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THE ISOLATION AND PROPERTIES OF THE CYTOPLASMIC MEMBRANE OF *MICROCOCCUS DENITRIFICANS*

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SUMMARY

If harvested under certain conditions of growth, the walls of all of the cells of a culture of *Micrococcus denitrificans* are susceptible to digestion by low concentrations of lysozyme. This digestion apparently weakens the cell walls in such a way that osmotic lysis of the spheroplasts formed releases relatively intact cytoplasmic membranes, which can be collected by centrifugation at 30000 × *g* for 45 min. Fragments of cell wall released are not sedimented under these conditions. The cytoplasmic membranes are similar to other cellular membranes in their dispersal by detergents and in their composition. Those from aerobically grown bacteria contain around 32 % lipid, 64 % protein and 0.6 % carbohydrate; membranes from cells grown anaerobically in the presence of nitrate have a slightly higher content of protein.

Granules with properties similar to, but not identical with, those of poly-β-hydroxybutyrate found in other bacterial species can also be separated from the lysates of lysozyme-treated cells.

INTRODUCTION

The respiratory chain systems of bacteria make interesting experimental material, since they present a wide variety of combinations of the different components and the bacteria synthesize varying ratios of these according to the conditions of growth[1]. Another attractive feature is localization of the respiratory chain system on the cytoplasmic membrane which underlies the cell wall, but can be separated from it. In Gram positive bacteria this localization was demontrated by removal of the cell wall from several species by lysozyme digestion of the exposed mucopolysaccharide which gives the wall rigidity, then lysis of the resulting osmotically active "protoplasts" and isolation of the membrane fraction[2–6]. The membranes have a composition similar to that of other cellular membranes, being largely composed of lipid and protein, with varying amounts of carbohydrate in some[4–7]. In the Gram negative bacteria so far studied, the rigid mucopolysaccharide is not exposed, but is buried under other layers and not accessible to lysozyme digestion except after special treatment[8–12]. Studies of the respiratory chain systems of these bacteria have usually been carried

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out with fragments from mechanically disrupted cells in which the cytoplasmic membrane remains intimately associated with cell wall[11,13,14].

The Gram negative bacterium *Micrococcus denitrificans* synthesizes a respiratory chain system with components and properties similar to those of the system on mammalian mitochondrial membranes[15]. The bacteria are difficult to rupture mechanically[16], and the procedures required produce extensive comminution of the membranes; thus a gentler procedure was sought for obtaining the membranes. This was made possible by the discovery that under some conditions of growth the bacteria are lysozyme sensitive and that osmotic lysis of the lysozyme-treated cells yields relatively intact cytoplasmic membranes. The conditions for obtaining these were ascertained and the chemical composition of the membranes studied.

METHODS AND MATERIALS

Growth of bacteria

M. denitrificans ATCC 13543 was grown aerobically in a medium containing 4 g peptone (Difco), 2 g yeast extract (Difco), 10 g K_2HPO_4 and 10 g glucose (sterilized separately) per l, adjusted to pH 6.8 with H_2SO_4. The bacteria were maintained on the same medium solidified with 2 % agar (Difco) (see VERNON[16]). Erlenmeyer flasks (vol. 500 ml) containing 100 ml of medium were given a 1 % inoculum from a 7-h culture and aerated on a rotary shaker at 200 rev./min at 30°. Under these conditions maximal growth of the organisms was reached after 20 h, as judged by turbidity measurements. Bacteria were harvested by centrifugation at 4000 × *g* for 15 min.

The bacteria were grown anaerobically in filled bottles in the same medium containing in addition 20 g KNO_3 per l. Cultures were first adapted to growth under anaerobic conditions with a 5 % inoculum of aerobically grown cells allowed to grow for 12–15 h. From these another 5 % inoculum was made; these reached maximal growth after 24–28 h at 28–30°.

Treatment of bacteria with lysozyme

Cells washed twice with 0.01 M phosphate buffer, pH 7.0, were suspended in the same buffer containing 0.5 M sucrose to make a suspension such that a 1–25 dilution in the buffer had an absorbance (turbidity) of 0.3 at 550 mμ, measured in a Coleman Junior spectrophotometer. Lysozyme (EC 3.2.1.17) (Sigma) was added in the amount of 1 ml of a solution containing 10 mg/ml for each 10 ml of bacterial suspension and the mixture incubated at 20–22°. It is possible to follow the formation of protoplasts of *Bacillus megaterium* by observing the change from rods to spheres with a light microscope[2], but no change can be observed in the appearance of *M. denitrificans* on incubation with lysozyme in the sucrose–phosphate. Thus the formation of osmotically sensitive bodies was followed by withdrawing 0.2-ml samples at various times and diluting them rapidly with 4.8 ml of 0.01 M phosphate buffer, pH 7.0, and reading the absorbance at 550 mμ after 30 sec in a Coleman Junior spectrophotometer[3] (see Fig. 1).

