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Isolation of Mycoplasma Membranes by Digitonin

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The cell membrane of *Mycoplasma hominis* was isolated by lysing the cells with digitonin. Electron microscopy and chemical, density gradient, and electrophoretic analyses of the membrane proteins showed the membranes so obtained, like those isolated by osmotic lysis, to be relatively free of cytoplasmic contaminants. Sensitivity to digitonin lysis depended on temperature but was not affected by Mg^{2+} ions and was only slightly affected by the age of the culture. Accordingly, it seems that digitonin may be used for the isolation of cell membranes from sterol-requiring mycoplasmas that tend to be fairly resistant to osmotic lysis.

Although osmotic lysis is the gentlest method devised so far for the isolation of mycoplasma membranes free of cytoplasmic contaminants (17), sensitivity of mycoplasmas to osmotic shock varies with the age of the cells (16) and is not the same in all *Mycoplasma* species (15). Although *Acholeplasma* strains are usually highly sensitive, the sterol-requiring *Mycoplasma* strains frequently resist osmotic lysis. Failure of osmotic lysis has led to the use of other techniques, such as alternate freezing and thawing (8, 26) or ultrasonic treatment (2, 8). Frequently, however, the bulk of the cells remains unlysed by alternate freezing and thawing (8), whereas by ultrasonic treatment, the membranes are fragmented into minute particles (9, 14). Therefore, an effective method evidently is needed for the isolation of membranes from sterol-requiring mycoplasmas, especially considering the recent findings that it is there that the major cell antigens as well as the transport and other enzymic systems are located.

The lysis of sterol-requiring mycoplasmas by digitonin has been described previously (18, 20, 25) and is attributed to the interaction of digitonin with cholesterol found in their membranes (17, 18, 25). This communication deals specifically with the use of digitonin for the isolation of membranes from these mycoplasmas.

MATERIALS AND METHODS

Organism and growth conditions. *M. hominis* strain 15056 was obtained from the American Type Culture Collection, Rockville, Md. The organism was grown in 500-ml volumes of Edward medium (15) supplemented with 2% (v/v) PPLO serum fraction (Difco) and 20 mM L-arginine. In some experiments 5 μCi of ^{3}H-cholesterol (500 mCi/mmole) or ^{3}H-oleic acid (4.9 Ci/mmole) was added to each liter of the growth medium. The *p*H of the medium was adjusted to 6.5 with 1 N HCl. The organisms were harvested after 16 to 20 hr of incubation at 37 C by centrifugation at 12,000 $\times$ *g* for 10 min, washed once in 10 ml of 0.25 M NaCl, and suspended in 2 to 4 ml of the same solution.

Assessment of sensitivity to digitonin. Portions (0.05 ml) of washed mycoplasma containing 400 μg of cell protein were added to 4-ml volumes of 0.25 M NaCl containing various concentrations of digitonin. The tubes were incubated at 37 C for 15 min and lysis was determined by measuring the change in turbidity of the cell suspension at 500 nm. Results were expressed as per cent lysis calculated in comparison with the turbidity of a control suspension without digitonin.

Sensitivity of the organisms to osmotic shock was determined as described before (15), and results were expressed as per cent lysis in 0.06 M NaCl calculated in comparison with the turbidity of a control suspension in 0.25 M NaCl.

Isolation of cell membranes. Cell membranes were isolated after lysis of the organisms by digitonin or by osmotic shock. For digitonin lysis, washed mycoplasma cell suspension (20 to 50 mg of cell protein) was added to an Erlenmeyer flask, containing 200 ml of 0.25 M NaCl and 25 μg of digitonin per ml, preheated to 37 C. After 15 min of incubation in a water bath at 37 C, membranes were collected by centrifugation at 34,000 $\times$ *g* for 60 min. The membranes were washed three times and suspended in 0.25 M NaCl. Isolation of membranes by osmotic cell lysis was carried out as described before (24).

