LOCALIZATION OF RESPIRATORY ENZYMES IN INTRACYTOPLASMIC MEMBRANES OF *AZOTOBACTER AGILIS*

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ABSTRACT

PANGBORN, J. (University of California, Davis), ALLEN G. MARR, AND S. A. ROBRISH. Localization of respiratory enzymes in intracytoplasmic membranes of *Azotobacter agilis*. J. Bacteriol. **84**:669–678. 1962.—Thin sections of the cells of *Azotobacter agilis* which have been disrupted by sonic treatment, by osmotic shock, or by ballistic disintegration reveal a network of internal membranes in the form of vesicles and tubules. The internal membranes are attached to the envelope. Treatment in a Mickle disintegrator of envelopes emptied of cytoplasm by osmotic shock results in the loss of the internal membranes and a concomitant release of reduced diphosphopyridine nucleotide oxidase from the envelopes. Thus, the intracytoplasmic membranes are the probable locus of the respiratory enzymes of the cell. Thin sections of whole cells show tubular intracytoplasmic membranes which are obscured by ribosomes and other dense cytoplasmic constituents.

When cells of *Azotobacter agilis* were emptied of cytoplasm by direct osmotic shock, the respiratory enzymes were recovered quantitatively in the pellet from low-speed centrifugation which contained the emptied envelopes. Examination of the envelopes by electron microscopy revealed extensive areas within the envelopes which were dense to the electron beam. This density was not due to occluded cytoplasm, since all of the soluble enzymes and the ribosomes were recovered in the supernatant liquid. Thin sections of the envelopes revealed, in addition to the cell wall and peripheral membrane, the presence of internal membranes which were responsible for the electron density of the envelope (Robrish and Marr, 1962).

This paper presents evidence that the internal membranes exist in the intact cell as intracytoplasmic membranes, which are either attached to or extensions of the peripheral membrane, and that the internal membranes are a locus of respiratory enzymes.

MATERIALS AND METHODS

Culture. A. agilis (*A. vinelandii* O) was grown in Burk's nitrogen-free medium under conditions described previously (Robrish and Marr, 1962). At the end of exponential growth, the cells were harvested by centrifugation and were washed free of slime by four successive centrifugations from distilled water at 2,000 × *g* for 45 min.

Disruption of cells. The pellet of washed cells was mixed with an equal volume of 3 M glycerol, allowed to equilibrate for 5 min, and ejected from a syringe into ten volumes of rapidly stirred buffer [0.05 M tris(hydroxymethyl)aminomethane chloride (pH 7.5) containing 0.001 M $MgSO_4$]. Less than 4% of the cells remained intact after osmotic shock with glycerol. Deoxyribonuclease (0.5 μg per ml) was added, and the mixture was allowed to stand at room temperature for 15 min to reduce the viscosity. The preparation was centrifuged at 10,000 × *g* for 15 min, which is sufficient to sediment the envelopes, and was washed once by centrifugation from buffer at 10,000 × *g* for 15 min.

A suspension (50 ml) of cells in buffer was treated at full power output (approximately 80 acoustical watts) in a Raytheon 10-kc sonic oscillator. The cup of the transducer was flushed with H_2 and the temperature of the sample was maintained at less than 5 C during treatment. The preparation was centrifuged and washed in the same manner as that described for disruption by osmotic shock.

A 5-ml suspension of cells in buffer (15.2 mg wet wt per ml) and 2 ml of glass beads (0.2 mm diam; Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were added to the cup of a Mickle disintegrator. The cups were shaken with

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a 7-mm peak-to-peak displacement. The cups were chilled periodically in an ice bath, so that the temperature of the contents did not exceed 20 C during the treatment.

Assay of reduced diphosphopyridine nucleotide (DPNH) oxidase. DPNH oxidase was assayed by measuring the decrease in absorbancy at 340 mμ resulting from the oxidation of DPNH. A reaction vessel with a 5-mm optical path contained 1.3 ml of 0.05 M phosphate buffer (pH 6.8) and 0.1 ml of a suitable dilution of the preparation containing DPNH oxidase. The temperature was maintained at 30 C during the reaction, and the absorbancy was recorded continuously (Marr and Marcus, 1961).

Hexosamine. Samples were hydrolyzed in 4 N HCl in a sealed tube at 100 C for 15 hr. The HCl was removed by vacuum distillation, and the hexosamine was determined by the Elson-Morgan reaction using glucosamine as a standard (Boas, 1953).

Electron microscopy. The pseudoreplica was prepared essentially according to the method of Hall (1956). The sample was sprayed on freshly cleaved mica, shadowed with Pt at an angle of 18°, and overlayered with a film of carbon. The carbon film was floated on water and transferred to copper grids for observation.

