Incorporation of Cholesterol by Membranes of Bacterial L-phase Variants

WITH AN APPENDIX

On the Determination of the L-phase Parentage by the Electrophoretic Patterns of Cell Proteins

By S. RAZIN AND ZEHAVAH SHAFER

*Department of Clinical Microbiology, The Hebrew University-Hadassah
Medical School, Jerusalem, Israel*

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SUMMARY

L-phase variants of *Streptobacillus moniliformis* and *Proteus mirabilis* resembled mycoplasmas in their ability to incorporate considerable amounts of exogenous cholesterol into their cell membranes. Much smaller quantities were incorporated by L-phase variants of *Streptococcus pyogenes* and *Staphylococcus aureus*, by the parent bacteria of all L-phase variants except *S. moniliformis*, and by a series of wall-covered bacteria, including their protoplasts and spheroplasts. Cholesterol-binding ability corresponded roughly to sensitivity to growth inhibition and lysis by digitonin. Like mycoplasma, the *S. moniliformis* L-phase had a non-enzymic cholesterol uptake mechanism, but its growth in a cholesterol-free medium was not improved by adding cholesterol. It is concluded that a bacterium, on transforming to its stable L-phase, does not necessarily develop the ability to bind cholesterol; if it does, the cholesterol bound is not essential for growth of the L-phase, unlike cholesterol-requiring mycoplasmas.

INTRODUCTION

Cholesterol, though an important constituent of membranes of eucaryotic organisms (Rothfield & Finkelstein, 1968), is not usually found in microbial membranes (Fiertel & Klein, 1959). Most mycoplasma species form an exception: cholesterol, supplied by the growth medium, is an essential component of their cell membrane (Razin, 1969). They also differ from other bacteria in that, like the stable L-phase variants of bacteria, they have no cell wall. As early as 1941 Partridge & Klieneberger reported the presence of large quantities of cholesterol in *Streptobacillus moniliformis* L-phase (L-1) cultures. Later, cholesterol originating from serum in the growth medium was detected in the L-phase of *Proteus mirabilis* (Rebel, Bader-Hirsch & Mandel, 1963) and in the L-phase of *Agrobacterium tumefaciens* (Santaolalla, 1966). Cholesterol was located in the cell membrane of the *S. moniliformis* L-phase by Razin & Boschwitz (1968).

The large quantities of cholesterol in L-phase membranes raise the question: is the transformation of a bacterium to its stable L-phase directly connected with the

microbial plasma membrane developing ability to incorporate cholesterol? Cholesterol has a stabilizing effect on artificial phospholipid + cholesterol membranes (Willmer, 1961; De Gier, Mandersloot & Van Deenen, 1969) and also on mycoplasma membranes (Razin, 1967). A fair hypothesis was that a capacity to integrate cholesterol into the microbial plasma membrane made for better adaptation to growth without a protective cell wall. To establish whether there is a connexion between cholesterol incorporation and L-phase transformation, cholesterol uptake was tested in a series of bacteria, protoplasts, spheroplasts, L-phase variants and their presumed parent bacteria and experiments were made to find whether the cholesterol, if in fact incorporated, was an integral part of the cell membrane and essential to the growth of the organism after it had lost its cell wall. The findings of Edward (1953), Smith & Rothblat (1960, 1962) and Rebel *et al.* (1963), which furnish considerable information on the matter, have thus been extended and re-evaluated.

METHODS

Organisms. *Streptobacillus moniliformis* ATCC 14647, *S. moniliformis* L-phase (L-1) ATCC 14075, *Proteus mirabilis* (strain 9) ATCC 14273, *P. mirabilis* L-phase (strain 'L 9') ATCC 14168, *Staphylococcus aureus* (Smith) ATCC 19636, and *S. aureus* L-phase (Smith L form) ATCC 19640 were obtained from the American Type Culture Collection (Rockville, Md. U.S.A.) through the courtesy of Dr R. G. Wittler. *Streptococcus pyogenes* and its derived stable L-phase variant were obtained from Dr C. Panos (Albert Einstein Medical Centre, Philadelphia, Penn., U.S.A.). *Mycoplasma mycoides* var. *capri* (PG 3) was obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. laidlawii* (oral strain) was isolated in our laboratory and *M. gallisepticum* (A 5969) was provided by Dr M. E. Tourtellotte (Department of Animal Diseases, The University of Connecticut, Storrs, Conn. U.S.A.). *Bacillus megaterium* (KM) and *Sarcina lutea* were the gift of Dr C. Weibull (Lund University, Sweden). All other bacteria were taken from our departmental collection.