Analytical methods. Protein was determined by

the method of Lowry et al. (10). Nucleic acids were extracted and determined as described before (19). Lipids were extracted from freeze-dried preparations with chloroform-methanol (2:1) by the method of Folch et al. (6). The lipid extracts were passed through a 1 by 5-cm column of Na_2SO_4, dried under a stream of nitrogen, and weighed. Total cholesterol in the lipid extracts was determined by the $FeCl_3$ method (27). When the organisms were grown with ^{3}H-cholesterol or ^{3}H-oleic acid, radioactivity in cells or in membrane preparations was determined in a Packard Tri-Carb liquid scintillation spectrometer by using toluene-dioxane scintillation liquor (24). Density gradient centrifugation was performed as described before (24) using 4.2 ml of a linear sucrose gradient of 28 to 45%.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of cells or membrane preparations solubilized by phenol-acetic acid-water (2:1:0.5, w/v/v) was carried out with gels containing 7.5% acrylamide, 35% acetic acid, and 5 M urea as previously described (22). The gels were stained with 0.5% amido black 10B for 30 min and the residual background stain was removed by immersing the gels for 3 to 4 days in 7% acetic acid. Densitometer tracings of the stained gels were made in a Gilford 2400 spectrophotometer equipped with a model 2410 scanner.

Electron microscopy. Negatively stained preparations were prepared as previously described (24). For the preparation of thin sections, pellets of sedimented membranes initially were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.4) for 4 hr at 0 C, washed in the phosphate buffer, and post-fixed in 2% OsO_4 in the same buffer for 16 hr. The material was dehydrated and embedded in Epon by the method of Luft (11). Sections were stained with uranyl acetate and lead citrate (21) and examined in a Phillips EM-300 electron microscope.

RESULTS

Good growth of *M. hominis* was obtained in the modified Edward medium (15) containing 20 mM L-arginine. The turbidity of the culture reached an absorbance of 0.35 to 0.50 at 600 nm after 18 hr of incubation when the inoculum level was 0.2% (v/v). Cell yields reached 60 to 100 mg of protein per liter of medium. The *p*H of the growth medium was adjusted to 6.5, being raised by the ammonia liberated by the hydrolysis of arginine during growth. When the *p*H of the medium reached 8.5, the cells began to lyse, so that the initial *p*H of the medium had to be lowered as much as possible and the organisms harvested before *p*H 8.0 was reached. Negatively stained preparations of *M. hominis* cells from a logarithmic culture showed long and highly branched filaments (Fig. 1).

M. hominis cells were sensitive to digitonin lysis in a 0.25 M NaCl solution which protects the mycoplasmas from osmotic lysis. Sensitivity to digitonin was temperature-dependent (Fig. 2) and decreased with the amount of cells in the testing mixture (Fig. 3). Whereas Mg^{2+} is highly effective in protecting *M. hominis* cells against osmotic lysis, it had no effect on digitonin lysis (Fig. 4). Sensitivity to lysis by digitonin decreased with the age of the culture, but less than the sensitivity to osmotic lysis (Table 1).

Thin sections of isolated *M. hominis* membranes obtained by digitonin lysis were relatively free of cytoplasmic contaminants such as ribosomes (Fig. 5). The isolated membranes contained 21 to 26% of the total cell protein and about 0.7 mg of lipid per mg of protein, with a very low nucleic acid content (Table 2). About 37 to 40% of the membrane lipid consisted of cholesterol. The strikingly similar gross chemical composition of the membranes obtained with digitonin and osmotic lysis may also be noted in Table 2. However, membranes obtained with digitonin consistently had a somewhat higher density than membranes obtained by osmotic lysis. The proteins of the two types of membranes also had very similar eletrophoretic patterns (Fig. 6), both being different from the protein pattern of whole cells.