Samples of all material to be sectioned, except the envelopes prepared by osmotic shock, were fixed by the method of Kellenberger, Ryter, and Sechaud (1958). Tryptone (0.1% final concentration) was added to the preparation of whole cells but was omitted from the preparations of envelopes during fixation. The envelopes prepared by osmotic shock were fixed by the method of Chapman (1959). All of the material sectioned was stained with uranium acetate either during fixation or after sectioning. The specimens were embedded in a 4:1 mixture of butyl-methyl methacrylate and sectioned with a Porter-Blum microtome, using a glass knife. The sections were floated on 20% acetone-water (v/v) and transferred to copper grids coated with Formvar.

All specimens were photographed with an RCA EMU 3-E microscope using a 75-μ objective aperture, when necessary, to increase contrast.

RESULTS

Since the morphology of the internal membranes may be altered in a manner related to the process of disruption, sections of envelopes prepared by ballistic disintegration, by sonic treatment, and by osmotic shock were examined by electron microscopy.

The appearance of envelopes prepared either by osmotic shock (Fig. 1) or by short-term ballistic treatment (Fig. 2) is similar. The peripheral structures of the envelope, the cell wall and peripheral membrane, are torn in some of the envelopes (Fig. 1, T). The cell wall (CW) appears in Fig. 1 as a laminate of alternating dense-light-dense-layers, which is typical of the appearance of sections of the cell walls of gram-negative bacteria (Kellenberger and Ryter, 1958). The peripheral membrane (PM) is retracted from the wall in some regions, but closely underlies the wall elsewhere. The interior of the envelope is filled with a system of membranes which appear most commonly as empty vesicles. Occasional vesicles appear to be attached to the peripheral membrane. Some regions in the interior of envelopes prepared by osmotic shock (Fig. 1) retain cytoplasmic matrix and seem to have suffered less distortion from the physical forces which disrupted the cell. In such regions, the internal membrane appears tubular rather than vesicular.

Prolonged shaking with glass beads is known to result in a loss in electron density of envelopes of gram-negative bacteria (Few, 1954). Sections of the envelopes, prepared by osmotic shock and subsequently treated for 15 min in the Mickle disintegrator, revealed a loss of most of the internal membranes (Fig. 3), which accounts for the loss of density of the envelopes to the electron beam. The wall and peripheral membrane show numerous rents, but do not appear to be appreciably comminuted.

Envelopes prepared by sonic treatment (Fig. 4) also show the wall, peripheral membrane, and internal membranes; however, the break in continuity of the peripheral structures is much more extensive than in other methods of disruption.

The three-dimensional appearance of the internal membranes in envelopes prepared by osmotic shock was investigated by examining serial sections. Several structures, which appear as vesicles in an individual section, could be traced through sequential sections from the interior to the periphery of the cell. The best demonstration of the three-dimensional appearance of the internal membrane was obtained