Growth conditions. The organisms were grown in a modified Edward medium (Razin, 1963) containing 3% (v/v) horse serum instead of Difco-PPLO serum fraction. For growth of the L-phase variants of the Gram-positive bacteria and their presumed parents NaCl was added to the medium to 3% (w/v). For satisfactory growth of *Streptobacillus moniliformis* and the L-phase variant of *Streptococcus pyogenes* the concentration of the serum in the medium had to be increased to 10% (v/v). The organisms were grown at 37° in 0.5 l. volumes dispensed in 1 to 2 l. flasks. The mycoplasma and L-phase cultures were incubated statically, while all other bacterial cultures were shaken. The organisms were harvested after 24 to 48 hr of incubation by centrifugation at 23,000 *g* for 20 min. Since growth of the L-phase of *Staphylococcus aureus* was slow it was usually harvested after 72 hr of incubation. The sedimented organisms were washed twice in 0.5M-NaCl at 4°. Cell protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as standard.

Preparation of protoplasts and spheroplasts and isolation of cell membranes. Protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* were prepared by using lysozyme (Gilby & Few, 1960) and protoplasts of *Bacillus megaterium* were obtained by the

method of Miller (1963). Spheroplasts of *Escherichia coli* were prepared by the combined action of lysozyme and EDTA (Birdsell & Cota-Robles, 1967). The mycoplasma and L-phase membranes were isolated by osmotic lysis of the organisms as described previously (Razin, Morowitz & Terry, 1965; Razin & Boschwitz, 1968). The membranes were washed twice in 0.01M-phosphate buffer (pH 7.5) containing 0.05M-NaCl.

Cholesterol uptake by growing organisms. The organisms were grown in 500 ml. volumes of Edward medium supplemented with 0.2 μ c of either [4-¹⁴C]cholesterol (specific activity 55.8 mc/m-mole) or cholesteryl-[1-¹⁴C]oleate (specific activity 10.7 mc/m-mole) (purchased from The Radiochemical Centre, Amersham, England). The organisms were harvested and washed as described above and their lipid extracted twice with chloroform + methanol (2 + 1, by vol.) first for 1 hr and then overnight at 4°. Radioactivity measurements showed that this extraction removed all the radioactive cholesterol or cholesteryl oleate from the organisms. Cholesterol and cholesteryl esters in the lipid extracts were separated by thin-layer chromatography and estimated by the FeCl₃ reaction and by radioactivity measurements (Argaman & Razin, 1965). Correlation between the two methods was good; in most experiments the radioactivity technique was used. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using 0.3% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis-2-(5-phenyl-oxazolyl)-benzene in toluene as the scintillation liquid. The efficiency of the ¹⁴C counting was 80%.

Cholesterol uptake by washed organisms and cell membranes. A mixture of 2 ml. cell or membrane suspension (containing 2 mg. protein), 0.5 ml. horse serum containing 0.05 μ c of labelled cholesterol or cholesteryl oleate, and 17.5 ml. of 0.5 M-NaCl in 0.067M-phosphate buffer (pH 7.5) was shaken in a 37° water bath; 2 ml. samples were withdrawn at various times and passed through membrane filters (type HA, 0.45 μ pore size, Millipore Corp, Bedford, Mass., U.S.A.). Filtration was by suction, through application of a negative pressure of 60 mm. of mercury. The filters were washed with 30 ml. of a cold 0.5M-NaCl solution containing 0.067M-phosphate buffer (pH 7.5) and 3% (v/v) horse serum. The filters were air-dried and their radioactivity determined. Mixtures without cells or membranes, which served as controls for the non-specific adsorption of radioactive cholesterol or cholesteryl oleate to the filters, showed very low radioactivity values.