Table 3 shows that the cholesterol content of the membranes was barely affected by increasing the concentration of digitonin used for cell lysis (12 to 200 μg of digitonin per mg of cell protein) so that no cholesterol is released from the membrane as a result of the digitonin-cholesterol interaction. An increase in the digitonin concentration up to 150 μg per mg of cell protein caused no lipid or protein components to be discharged from the membranes since no radioactivity was released from membranes labeled with ^{3}H-oleic acid, and the lipid-to-protein ratio of the membranes as well as the electrophoretic patterns of membrane proteins remained unchanged. At 200 μg of digitonin per mg of cell protein, however, a release of about 20% of membrane protein was observed, resulting in an increase in the lipid to protein ratio. Yet, despite the decrease in total protein content, the electrophoretic pattern remained unchanged.

DISCUSSION

Cell lysis by digitonin, here shown to be effective in the isolation of *M. hominis* membranes, also appears to be applicable to other sterol-requiring mycoplasmas. Preliminary experiments show that *M. gallisepticum*, which is more resistant to osmotic lysis than other mycoplasmas (15, 24), can be most effectively lysed by digitonin.

The great advantages of digitonin lysis are

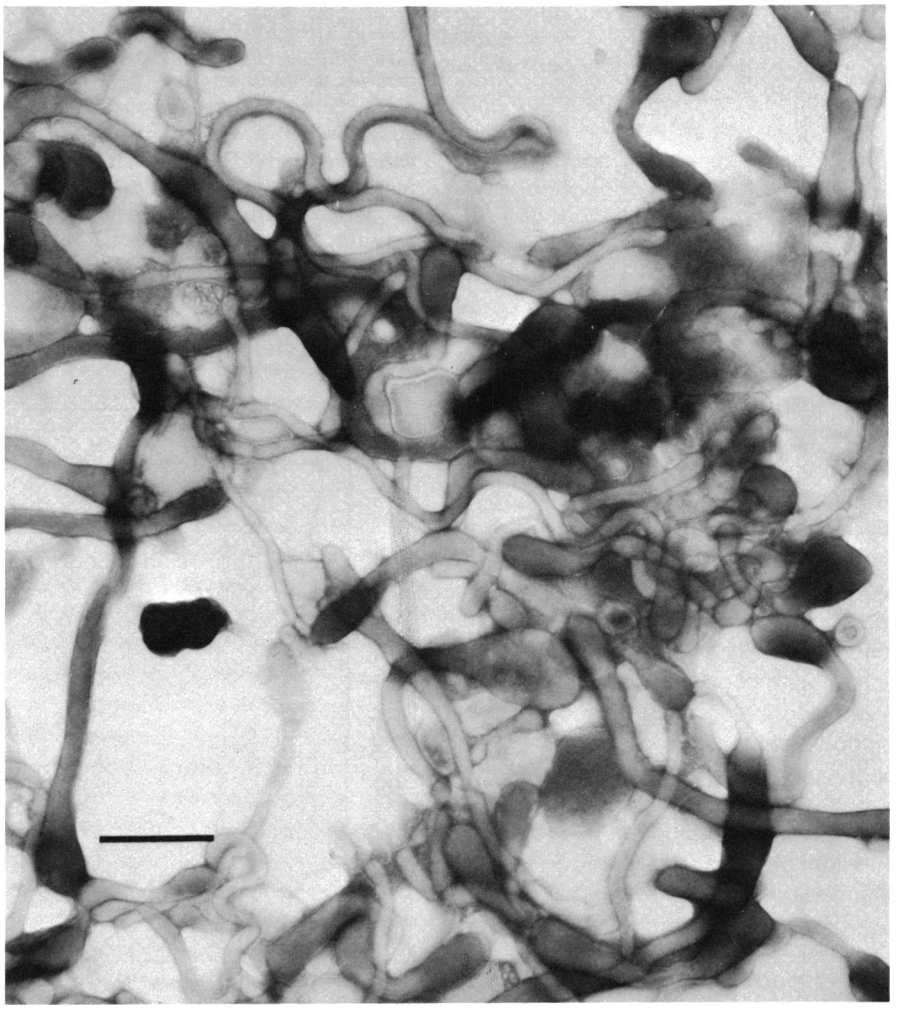


FIG. 1. *Mycoplasma hominis cells negatively stained with 1% phosphotungstate. Scale marker = 500 nm.*