Isolation of cell membranes

Lysozyme-treated cells can be centrifuged at 30000 × *g* for 30 min to obtain a well-packed pellet, then resuspended in 0.05 M phosphate buffer, pH 7.0, with

little or no lysis for several minutes; the bacteria from each 10 ml of the original suspension in sucrose–phosphate were suspended in 2 ml of the buffer. Deoxyribonuclease (EC 3.1.4.5) (Worthington) was then added (0.2 ml containing 0.02 mg/ml for each ml of bacterial suspension) and the mixture lightly homogenized and diluted 1:5 with cold distilled water to lyse the cells. The DNA released upon lysis is rapidly

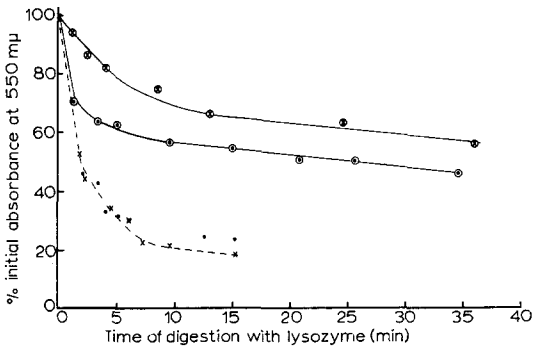


Fig. 1. Osmotic lysis of lysozyme-treated cells. Bacteria from the lysozyme digests were diluted as described in METHODS and the change in absorbance followed at 550 mμ. Cells grown aerobically for 12 h, ×---×; for 14 h, ●---●; for 24 h, ⊙—⊙; for 36 h, ⊗—⊗.

hydrolyzed and centrifugation at 30000 × *g* for 45 min in a refrigerated centrifuge yields a double-layered residue and a slightly opalescent supernatant fluid. The upper layer of the residue (RR) is rich in cytochromes and can be easily suspended, while the lower white layer (WR) is difficult to suspend. If RR is carefully suspended after a wash in 0.01 M phosphate buffer it can be separated from traces of WR; alternatively, the heavy WR particles can be removed at a very low centrifugal force (500 × *g*). Conversely, the white layer can be obtained free of protein (as judged by the biuret reaction) if it is allowed to settle repeatedly at 4° in distilled water or dilute buffer. Under conditions where lysis is incomplete (see RESULTS), whole cells are associated with the white layer, and further washings may be required to obtain uncontaminated membrane fractions.

Analysis of the membrane fraction

Before analysis the membranes were washed five times with glass-distilled water, then lyophilized and stored over Drierite. Protein was estimated by the biuret method[17] in the presence of 0.1 % sodium deoxycholate, with a correction made for the slight turbidity of the samples by subtracting a blank made with 3 % NaOH in place of the biuret reagent. Protein was also assayed by determining the nitrogen content with the method of MARKHAM[18] and multiplying these values by the factor 6.25. Lipid content was measured by ether extraction after digestion of the membranes with 6 M HCl for 4 h[19]. Carbohydrate was assayed by the anthrone method, with glucose as standard[20].

Electron microscopy

We are indebted to Dr. ELIZABETH GANTT for all of the electron microscopy. Intact cells and lysozyme-treated cells were fixed either (a) in OsO_4 according to KELLENBERGER, RYTER AND SECHAUD[21] or (b) by a 2-h suspension in glutaraldehyde

in a concentration of 4 % in the suspending medium (whole cells in 0.01 M phosphate buffer, lysozyme-treated cells in sucrose–phosphate), followed by several rinses in 0.1 M phosphate buffer, pH 6.8, and a 2-h post-fixation with 1 % OsO_4. Preparations fixed by both methods were dehydrated and embedded as described by LUFT[22] and stained by the method of MILLONIG[23], then examined with a Phillips 200 electron microscope.

Fraction WR was fixed by the method of KELLENBERGER, RYTER AND SECHAUD[21]. Fraction RR was negatively stained using 1 % phosphotungstic acid in 0.1 M ammonium acetate, pH 6.8. The preparations were applied to carbon-coated formvar-covered grids and dried in air prior to examination in the electron microscope.

Chemicals

Analytical reagents were used throughout. The phosphate buffers were prepared from Na_2HPO_4 and KH_2PO_4.

RESULTS

The action of lysozyme on M. denitrificans

(1) *Changes in sensitivity to lysozyme.* When bacteria are grown aerobically to an absorbance (turbidity) less than 0.6 at 550 mμ in the Coleman Junior spectrophotometer (about 14 h growth), incubation of thick suspensions with lysozyme for 15–20 min yields cells which can be completely lysed osmotically, as judged by the absence of whole cells when the diluted suspension is examined with a microscope. If the bacteria are grown for longer times, lysis is incomplete. Fig. 1 illustrates the change in lysozyme sensitivity with the age of cultures. In cultures grown aerobically for 24 or 36 h, a fraction of the cells can be lysed after digestion for 15 min, but the remainder appear to be highly resistant to lysozyme treatment under the conditions employed. Increased resistance to lysozyme with increased growth periods is also seen in anaerobically grown cells; VERNON[16] described cells grown anaerobically for several days as insensitive to lysozyme.