Cholesterol exchange. The method of Rothblat *et al.* (1966) was used in a modified form. The organisms were grown in Edward medium containing [4-¹⁴C]cholesterol, washed twice and suspended in the uptake mixture described above which contained [1 α -³H]cholesterol (specific activity 500 mc/m-mole, The Radiochemical Centre, Amersham, England) instead of [4-¹⁴C]cholesterol. The suspension was shaken in a 37° water bath and 2 ml. samples withdrawn at various times and filtered. The filters were assayed for both ¹⁴C and ³H by liquid scintillation spectrometry. The efficiency of ¹⁴C counting was 54% and that of ³H was 20%.

Inhibition of growth and lysis of micro-organisms by digitonin. Inhibition of growth was tested in Edward medium containing 3% (v/v) horse serum. An ethanolic solution (0.5 ml.) of digitonin (40 mg./100 ml.) was added to 9.7 ml. of Edward medium without serum. The ethanol was then evaporated by boiling the medium, and 0.3 ml. horse serum was added after cooling. Serial two-fold dilutions of this medium were made in 5 ml. quantities of the serum-containing Edward medium. Each test-tube received 0.1 ml. of a young culture of the test organism and the tubes were incubated at 37°. Lysis

by digitonin was examined in test-tubes containing different concentrations of digitonin in 3 ml. of 0.5M-NaCl. Each test-tube received 0.1 ml. of a washed suspension of organism and the extinction was read at 500 m μ after 30 min. incubation at 37°.

Table 1. *Uptake of cholesterol by growing or washed organisms and their sensitivity to digitonin**

Organism	[4- ¹⁴ C]cholesterol uptake (counts/min./mg. cell protein)		Minimal concentration of digitonin inhibiting growth§ (μ g./ml.)
	Growing organisms†	Washed organisms‡	
<i>Streptobacillus moniliformis</i>			
L-phase	3515	19,820	15
Bacterial phase	1674	17,000	60
<i>Proteus mirabilis</i>			
L-phase	2032	3200	120
Bacterial phase	152	250	> 2000
<i>Streptococcus pyogenes</i>			
L-phase	148	1115	1000
Bacterial phase	161	1100	> 2000
<i>Staphylococcus aureus</i>			
L-phase	442	195	120
Bacterial phase	541	540	> 2000
<i>Mycoplasma gallisepticum</i>	4372	3180	30
<i>M. mycoides</i> var. <i>capri</i>	2154	5530	30
<i>M. laidlawii</i>	1224	2050	500
<i>Diplococcus pneumoniae</i>	627	ND	500
<i>Mycobacterium phlei</i>	90	125	> 2000
<i>Corynebacterium diphtheriae</i>	612	1725	> 2000
<i>Pseudomonas aeruginosa</i>	37	1500	2000
<i>Escherichia coli</i> K 12			
Bacterial phase	102	1430	> 2000
Spheroplasts	62	1500	ND
<i>Bacillus megaterium</i>			
Bacterial phase	9	1360	> 2000
Protoplasts	11	1400	ND
<i>Micrococcus lysodeikticus</i>			
Bacterial phase	102	1745	> 2000
Protoplasts	74	1520	ND
<i>Sarcina lutea</i>			
Bacterial phase	15	1260	> 2000
Protoplasts	8	1630	ND

* Each result represents the average of 3-5 experiments.

† Grown in Edward medium containing 0.2 μ c of [4-¹⁴C]cholesterol/500 ml. of medium.

‡ Uptake determined after 3 hr incubation at 37° in a reaction mixture containing [4-¹⁴C]cholesterol as described in Methods.

§ Tested in Edward medium containing 3% (v/v) horse serum. Results read after 48 hr incubation at 37°. *S. moniliformis* and *S. pyogenes* bacterial and L-phase organisms were tested in Edward medium containing 10% (v/v) horse serum.

ND, not done.