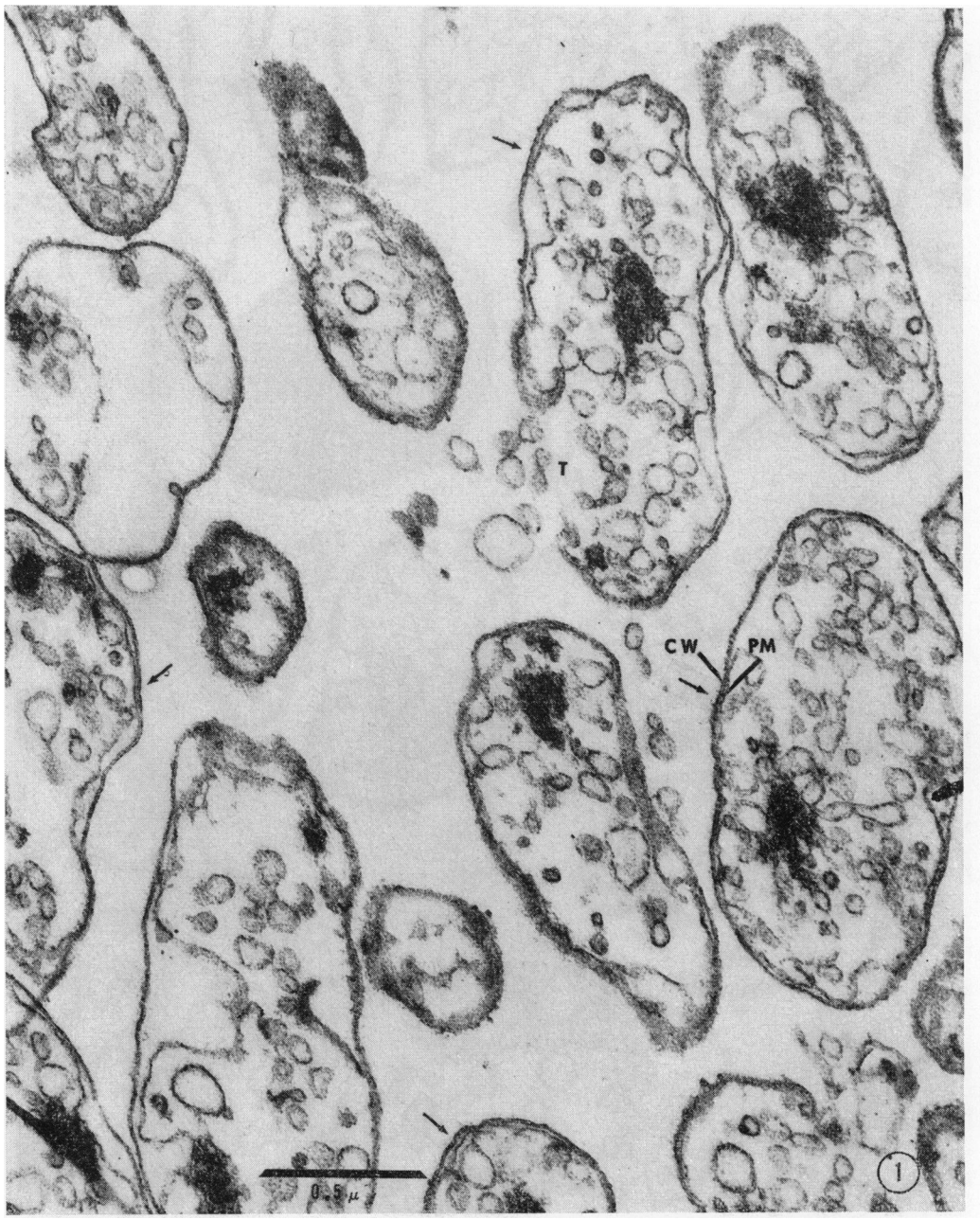


FIG. 1. *Section of envelopes of Azotobacter agilis prepared by osmotic shock. Note that the internal membranes appear tubular, rather than vesicular, where the electron-dense cytoplasm has been retained. The arrows indicate regions in which the peripheral membrane* (PM) *is distinct from, but closely underlies, the cell wall* (CW). *The peripheral membrane is frequently separated from the wall. A large tear* (T) *is apparent in the wall of one of the envelopes.* × *47,000.*

