement and hence would suggest that the T-mycoplasmas be ranked as a separate and third genus in the order *Mycoplasmatales*, class *Mollicutes*.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service contract PH 43-68-1256 within the Special Virus Cancer Program, National Cancer Institute; L. Hayflick is Public Health Service Career Development Awardee 7KO4 CAO5938-07.

We thank G. B. Haydon of the Department of Pathology, Stanford University School of Medicine, for the use of the electron microscope and E. Stanbridge and K. F. Tam of the Department of Medical Microbiology, Stanford University School of Medicine, for their assistance in preparing the samples for electron microscopy.

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