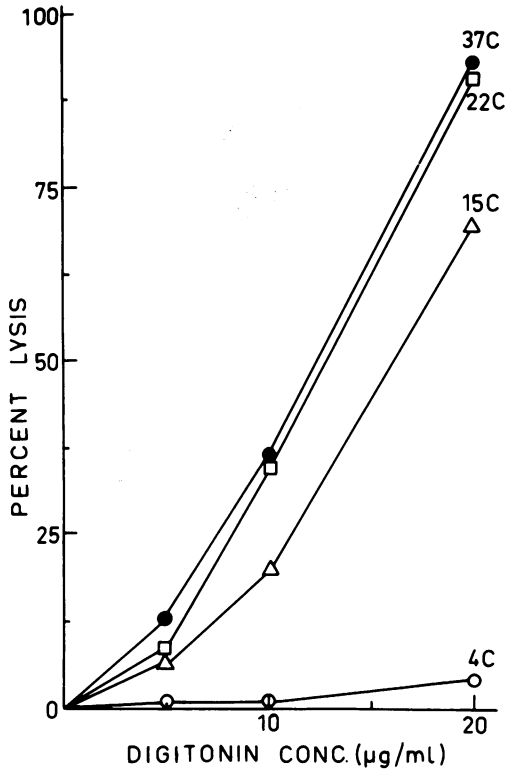
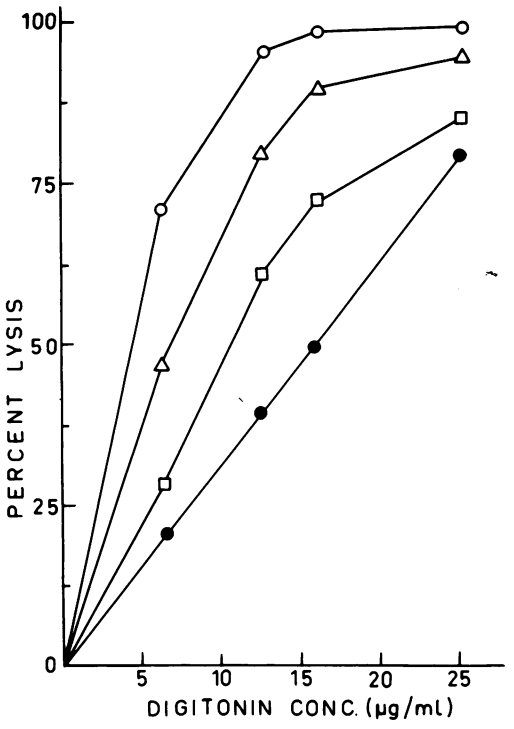


FIG. 2. *Effect of temperature on lysis of Mycoplasma hominis cells by digitonin.*



that, unlike osmotic lysis, it takes place in the presence of Mg^{2+} and does not depend so much on the age of the culture. Its relative independence of the age of the culture makes it a useful technique for the isolation of membranes from slow-growing mycoplasmas where the difficulty of determining the right harvesting time constitutes a handicap to osmotic lysis. It may also prove useful that membranes can be isolated in the presence of Mg^{2+} because microbial membranes may lose some enzymes being washed in media devoid of divalent cations (1, 13). Although the mycoplasma membranes obtained with digitonin so far have not been tested for immunological activity, the chemical data (Table 2, Fig. 6) leave little doubt that they will not differ in this respect from membranes obtained by osmotic lysis.