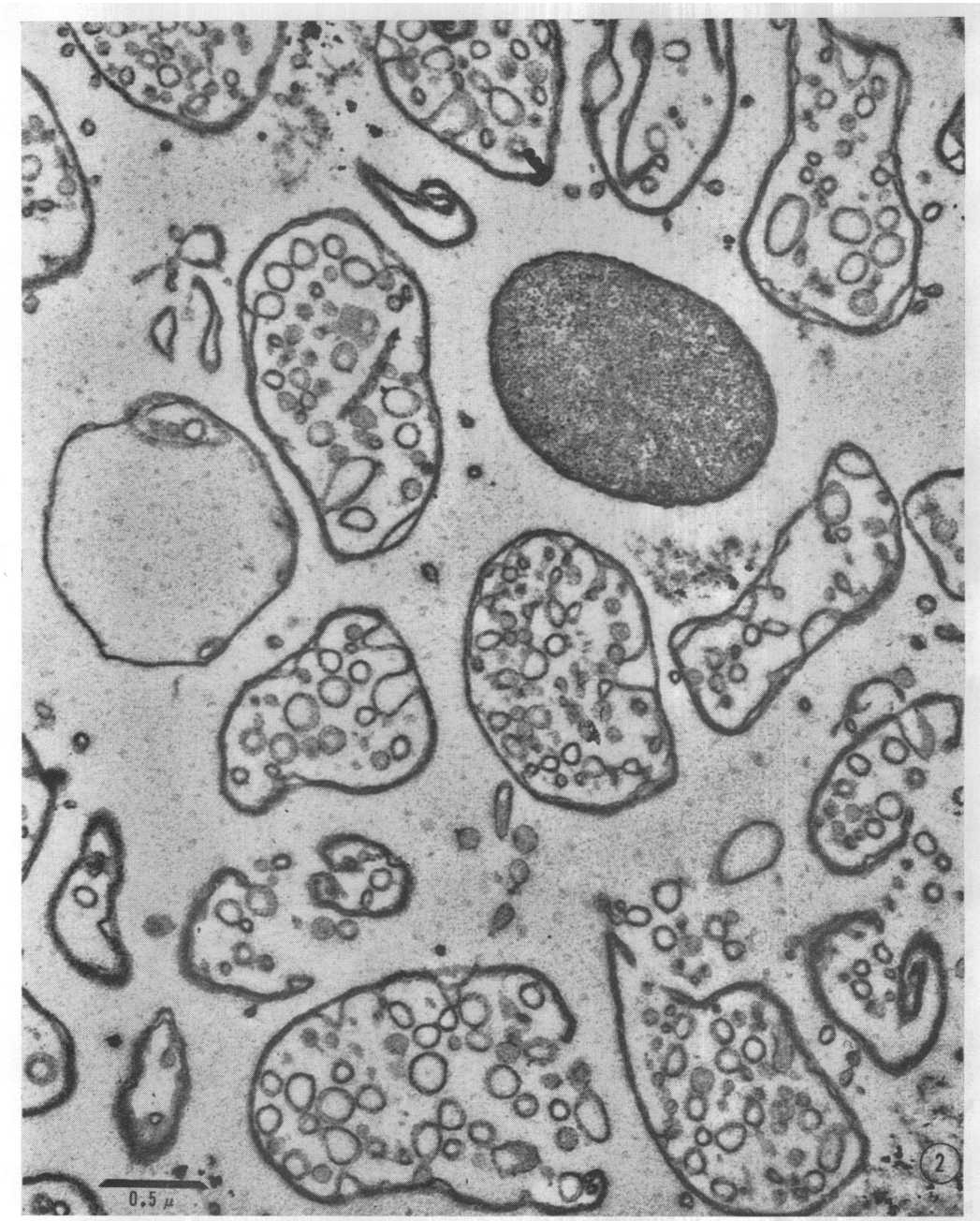


FIG. 2. *Section of envelopes of Azotobacter agilis prepared by brief (2 min) ballistic disintegration. Numerous internal membranes appear within the envelope. Tears are apparent in the walls of several envelopes. One cell in the field has escaped disruption and has retained its cytoplasm.* × *31,000.*

with a shadowed preparation of the envelopes opened by sonic treatment (Fig. 5). In both of the envelopes shown, about one-half of the cell wall has been disintegrated by the sonic treatment. The internal membranes extend from the opening and appear as a network of branched tubes. Sections through this network would be expected to have the appearance of the vesicles observed in Fig. 4. Wet mounts of the envelopes prepared by brief sonic treatment and observed