RESULTS

Incorporation of cholesterol by growing organisms. The L-phase variants of the Gram-negative bacteria resembled mycoplasmas in their marked ability to incorporate cholesterol from the growth medium (Table 1). The L-phase of *Proteus mirabilis*

incorporated far more cholesterol than its parent bacterium, while in *Streptobacillus moniliformis* the difference was less pronounced. Phase-contrast microscopy of *S. moniliformis* cultures showed both rods and spherical forms resembling those of the L-phase variant. The percentage of the spherical forms was very variable. The ability of the L-phase variants of the Gram-positive bacteria to incorporate cholesterol was low and resembled that of their presumed parents and other wall-covered bacteria investigated. Cholesterol uptake by wall-covered bacteria was generally low, but somewhat higher in *Diplococcus pneumoniae*, *Corynebacterium diphtheriae* and *Staphylococcus aureus* (Table 1). When the cell wall of bacteria grown with [4-¹⁴C]-cholesterol was completely or partly removed at least half of the radioactivity remained in the resulting protoplasts or spheroplasts (Table 1). Chromatographic analysis of the extracted cell lipids showed that in most organisms grown with [4-¹⁴C]cholesterol, it was incorporated unchanged, except in *Mycobacterium phlei* and *Escherichia coli*, where 23 to 50% was esterified.

Table 2. Uptake of cholesterol by L-phase and mycoplasma membranes

Membranes	Incubation time (min.)	[4- ¹⁴ C]cholesterol uptake (counts/min./mg. membrane protein)
L-phase of <i>S. moniliformis</i>	0	700
	60	13,100
	180	19,400
	240	23,200
L-phase of <i>P. mirabilis</i>	0	30
	60	1050
	180	2000
	240	3400
L-phase of <i>S. pyogenes</i>	0	300
	60	800
	180	1600
	240	1600
<i>M. laidlawii</i>	0	80
	60	700
	180	2020
	240	3300

Incorporation of cholesterol by washed organisms and cell membranes. Generally, cholesterol uptake by washed organisms resembled that of growing organisms, but washed organisms of *Streptobacillus moniliformis* L-phase incorporated cholesterol much better than all the other organisms tested, including the mycoplasmas (Table 1). The wall-covered bacteria and their protoplasts and spheroplasts bound only small amounts of cholesterol, and the uptake usually was instantaneous. Cholesterol uptake of the L-phase variants of *S. moniliformis*, *Proteus mirabilis* and the mycoplasmas depended on time (Fig. 1). *S. moniliformis* L-phase incorporated cholesterol faster than any of the mycoplasmas, and usually reached saturation after 1 hr (Fig. 1). Table 2 shows that isolated L-phase and mycoplasma membranes incorporated cholesterol; the membranes of the *S. moniliformis* L-phase showed the highest values. Growing L-phase variants of *S. moniliformis*, *P. mirabilis*, *M. laidlawii* and *M. mycoides* var. *capri* incorporated much less cholesteryl oleate than free cholesterol (Table 3).

Growth inhibition and lysis by digitonin. The sensitivity of organisms to growth inhibition by digitonin roughly paralleled their ability to incorporate cholesterol. Thus,

Streptobacillus moniliformis L-phase and the parasitic mycoplasmas were the most sensitive, while the other L-phase variants and *Mycoplasma laidlawii* were less sensitive (Table 1). All the wall-covered bacteria except *Diplococcus pneumoniae* were essentially resistant to digitonin. The organisms whose growth was inhibited by digitonin were also lysed by it (Fig. 2).

Factors influencing cholesterol uptake. *Streptobacillus moniliformis* L-phase, having the highest cholesterol uptake, was chosen for this part of the study. Cholesterol

Table 3. Uptake of cholesteryl oleate by growing organisms compared to free cholesterol uptake

Organism*	Radioactivity (counts/min./mg. cell protein)	
	Cholesteryl-[1- ¹⁴ C]oleate uptake	[4- ¹⁴ C]cholesterol uptake
L-phase of <i>S. moniliformis</i>	234	3095
L-phase of <i>P. mirabilis</i>	98	1170
<i>S. pyogenes</i>		
L-phase	53	97
Bacterial phase	75	97
<i>M. mycoides</i> var. <i>capri</i>	192	1987
<i>M. laidlawii</i>	14	1607
<i>M. lysodeikticus</i>	10	77
<i>M. phlei</i>	4	92

* The organisms were grown in Edward broth containing 0.2 µc of either cholesteryl-[1-¹⁴C]-oleate or [4-¹⁴C]cholesterol/500 ml. medium.