Mycoplasma resistance to both digitonin

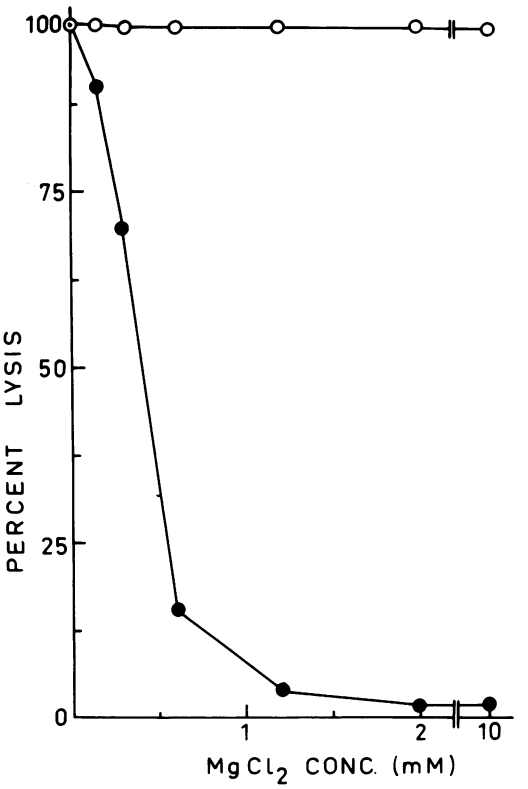


FIG. 4. *Effect of Mg^{2+} on lysis of Mycoplasma hominis cells by 20 μg of digitonin per ml (○) or in 0.06 M NaCl (●).*

FIG. 3. *Effect of the amount of Mycoplasma hominis cells in the test mixture on their sensitivity to lysis by digitonin. Symbols: ○, 100 μg of cell protein per ml; △, 200 μg of cell protein per ml; □, 400 μg of cell protein per ml; ●, 600 μg of cell protein per ml.*

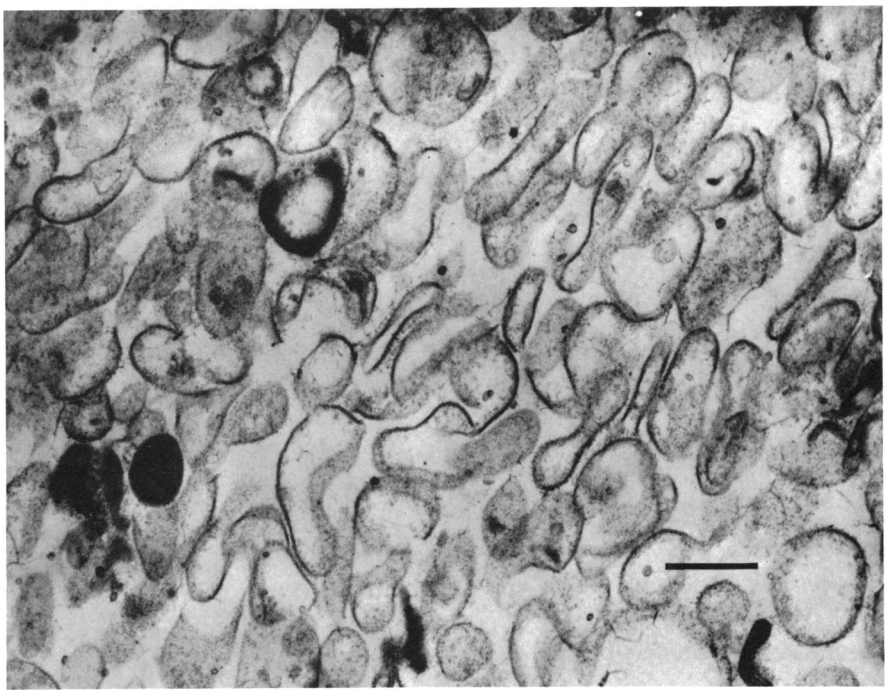


FIG. 5. *Thin sections of Mycoplasma hominis membranes isolated by digitonin. Scale marker = 500 nm.*

TABLE 1. *Osmotic and digitonin lysis of Mycoplasma hominis cells harvested at different ages of culture*[a]

Age of culture (hr)	Sensitivity to lysis by:	
	Osmotic shock[b]	Digitonin[c]
18	63	60
26	40	53
42	23	40

[a] Suspensions tested for lysis contained 100 μg of cell protein per ml.