Autolysis appears to play no significant part in the lysis of *M. denitrificans* described above, since controls without added lysozyme show no drop in turbidity on dilution over the period of digestion employed.

(2) *Electron microscopy of whole cells and lysozyme-treated cells.* Figs. 2A and 2B are electron micrographs of sections of cells made before and after treatment with lysozyme, prefixed with glutaraldehyde and fixed with osmium. The cell wall is clearly visible in both preparations, but the underlying cytoplasmic membrane is not well defined. The patterned effect of the cell wall, only seen in preparations prefixed with glutaraldehyde, resembles that described by HOUWINK[24] and SALTON[9] in other organisms. The cytoplasmic membrane is well defined in preparations not prefixed with glutaraldehyde (Figs. 2C and 2D), but the cells are less well preserved and the walls are often broken in the lysozyme-treated preparations. The cell wall and cytoplasmic membrane are seen as two distinctly separate structures; this is particularly clear in sections of lysozyme-treated cells. Although the cell wall shows a quite different appearance in micrographs made after the two different fixation procedures, we were unable to detect any specific differences between the walls of untreated and lysozyme-digested cells. The osmotic sensitivity of the digested bac-

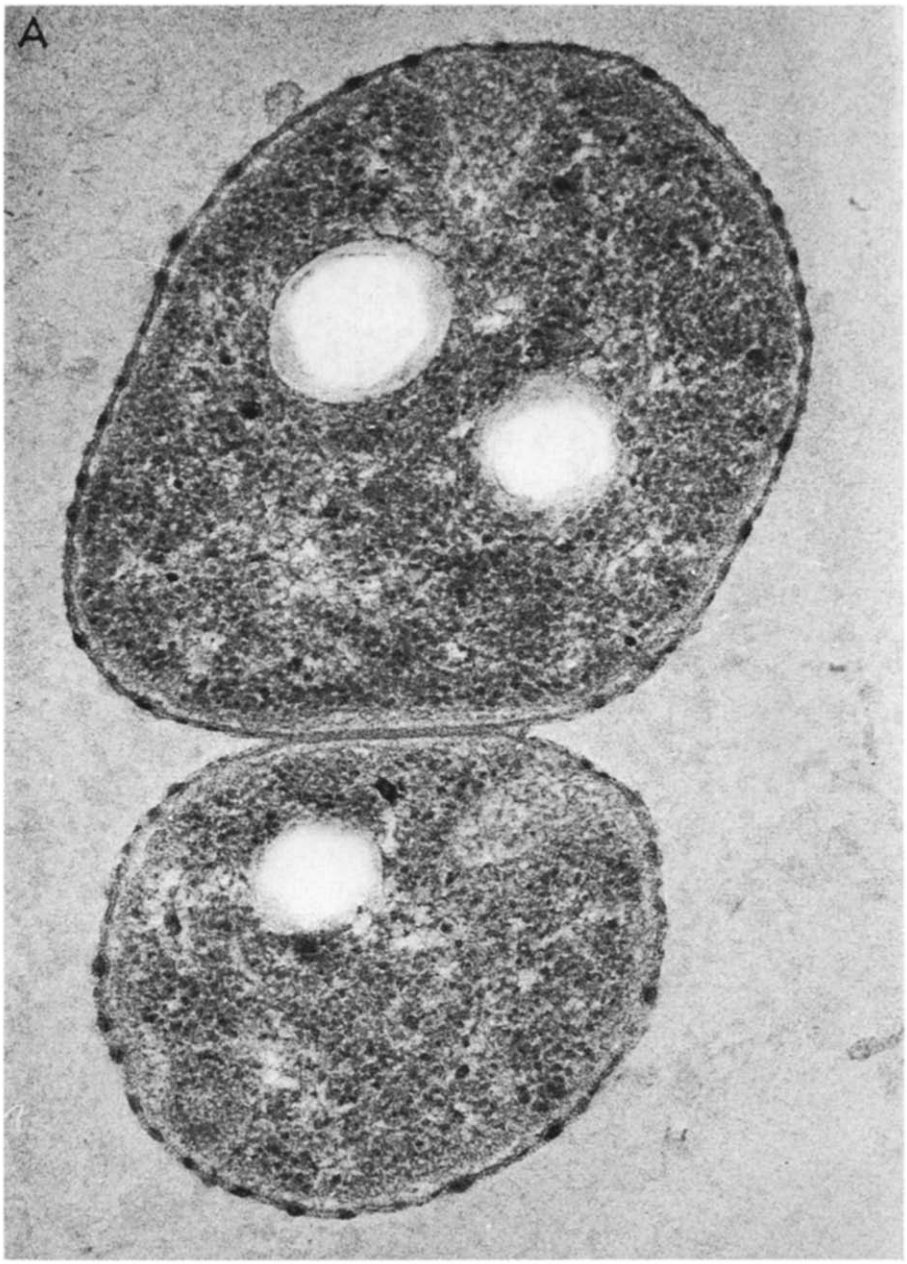
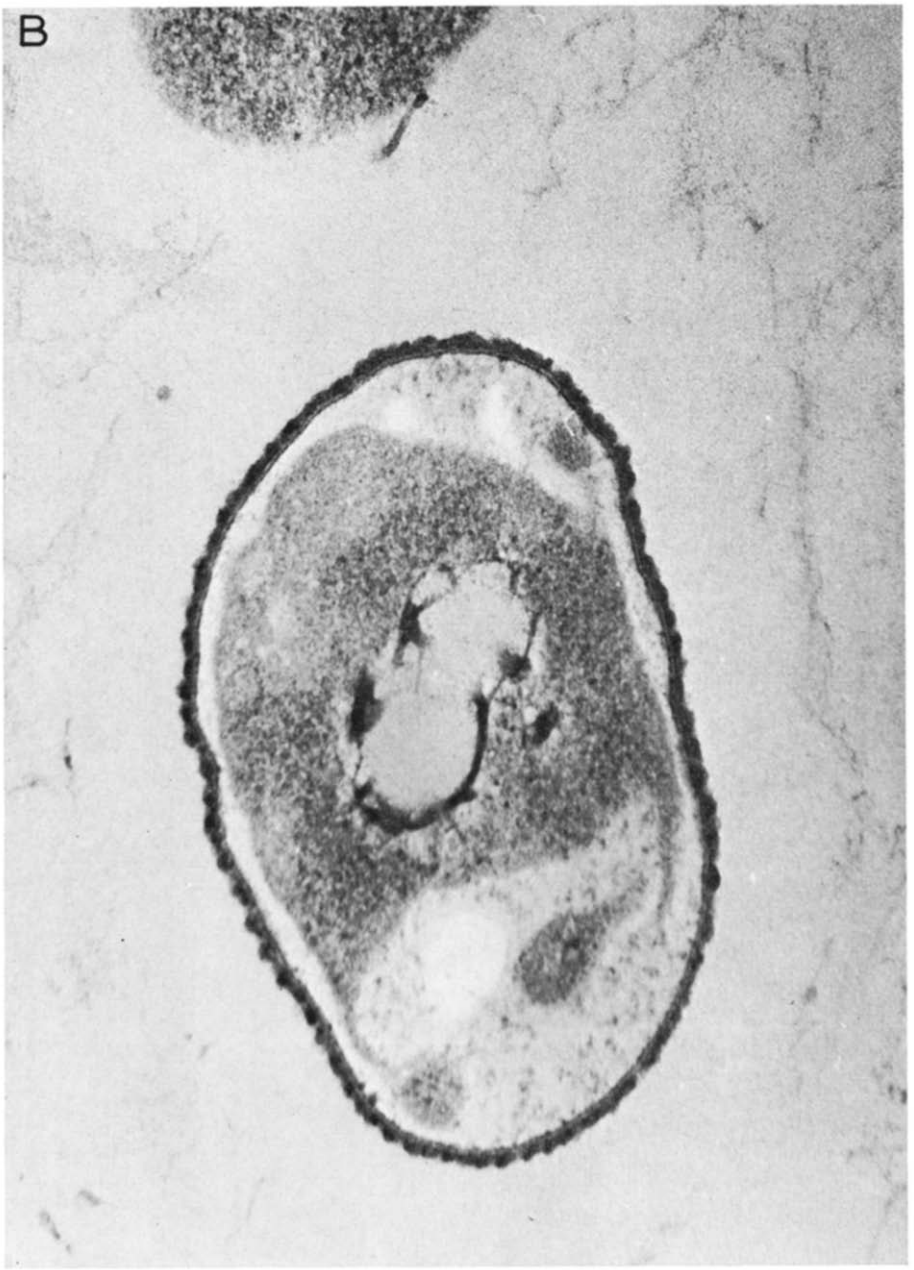
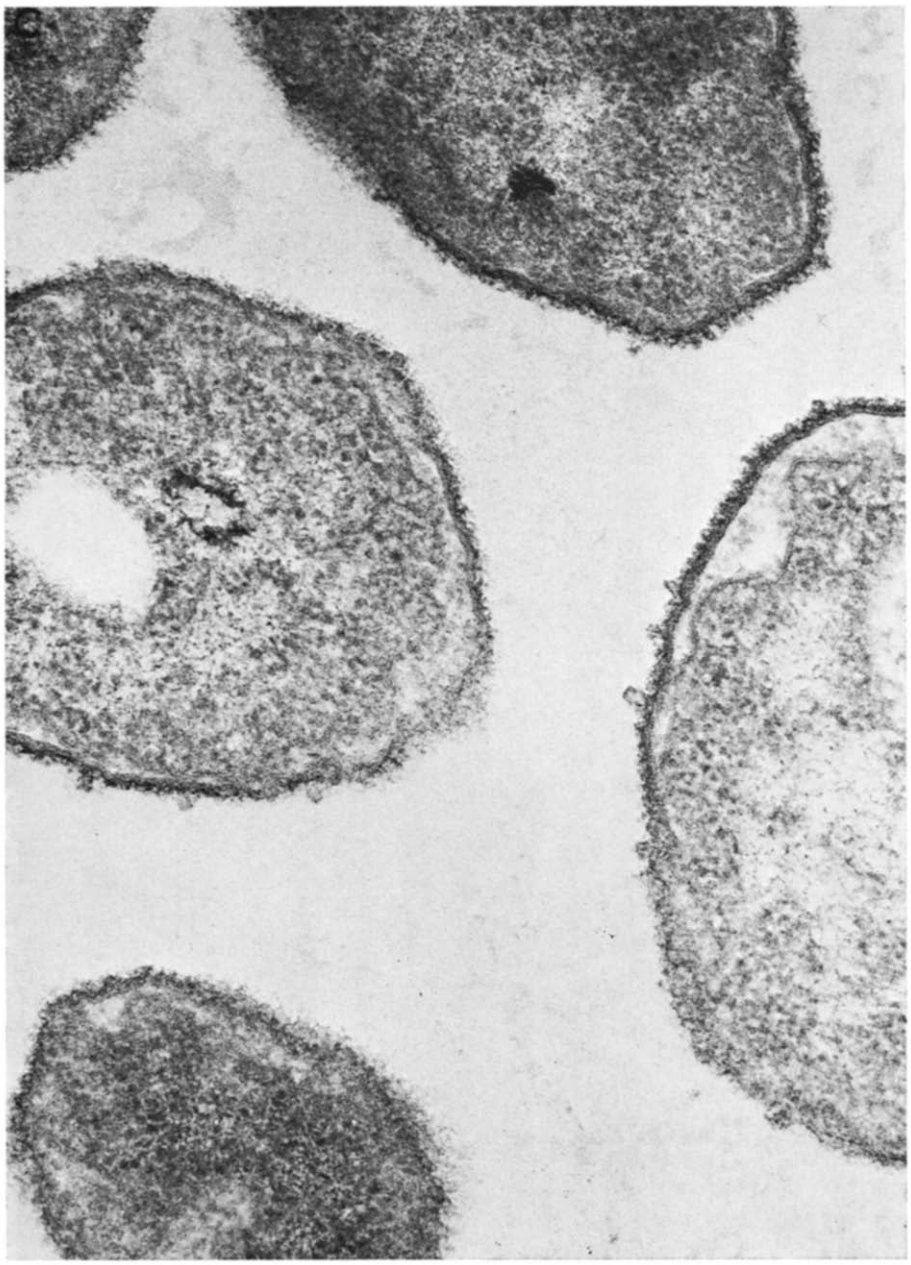