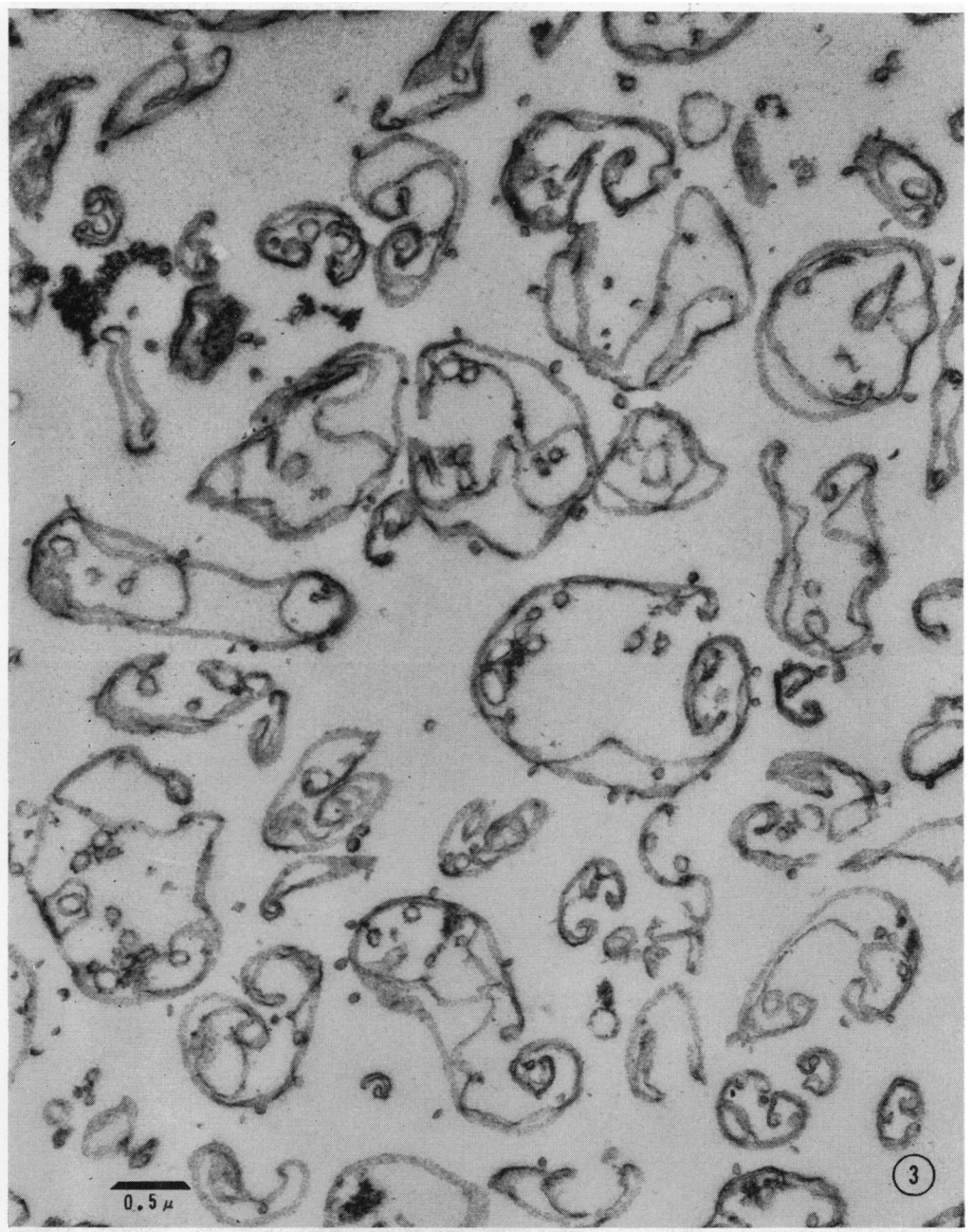


FIG. 3. *Section of envelopes of Azotobacter agilis prepared by osmotic shock and subsequently treated in the Mickle disintegrator for 15 min. Prolonged ballistic distintegration has removed most of the internal membranes without appreciably comminuting the peripheral structure. Compare with Fig. 1.* × 22,000.

by phase-contrast microscopy revealed the membranes extending from the opening in the cell wall in violent Brownian motion.

Sections of intact cells show a network of fine tubules throughout the cytoplasm (Fig. 6), rather than the large vesicles observed in emptied envelopes. The intracytoplasmic membranes in sections of whole cells are obscured by the electron density of the ribosomes and cytoplasmic matrix. By using a lightly printed micrograph,