Fig. 1. Cholesterol uptake by washed organisms of the L-phase of *Streptobacillus moniliformis* (●); the L-phase of *Proteus mirabilis* (■); *Mycoplasma mycoides* var. *capri* (○); *Mycoplasma gallisepticum* (△); *Mycoplasma laidlawii* (□); *Escherichia coli* (▲).

Fig. 2. Lysis of washed organisms by digitonin. L-phase of *Streptococcus pyogenes* (○); L-phase of *Proteus mirabilis* (▲); L-phase of *Streptobacillus moniliformis* (●); *Mycoplasma laidlawii* (△).

uptake of washed organisms depended on temperature (Fig. 3), but after heating at 60° for 30 min. they still incorporated about two-thirds of the amount incorporated when unheated. The decrease was apparently due to the organisms aggregating when heated so that the cell surface available for cholesterol binding was smaller. Within the range of pH 5.0 to 9.0 the pH value had little effect on the cholesterol uptake. At more acid pH values the serum proteins carrying the cholesterol precipitated and the organisms agglutinated, decreasing the amount incorporated. Cholesterol uptake by

Fig. 3. Temperature dependence of the cholesterol uptake by washed organisms of the *Streptobacillus moniliformis* L-phase.

washed organisms of the *S. moniliformis* L-phase, *Mycoplasma gallisepticum* and *Streptococcus pyogenes* was unchanged when the NaCl concentration in the mixture used for measuring uptake was changed from 0 to 0.5M, but was lower when Tween 80 was used as emulsifier and carrier of cholesterol instead of serum.

The cholesterol incorporated was firmly bound to the cell components. During incubation of the *Streptobacillus moniliformis*-phase, prelabelled with [4-¹⁴C]cholesterol, in a fresh medium containing [1 α -³H]labelled cholesterol very little ¹⁴C was lost but ³H was incorporated (Fig. 4). The results with *Mycoplasma gallisepticum* were essentially the same.

The effect of cholesterol on the growth of the Streptobacillus moniliformis L-phase. Since the *Streptobacillus moniliformis* L-phase resembles the parasitic mycoplasmas

in its ability to incorporate appreciable amounts of cholesterol into its membrane (see also Razin & Boschwitz, 1968) we re-examined the role of cholesterol in the growth of this L-phase variant. The *S. moniliformis* L-phase could not be grown in the partially defined medium of Razin & Cohen (1963) suitable for *Mycoplasma laidlawii* growth, even when this was supplemented with yeast extract. It could, however, be grown in Edward broth in which the serum component had been replaced by 0.01% (w/v) Tween 80 + 1% (w/v) bovine serum albumin fraction V. No cholesterol could be detected by chromatographic analysis in the lipid (98 mg.) extracted from the constituents of this serum-free medium (sufficient to make up 200 ml.). Figure 5 shows that

Fig. 4

Fig. 5

Fig. 4. Uptake and loss of cholesterol by washed organisms of the *Streptobacillus moniliformis* L-phase. The organisms were grown in Edward medium containing [4-¹⁴C]cholesterol, harvested, washed and tested for additional [1-³H]cholesterol uptake (●) and [4-¹⁴C]-cholesterol loss (○) as described under Methods.

Fig. 5. The effect of cholesterol on growth of the *Streptobacillus moniliformis* L-phase (○) and *Mycoplasma gallisepticum* (●). The organisms were grown in Edward broth devoid of serum, but with 0.01% (w/v) Tween 80, 1% (w/v) bovine serum albumin fraction V, 12 μg/ml. palmitic acid and various concentrations of cholesterol solubilized in Tween 80. The organisms were harvested after 120 hr incubation at 37°, washed twice in 0.5 M-NaCl and their cell protein was determined.

cholesterol added to this medium did not improve the growth of *S. moniliformis* L-phase, but had a most pronounced effect on the growth of *M. gallisepticum*. The *S. moniliformis* L-phase could be subcultured several times in serum-free medium with no apparent decrease in growth, and after their second subculture no cholesterol could be detected in the lipid fraction (3.1 mg.) of the L-phase cells. The minimal amount of cholesterol detected by the thin-layer chromatographic method used was 1 μg. included in a spot of 4 mm. diameter.