Fig. 2. Electron micrographs of bacteria before and after treatment with lysozyme. A and C are intact cells fixed with (A) and without (C) glutaraldehyde treatment; B and D are lysozyme-digested cells with (B) and without (D) glutaraldehyde pretreatment. All × 99000.

teria demonstrates that the cell walls have been changed, but the change is not demonstrable in the electron micrographs. This seems to indicate that some of the linkages of the mucopolysaccharide have been broken, but that the structure is

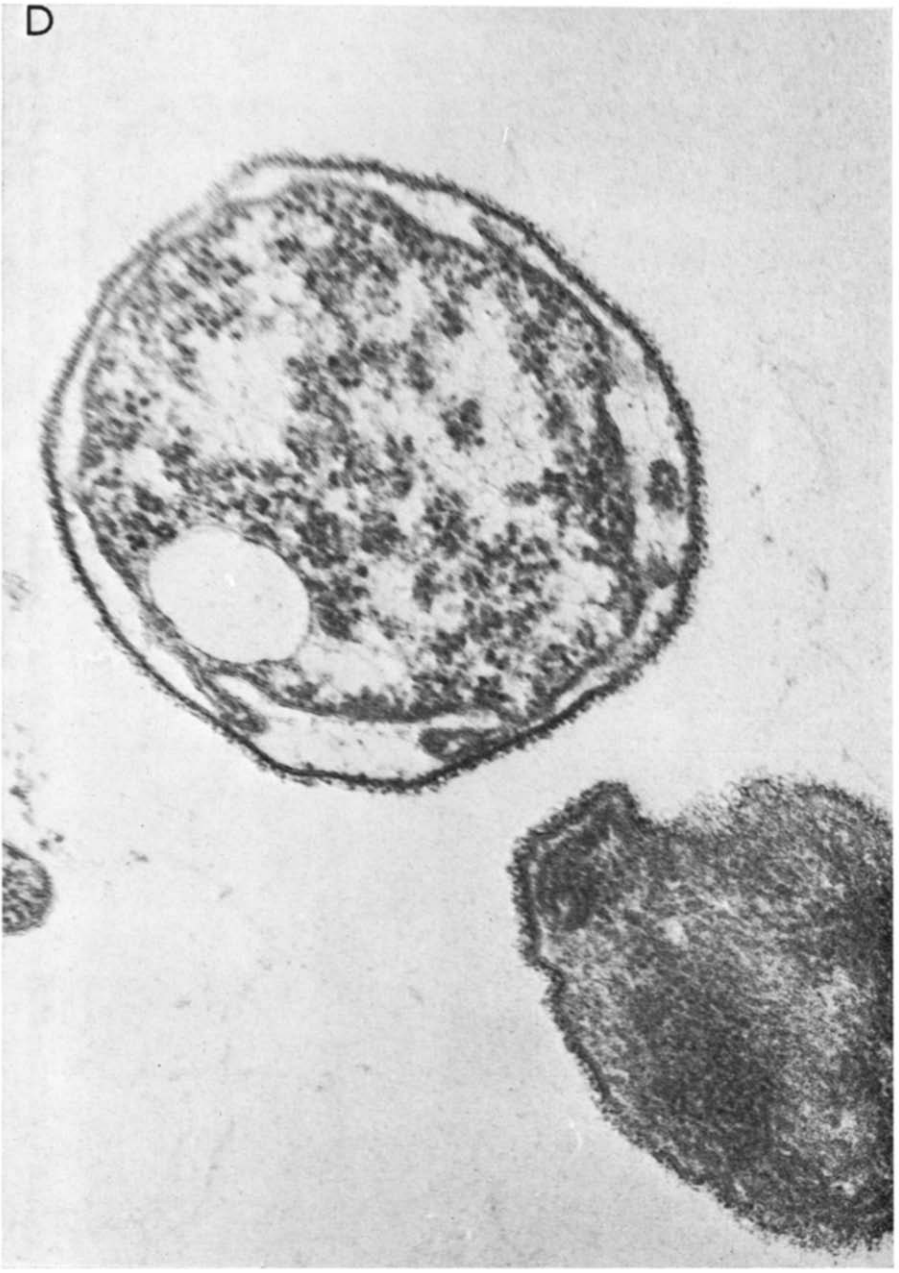


held together by other linkages as long as proper osmotic pressure relationships are maintained (see DISCUSSION). The osmotically active bodies from *M. denitrificans* would fall into the category designated "spheroplasts" rather than "protoplasts"[25], since they are bacteria with the cell walls weakened or depleted, but not removed.



Some properties of the spheroplasts

Like protoplasts or spheroplasts of other organisms, spheroplasts of *M. denitrificans* swell and finally burst as the osmotic pressure is decreased in the suspending medium containing an impenetrable solute. When suspended in 0.01 M phosphate buffer containing sucrose in excess of 0.1 M, there is very little rupture of the cells,



as measured by release of material absorbing at 260 and 280 mμ. The absorbance at 550 mμ of a suspension of spheroplasts decreases as the concentration of sucrose in the medium is lowered from 0.8 to 0.1 M, which must result from changes in light scattering as the spheroplasts swell; these changes are less than the large decrease seen on rupture of the cells. Similar observations are made when sucrose in the sus-

pending medium is replaced by NaCl or KCl, but then the spheroplasts are less well preserved. In agreement with observations with other bacteria[3], iso-osmotic phosphate buffers or glycerol produce lysis of the spheroplasts. The stability of the spheroplasts does not seem to be unduly influenced by increasing the pH from 7.0 to 7.6.

Protoplasts are very sensitive to lysis by detergents, which is not surprising in view of the lipoprotein nature of membranes. Spheroplasts of *M. denitrificans* suspended in an osmotically stable medium are rapidly dispersed by Triton X-100, sodium deoxycholate or sodium dodecyl sulfate. However, these spheroplasts are only slowly lysed by commercial preparations of pancreatic or microbial lipase (EC 3.1.1.3); with the same lipases and the same experimental conditions, protoplasts of *B. megaterium* are rapidly lysed[26].

TABLE I

COMPOSITION OF WASHED MEMBRANE FRAGMENTS FROM AEROBICALLY AND ANAEROBICALLY GROWN BACTERIA

Cells were cultured either for 12 h aerobically at 30° or for 20 h anaerobically at 28–30° and the membrane fragments prepared, washed and analyzed as described in the METHODS section. The values are expressed as % dry weight. Individual values were obtained with preparations from different batches of bacteria.