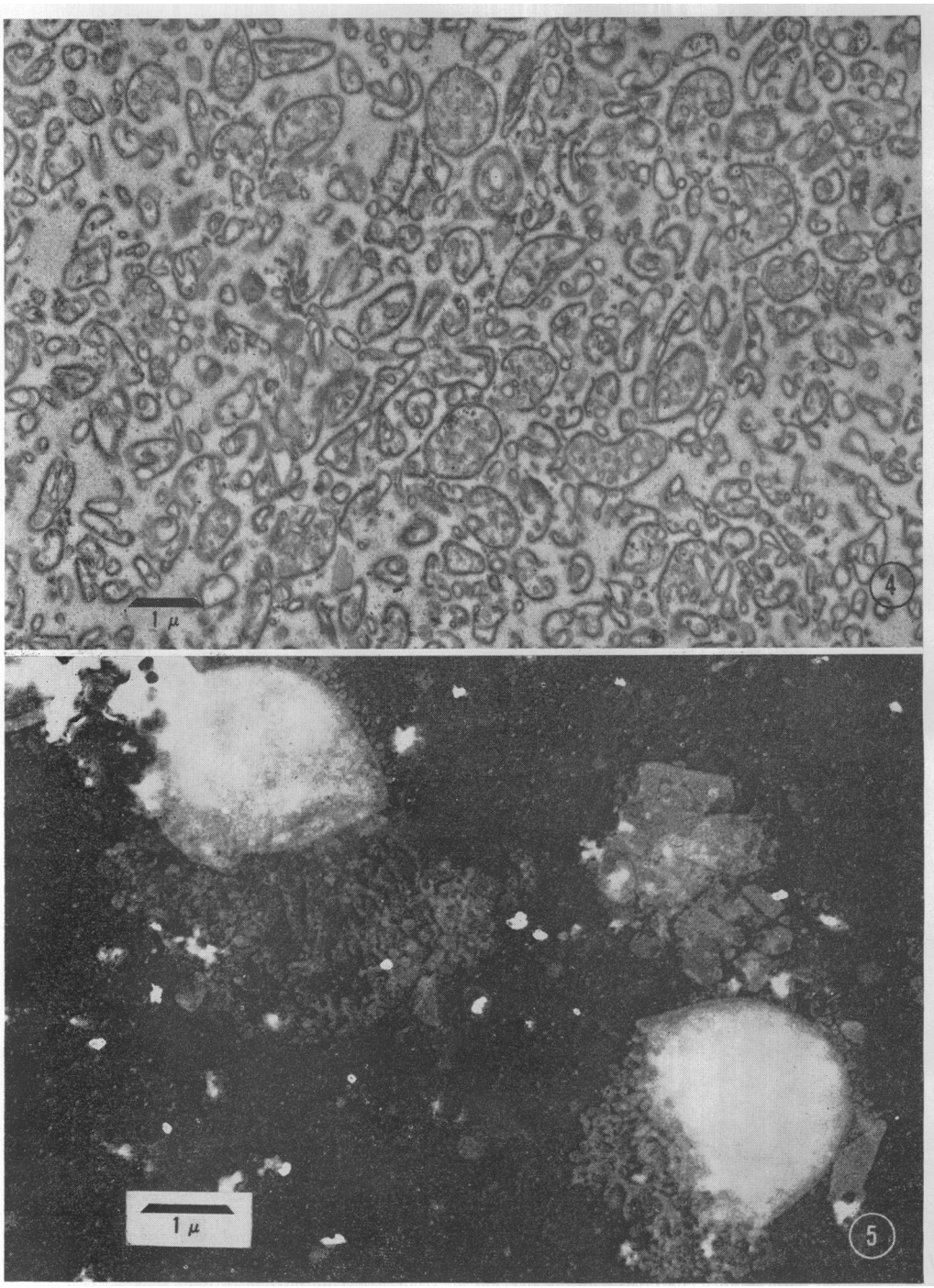
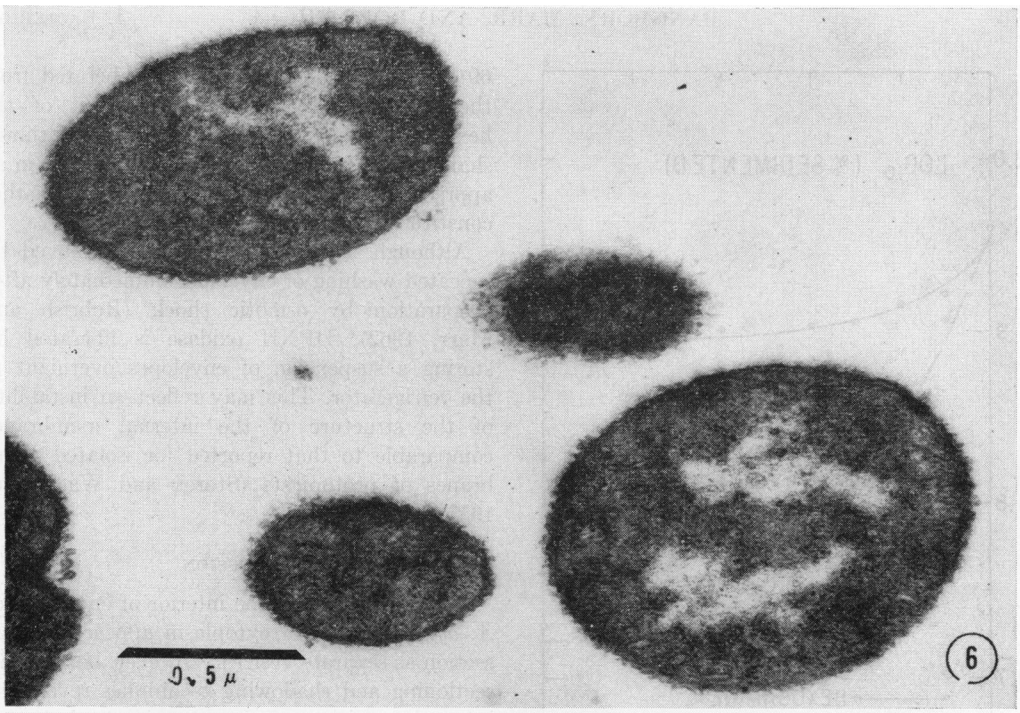


FIG. 4. *Section of envelopes of Azotobacter agilis prepared by sonic disintegration. Damage to the envelopes is extensive; however, a few entire envelopes with internal membranes are apparent.* × *11,000.*

FIG. 5. *Shadowed preparation of envelopes of Azotobacter agilis prepared by sonic disintegration. Both envelopes in the micrograph have lost about one-half of their peripheral structures. The membranes extend through the opening in the wall and appear as a network of tubules.* × *13,000.*