DISCUSSION

Our results show that the transformation of a bacterium to its L-phase is correlated with a cholesterol-binding capacity in *Proteus mirabilis* but not in the Gram-positive cocci. Bacterial-phase cultures of *Streptobacillus moniliformis* incorporated slightly less cholesterol than did the L-phase. However, here the transformation to the L-phase occurs spontaneously (Dienes, 1939), so that bacterial-phase cultures always contained a high percentage of L-phase forms, which may account for their high cholesterol incorporation.

Chemical analyses of isolated cell membranes (Razin, 1967; Razin & Boschwitz, 1968) have suggested that the cholesterol taken up by mycoplasmas and L-phase variants is located in the cell membrane and we have now shown marked cholesterol uptake by isolated L-phase and mycoplasma membranes. The sensitivity of mycoplasmas to lysis by digitonin (Smith & Rothblat, 1960, 1962; Razin & Argaman, 1963) suggests that the cholesterol becomes an integral part of their membrane. Saponin, which resembles digitonin in action and is known to form holes in biological and artificial cholesterol-containing membranes, has recently been shown to produce holes in *Mycoplasma mycoides* var. *mycoides* membranes (Chu & Horne, 1967). Our study extends these previous observations by showing that digitonin inhibits mycoplasma and L-phase growth. Since the sensitivity of the various organisms to growth inhibition by digitonin roughly paralleled their cholesterol-binding capacity, this method may be useful in comparing the cholesterol content of different microbial membranes.

Smith & Rothblat (1962) reported that digitonin did not lyse the *Streptobacillus moniliformis* L-phase but caused agglutination of the organisms. Since we were able to show distinct lysis of this L-phase by digitonin, it seems probable that Smith & Rothblat were using organisms which had already been lysed during washing before being tested for digitonin sensitivity. They used physiological saline (0.15 M-NaCl) and not 0.5 M-NaCl which in our experience protects the fragile L-phase organisms much better against lysis during washing. The sensitivity of *Diplococcus pneumoniae* to growth inhibition and lysis by digitonin distinguishes it from the other wall-covered bacteria. Digitonin sensitivity depends on the pneumococcus being grown in the presence of cholesterol (Klein & Stone, 1931; Smith & Rothblat, 1960). Probably the mechanism of lysis involves activation of autolytic enzymes which solubilize the rigid cell wall, as postulated to explain the lysis of this bacterium by surface-active agents (MacLeod, 1965).

Smith & Rothblat (1960) with mycoplasmas and Rothblat *et al.* (1966) with tissue-culture cells, noted that the adsorbed cholesterol was firmly attached to the cells and that there was no exchange with exogenous cholesterol. Graham & Green (1967), on the other hand, claimed that cholesterol in a variety of animal cell membranes was exchanged with plasma cholesterol. However, the rate of exchange as shown by Bruckdorfer, Graham & Green (1968) was rather slow. Our experiments also showed a slow rate of exchange of the cholesterol bound to the *Streptobacillus moniliformis* L-phase and *Mycoplasma gallisepticum* with exogenous cholesterol, but so slowly that serum could be used in the washing fluid to remove radioactive cholesterol weakly adsorbed to the cells and to the membrane filters.

The present findings corroborate the conclusion of Smith & Rothblat (1960) and of Rothblat *et al.* (1966) that the mycoplasma and L-phase cells take up cholesterol by a

physical adsorptive non-enzymic mechanism. Both the L-phase variants and the mycoplasmas investigated here incorporated much less cholesteryl oleate than free cholesterol as had been found with mycoplasmas (Argaman & Razin, 1965), the L-phase of *Proteus mirabilis* P 18 (Rebel *et al.* 1963), and tissue-culture cells (Rothblat *et al.* 1966). The cholesterol in biological membranes is found almost exclusively in the free state (Ashworth & Green, 1966; Nelson, 1967; Pfeiffer, Anderson & Snyder, 1968). The rather tight packing of lipids in cellular membranes, due to the presence of protein septa might, according to Bruckdorfer *et al.* (1968), explain the rejection by the membranes of sterols with bulky side-chains and the absence of cholesteryl esters in them.