	Lipid	*Protein*		*Carbohydrate*	*Recovery*
	A	*B* (*by biuret method*)	*C* (*by Kjeldahl N method*)	*D*	*A* + *C* + *D* (%)
Aerobically grown bacteria	33.2	61.0	64.8	0.623	96.2
	32.8	59.2	62.3		
	30.1	60.5			
Average	32.0	60.2	63.6		
Anaerobically grown bacteria	32.3	67.6	63.3	0.314	97.7
	31.1	65.2	67.6		
	30.1	64.2	67.6		
Average	31.2	65.5	66.2		

Properties of the membranes and granules isolated after osmotic lysis of spheroplasts

The WR fraction, when washed free of protein, represents only about 1 % of the dry weight of the cell under the conditions of growth employed. The granules can be observed in intact cells and spheroplasts (Fig. 2) and have the same appearance in electron micrographs of the WR fractions. The isolated granules are resistant to mineral acid and largely soluble in alkali. In contrast to similar material isolated from *Micrococcus halodenitrificans*[27], only about 20 % of the granule fraction from *M. denitrificans* resembles poly-β-hydroxybutyric acid in being resistant to alkaline hypochlorite (1 h) then soluble in hot chloroform after acetone and ethanol extraction. Of the remainder approximately 40 % escapes digestion with hypochlorite but is soluble in acetone and ethanol.

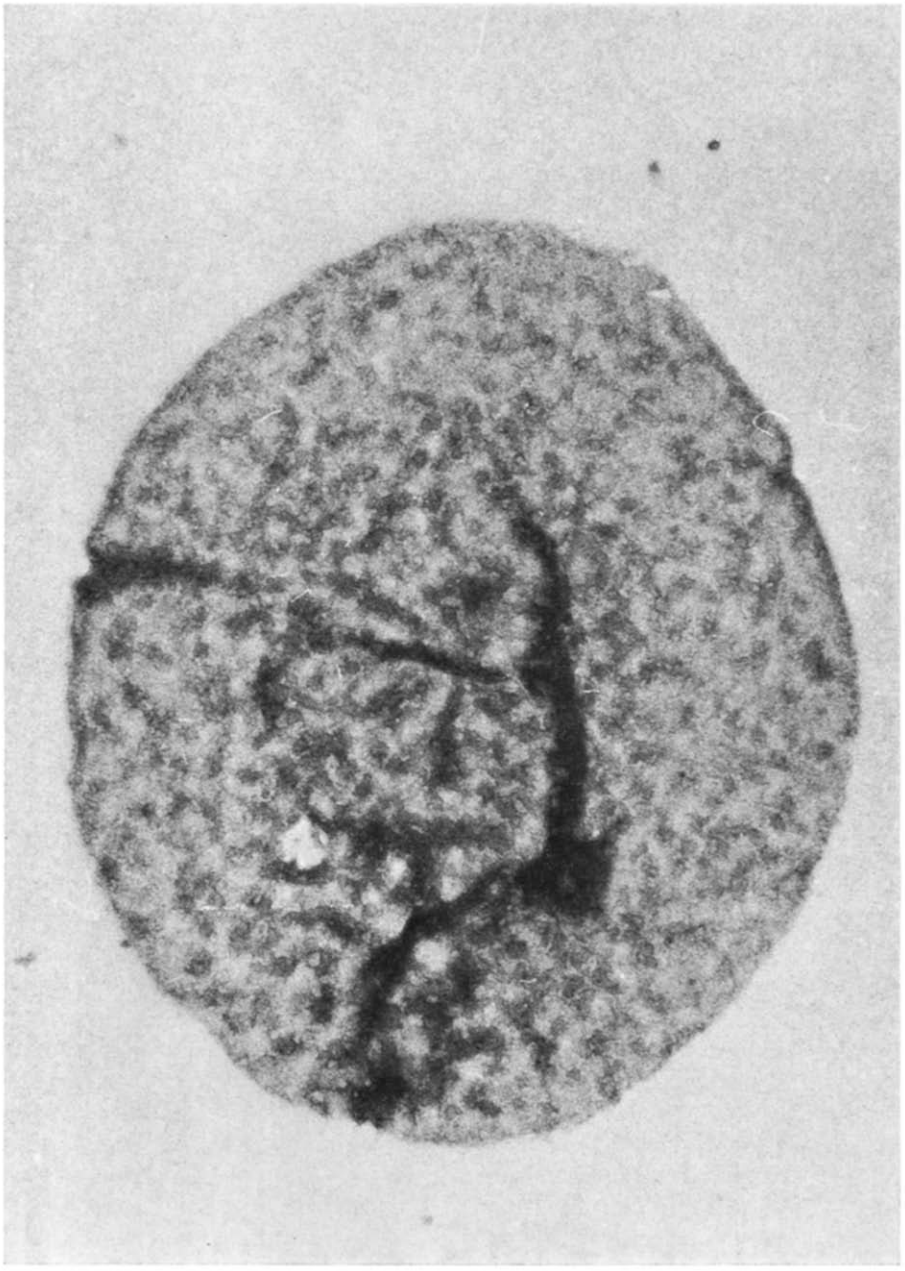


Fig. 3. Electron micrograph of membrane from osmotic lysate of *M. denitrificans*, negatively stained with phosphotungstate. × 50000.