[b] Per cent lysis in 0.06 M NaCl.

[c] Per cent lysis in 6.25 μg of digitonin per ml.

and osmotic lysis in the cold (16) may be due to the cytoplasm at a low temperature being in gel form (5), so that it cannot be released through the holes formed in the membranes. The low temperature also may reduce the fluidity of the lipid bilayer which apparently forms the backbone of the membrane (23), changing its mechanical properties and probably also those governing its ability to combine with digitonin.

The mechanism of digitonin lysis has not yet been completely elucidated. It is clear that digitonin acts only on cholesterol-containing membranes (18). As it forms cholesterol-digitonin complex in vitro, a similar complex may also be formed upon the interaction of digi-

TABLE 2. *Chemical composition of Mycoplasma hominis membranes obtained by osmotic or digitonin lysis of the cells*

Prepn	Lipid (μg/mg of protein)		RNA (μg/mg of protein)	DNA (μg/mg of protein)	Density (g/cm³)
	Total	Cholesterol			
Membranes (from digitonin-lysed cells)	674	250	14	11	1.171
Membranes (from osmotically lysed cells)	695	278	10	8	1.166
Whole cells	180	70	113	55	1.202[a]

[a] Sedimented to the bottom of gradient.

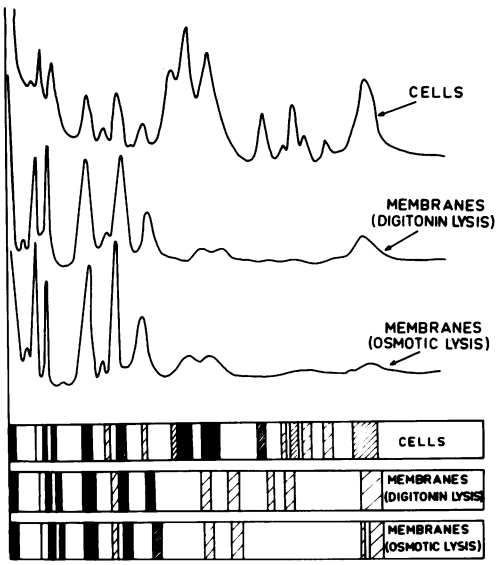


FIG. 6. *Densitometer tracings and schematic representation of the electrophoretic pattern of proteins of Mycoplasma hominis cells and membranes isolated by osmotic or digitonin lysis.*

TABLE 3. *Cholesterol in Mycoplasma hominis membranes obtained after lysis of cells by various digitonin concentrations*[a]

Digitonin concn (μg/ml)	^{3}H-cholesterol (counts/min)	
	Membrane fraction	Soluble fraction
12	100,124	432
25	99,410	684
50	98,780	972
200	99,280	1,056

[a] Cells were grown in the presence of ^{3}H-cholesterol.

tonin with cholesterol in the membrane. This would account also for the increased concentration of digitonin needed for lysis the bigger the amount of cells used (Fig. 3). The formation of a huge cholesterol-digitonide complex in the membrane would inevitably be bound up with extensive rearrangements of the membrane lipids and increased membrane permeability, possibly leading to cell lysis. It is well documented, for instance, that holes or pits are formed in biological membranes, including those of mycoplasma, by their interaction with saponins, which likewise interact with sterols (3, 4, 7). The appearance of these holes was attributed by Glauert et al. (7) to an extensive rearrangement of membrane lipids rather than to a loss of cholesterol from the membrane. In the case of digitonin too, it seems that the cholesterol is not removed from the membranes (Table 3), as reported by Martonosi (12) for sarcoplasmic reticulum membranes, but that a rearrangement of lipids leading to cell lysis takes place.

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