Our findings support the suggestion of Edward (1953) that the requirement of cholesterol for growth is a valid criterion for differentiating between bacterial L-phase variants and mycoplasmas. The ability of the L-phase variants of *Streptococcus pyogenes* (van Boven, Kastelein & Hijmans, 1967), *Listeria monocytogenes* (Edman, Pollock & Hall, 1968), *Bacillus subtilis* (Burmeister & Hesseltine, 1968), *Proteus mirabilis* (Rebel *et al.* 1963) and *Agrobacterium tumefaciens* (Santaolalla, 1966) to grow in cholesterol-free media proves their independence of cholesterol, while most mycoplasmas are dependent on it for growth. One of our main objectives was to show that the *Streptobacillus moniliformis* L-phase also conforms to this rule, although it incorporates large quantities of cholesterol into its cell membrane. Edward (1953) could not show a requirement for cholesterol by the *S. moniliformis* L-phase but found that the serum had to be replaced by acetone-insoluble lipid from egg yolk and bovine serum albumin. Smith & Rothblat (1962) argued that the complete absence of sterol from Edward's experiments was not proved. Our study supports and strengthens Edward's observations by demonstrating the absence of cholesterol from the serum-free medium, and in the L-phase organisms grown in it. The L-phase of *S. moniliformis* thus resembles all the other L-phase variants investigated in so far as it does not depend on cholesterol for growth, although it has a marked ability to incorporate it. It may be concluded that in none of the L-phase membranes tested does cholesterol play the essential role which it does in the mycoplasma membrane.

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APPENDIX

On the Determination of the L-phase Parentage by the Electrophoretic Patterns of Cell Proteins

The electrophoretic technique devised by us for the identification of mycoplasmas (Razin & Rottem, 1967; Razin, 1968) and Gram-negative bacteria (Sacks, Haas & Razin, 1969) was used to determine whether the L-phase variants are the descendants of the bacteria presumed to be their parents. Washed organisms were dissolved in phenol + acetic acid + water (2 + 1 + 0.5, by vol.) and the dissolved material was run in polyacrylamide gels containing 35% acetic acid and 5M-urea. The gels were stained with 1% Amido Black 10B (Razin & Rottem, 1967) and densitometer tracings of the stained gels were made in a Gilford Model 2410 Scanner. Figure 1 (A to C) shows that the electrophoretic patterns of the *Streptobacillus moniliformis*, *Proteus mirabilis* and *Streptococcus pyogenes* L-phase variants were very close to those of their presumed parents. The differences were quantitative rather than qualitative, for almost all protein bands appeared both in the L-phase and bacteria patterns. The electrophoretic pattern of the L-phase of *Staphylococcus aureus*, however, differed fundamentally from that of its presumed parent (Fig. 1D). This strongly indicates that *S. aureus* (Smith) ATCC 19636 cannot be the parent of the *S. aureus* (Smith) L-phase ATCC 19640, since the genetic identity of micro-organisms seems to be reflected in the electrophoretic patterns of their cell proteins (Razin & Rottem, 1967) and the L-phase variant should be genetically indistinguishable from its parent bacterium (McGee, Rogul & Wittler, 1967).

The electrophoretic technique accordingly seems to be useful in determining the parentage of L-phase variants, and might well replace the far more complex nucleic acid hybridization techniques. Its use for the identification of L-phase variants is also advocated in the recent report of Theodore, King & Cole (1969).

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Fig. 1. Densitometer tracings and schematic representation of the electrophoretic patterns of cell proteins of bacterial L-phase variants and their presumed parents. (A) *Streptobacillus moniliformis* ATCC 14647 (upper diagram and curve) and the L-phase of *S. moniliformis* (L-1) ATCC 14075 (lower diagram and curve). (B) *Proteus mirabilis* 9, ATCC 14273 (upper diagram and curve) and the L-phase of *P. mirabilis* (L-9) ATCC 14168 (lower diagram and curve). (C) *Streptococcus pyogenes* (upper diagram and curve) and the L-phase of *S. pyogenes* (lower diagram and curve). (D) *Staphylococcus aureus* Smith ATCC 19366 (upper diagram and curve) and the L-phase of *S. aureus* (Smith-L) ATCC 19640 (lower diagram and curve).