The electron micrograph (Fig. 3) shows that fraction RR consists largely of structures somewhat larger than whole cells with no evidence of cell wall material. These membranes are similar in appearance to the "ghost" structures isolated from

B. megaterium and *Micrococcus lysodeikticus* following similar procedures. To confirm the absence of cell wall material, we have analyzed membranes from both aerobically and anaerobically grown bacteria (Table I). Over 90 % of the membrane material can be accounted for as protein and lipid, and the carbohydrate content is very low. Thus the composition of the membranes is similar to that of membranes from other sources; it is particularly close to that reported for membranes from lysates of spheroplasts of *Pseudomonas aeruginosa*[28]. Although the results do not preclude the presence of small amounts of carbohydrate-containing cell wall material in the membrane fraction, if present it could represent only a small proportion of the total.

DISCUSSION

M. denitrificans are unusual in that they are Gram negative bacteria which can be as sensitive to lysozyme as Gram positive bacteria such as *B. megaterium*. In other Gram negative organisms studied the wall and the cytoplasmic membrane are intimately associated and the rigid mucopolysaccharide lies buried under other cell wall components[8–10,11,14] and is not accessible to digestion with lysozyme. Treatment of *M. denitrificans* with lysozyme does not make a difference in the cell wall structure which is apparent in electron micrographs of spheroplasts suspended in sucrose–phosphate. However, the digestion must rupture enough bonds of the polymer so that the pressure generated on osmotic lysis disrupts the wall and releases the relatively intact cytoplasmic membrane. It has often been observed that when the rigid mucopolysaccharide is weakened or destroyed, the cells are susceptible to disruption by osmotic pressure. In the case of *M. denitrificans* they are broken in such a way that the cytoplasmic membranes are released free of the mucopolysaccharide, as shown by the very low content of carbohydrate. Since the wall and the membrane appear to be separated in the spheroplasts, and since electron micrographs of membranes stained with phosphotungstic acid show no evidence of cell wall, it seems most likely that all of the cell wall components are missing from the isolated membranes. The pieces of cell wall released on osmotic lysis of the spheroplasts are of a size and density not sedimented by centrifugation at 30000 $\times$ *g* for 45 min. The cytoplasmic membranes released appear to be relatively intact or to be broken mostly into large fragments.

The sensitivity to lysozyme is critically dependent upon the time of growth of cultures of *M. denitrificans* and this differs for anaerobically as compared with aerobically grown cells. Changes of lysozyme sensitivity with growth stage have also been observed with other bacteria[29,30,12]. Such changes must result from decreased accessibility of lysozyme to its substrate due to either increased coverage of the mucopolysaccharide by other wall components or to chemical changes in the polymer. The latter appears to be the explanation for lysozyme resistance seen in some Gram positive organisms[31,32]. However, the former may be the reason for the change in sensitivity to lysozyme in *M. denitrificans*, since in Gram negative bacteria the mucopolysaccharide can be completely covered by other layers of cell wall components (some covalently linked, others attached by weaker bonds[10]). In early stages of growth the cell wall structure of *M. denitrificans* may be incomplete, with parts of mucopolysaccharide exposed to digestion with lysozyme.

The fortunate result of the observations with *M. denitrificans* is that if the

bacteria are harvested in the proper condition, relatively intact cytoplasmic membranes can be isolated and used for studies of composition and of function of the constituent enzyme systems. Relatively intact membranes can be isolated from lysates of protoplasts of *B. megaterium* if Mg^{2+} is added[33].

The properties of the spheroplasts of *M. denitrificans* show the membranes to resemble those of other bacteria in being relatively impenetrable to sucrose and NaCl and KCl and in behaving as osmometers in the presence of these substances and in being penetrated and lysed by substances such as phosphate or glycerol. They are also similar in being disrupted by detergents. The membranes of *M. denitrificans* are less rapidly digested by commercial preparations of lipase than are those from *B. megaterium*. The lipase preparations are not pure, and thus the identity of the enzyme active in this digestive process is not known. The electron micrographs of the negatively stained intact membranes show some fine structure, but no evidence of the "knobs" seen along mitochondrial membranes[34] or membranes of some bacteria stained similarly[35,36].

The cytoplasmic membranes of both aerobically and anaerobically grown *M. denitrificans* are composed almost entirely of lipid and protein and the ratio of these is similar to that seen in other kinds of membranes. The respiratory chain system of these bacteria is completely localized in the membranes; the constituent pigments are qualitatively similar to those on mammalian mitochondrial membranes and DPNH, succinate and mammalian and bacterial cytochromes *c* are rapidly oxidized. These components and activities are described in the following paper, which will also describe the quantitative variations in the different pigments under different growth conditions, particularly in anaerobic as compared with aerobic growth. In this regard it is interesting that the protein content of the membranes from anaerobically grown cells is slightly higher.

The identity of the white granules seen in intact cells and isolated from the lysates is not established. They are free of protein and have properties similar to, but not identical with, the poly-β-hydroxybutyric acid which appears to serve as storage material in other bacteria.

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