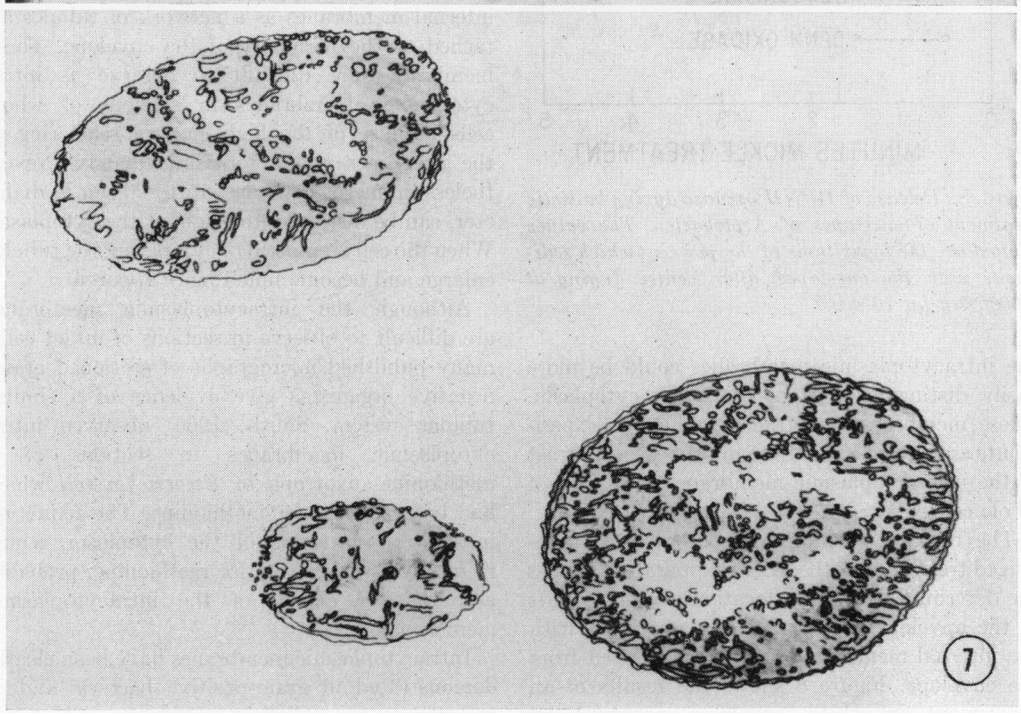


FIG. 6. *Section of undisrupted cells of Azotobacter agilis showing the typical electron-dense cytoplasm surrounding the nuclear vacuole. Upon close inspection, the intracytoplasmic membranes may be seen in a few regions. Compare with Fig. 7.* × *50,000.*

FIG. 7. *Tracing of the intracytoplasmic membranes observed in a light printing of the micrograph shown in Fig. 6. The membranes near the cell periphery appear as transversely sectioned tubules, whereas those more centrally located appear as cross-sectioned tubules. The membranes are particularly numerous along the margin of the nuclear vacuole, although the vacuole itself is devoid of membranes.*

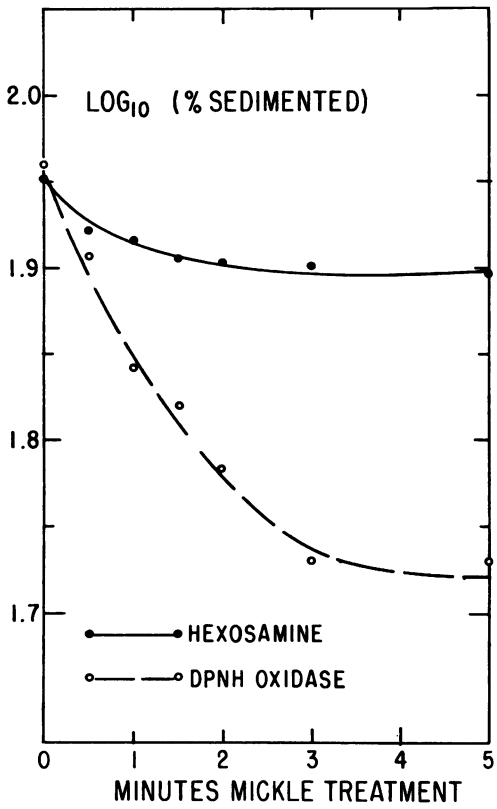


FIG. 8. *Release of DPNH oxidase during ballistic treatment of envelopes of Azotobacter. The values plotted are the logarithms of the per cent which sediments with the envelopes after centri fuging at 5,000 × g for 10 min.*

the intracytoplasmic membranes could be more easily distinguished from the dense cytoplasm. These membranes are traced in Fig. 7. Experiments are now in progress to improve the contrast of the intracytoplasmic membrane in sections of whole cells.

The removal of internal membranes by prolonged treatment in the Mickle apparatus permits the determination of the location of constituents of the envelope. Any substance associated with the internal membranes should be released from the envelope. Figure 8 shows the results of an experiment in which envelopes prepared by osmotic shock were shaken with glass beads for various intervals. Each sample was centrifuged at 5,000 × *g* for 10 min, and the amount of DPNH oxidase and hexosamine still sedimentable was determined. After 3 min of shaking, almost 60% of the DPNH oxidase was liberated from the sedimentable material, and most of the hexosamine remained sedimentable. Continued shaking for a total of 15 min did not result in an appreciable change in the amounts of either constituent sedimentable.

Although DPNH oxidase is not removed by repeated washing of envelopes immediately after preparation by osmotic shock (Robrish and Marr, 1962), DPNH oxidase is liberated by storing a suspension of envelopes overnight in the refrigerator. This may reflect an instability of the structure of the internal membranes comparable to that reported for isolated membranes of protoplasts (Storck and Wachsman, 1957).

DISCUSSION

The membranes in the interior of envelopes of *A. agilis* emptied of cytoplasm appear in cross-section as separate vesicles; however, both serial-sectioning and shadowing techniques reveal the internal membranes as a network of tubules attached to the periphery of the envelope. These membranes are difficult to observe as intracytoplasmic membranes in sections of whole cells because of the high electron-scattering of the ribosomes and cytoplasmic matrix. Nevertheless, numerous tubules, 15 to 20 mμ in diameter, can be discerned throughout the cytoplasm. When the cell is emptied of cytoplasm, the tubules enlarge and become much more apparent.

Although the intracytoplasmic membranes are difficult to observe in sections of intact cells, many published micrographs of sectioned gram-negative organisms give evidence of a similar tubular system. Smith (1960) observed intracytoplasmic membranes in sections of a methionine-auxotroph of *Escherichia coli* which had been starved for methionine. The reduction in electron-scattering of the cytoplasm, which results from starvation for methionine, probably accounts for clarity of the intracytoplasmic membranes.

Intracytoplasmic membranes have been clearly demonstrated in gram-positive bacteria and in streptomycetes (Glauert, Brieger, and Allen, 1961; Glauert and Hopwood, 1959). The intracytoplasmic membranes in *Bacillus*, which appear as concentric rings in cross-section, have been termed "mesosomes" by Fitz-James (1960). Mesosomes differ in morphology and distribution

from the intracytoplasmic membranes of *A. agilis* and have been assumed to be specialized organelles associated with cross-wall formation or sporogenesis.

The loss of internal membranes from the envelopes subjected to prolonged shaking with glass beads can be ascribed to the shearing forces which occur in this treatment. The network of internal membranes which protrudes through the initial breach in the wall is torn away from the envelope by this shearing action. Isolated envelopes of gram-negative bacteria are more electron dense than are the cell walls isolated from gram-positive bacteria (Salton and Horne, 1951). The correlation of the loss of internal membranes with the loss of electron density suggests that the internal membranes are largely responsible for this higher density.

The conclusion that the intracytoplasmic membranes are the locus of respiratory enzymes is based on the correlation of the release of DPNH oxidase and the loss of internal membranes from the envelopes during treatment in the Mickle disintegrator. The lack of release of hexosamine confirms the microscopic observation that the peripheral structure of the envelope is not significantly comminuted during this treatment. Approximately 40% of the DPNH oxidase remains sedimentable even after treatment for 15 min. This substantial residue may reflect a mechanical protection of the residue of internal membranes by the envelope or a location of some of the respiratory enzymes in the peripheral membrane, which is still apparent in envelopes after prolonged treatment.

Lukoianova, Gelman, and Biriusova (1961) recovered the succinic oxidase system of *Micrococcus lysodeikticus* in a fraction which contained the ghosts of protoplasts. Thin sections of this material showed a limiting membrane and "mesosomes" within the ghost. These authors assumed that the "mesosomes" contain all or part of this enzyme system. Thus, "mesosomes," although morphologically distinct, may be functionally related to the intracytoplasmic membranes of *A. agilis* and other gram-negative bacteria.

The location of respiratory enzymes in tubular intracytoplasmic membranes originating from, or attached to, the peripheral membrane of *A. agilis* is strikingly similar to the locus of the respiratory enzymes in the mitochondria of higher forms. The location of the respiratory apparatus in the membrane of a small, closed compartment may be significant in oxidative phosphorylation (Mitchell, 1961).

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