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Intracytoplasmic Membrane Formation and Increased Oxidation of Glycerol During Growth of *Gluconobacter oxydans*

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Gluconobacter oxydans is well known for the limited oxidation of compounds and rapid excretion of industrially important oxidation products. The dehydrogenases responsible for these oxidations are reportedly bound to the cell's plasma membrane. This report demonstrates that fully viable *G. oxydans* differentiates at the end of exponential growth by forming dense regions at the end of each cell observed with the light microscope. When these cells were thin sectioned, their polar regions contained accumulations of intracytoplasmic membranes and ribosomes not found in undifferentiated exponentially growing cells. Both freeze-fracture-etched whole cells and thin sections through broken-cell envelopes of differentiated cells demonstrate that intracytoplasmic membranes occur as a polar accumulation of vesicles that are attached to the plasma membrane. When cells were tested for the activity of the plasma membrane-associated glycerol dehydrogenase, those containing intracytoplasmic membranes were 100% more active than cells lacking these membranes. These results suggest that intracytoplasmic membranes are formed by continued plasma membrane synthesis at the end of active cell division.

The newly recognized genus *Gluconobacter* (9) is composed of certain obligately aerobic gram-negative rods previously assigned to the genus *Acetobacter*. Unlike *Acetobacter*, *Gluconobacter* has a deficient tricarboxylic acid cycle (16) and consequently lacks the ability to completely oxidize acetate and lactate (3, 9). *Gluconobacter* is also distinguished from the *Acetobacter* by its ability to rapidly carry out limited oxidation of organic compounds and to quantitatively excrete the oxidation products into the growth medium (3). *Gluconobacter* will partially oxidize over 80 different compounds (3, 37), and the products of several of these oxidations are currently being used by the cosmetic and pharmaceutical industries (38).

One of the most frequently studied species of this genus is *Gluconobacter oxydans* subspecies *suboxydans*, formerly classified as *Acetobacter suboxydans*. Of the large number of polyhydroxy compounds partially oxidized by *G. oxydans*, the two groups most extensively studied are the aliphatic glycols and the sugar alcohols. More than two-thirds of the glycols and sugar alcohols are reportedly catalyzed by particulate-bound dehydrogenases. Since the glycol dehydrogenases can be removed from the particulate fraction by treatment with Triton X-100, De Ley and Kersters (14) concluded that these enzymes are bound to membrane. They proposed that oxidation of glycols by intact cells is mainly, if not solely, due to enzymatic activity of the plasma membrane. A similar study by Kersters et al. (20) prompted the conclusion that oxidation of sugar alcohols (polyols) by growing *G. oxydans* is also due to dehydrogenases located on the plasma membrane.

Batzing and Claus (6) recently reported that early stationary-phase *G. oxydans* grown on a minimal medium contained intracytoplasmic membranes, and that these membranes were essentially absent in exponentially growing cells. In their recent review, Reaveley and Burge (29) conclude that intracytoplasmic membranes are formed by invaginations of the plasma membrane. We reasoned that if the dehydrogenases of *G. oxydans* are synthesized and incorporated into the membrane as a normal consequence of plasma membrane synthesis and if newly formed intracytoplasmic membranes are the result of continued plasma membrane synthesis, then cells containing intracyto-

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plasmic membranes should also have more dehydrogenases and consequently exhibit a greater rate of oxidation. If increases in oxidation rate could be shown to accompany intracytoplasmic membrane formation in *G. oxydans*, this would suggest a functional role for these membranes and justify a more rigorous fine-structural study.

This report describes experiments designed to (i) determine if intracytoplasmic membranes are formed under nutritional conditions other than those previously described, (ii) assess the effect of intracytoplasmic membranes on the cell's oxidative activity, (iii) ascertain the amount of early intracytoplasmic membrane development, and (iv) determine to what extent intracytoplasmic membranes are associated with the cell's plasma membrane.

MATERIALS AND METHODS

Preparation of stock cultures. The organism used in this study was *A. suboxydans* ATCC 621 obtained from the American Type Culture Collection in 1971. Cells were grown in a medium containing (wt/vol) 5% glycerol, 1% yeast extract, 1% peptone, 1.25% (0.55 M) *cis,cis,cis,cis*-1,2,3,4-cyclopentanetetracarboxylic acid buffer, and 0.1% GE-60 silicon antifoam to retard cell clumping during growth (5). This will hereafter be referred to as the glycerol medium. After adjusting the pH to 6.0 with KOH, 50 ml of the glycerol medium was placed in a 500-ml Bellco Nephelo culture flask (Bellco Glass, Vineland, N.J.) fitted with a cuvette (14 by 130 mm) and subjected to heat sterilization. The medium was inoculated, and cultures were incubated at 28 C with shaking (200 strokes per min; 1.5-inch [3.81 cm] stroke amplitude) until reaching 1.0 optical density at 620 nm (OD_{620}). The cell concentration at this time was about 1.9×10^9 cells per ml. Portions (1.0 ml) of the culture were each placed in sterile 5-ml screw-capped vials containing 2 ml of glycerol. The resulting stock-culture suspensions were mixed well and stored without freezing at −20 C for later use as inocula.

Cell growth. To prepare cells for study, 50 ml of the glycerol medium was inoculated with 0.2 ml of the glycerol stock-culture suspension. These cultures were incubated under identical conditions to those used to prepare stock cultures. Increase in culture turbidity was followed at 620 nm with a Bausch and Lomb Spectronic-20 spectrophotometer. Culture turbidity versus time could be closely predicted during growth if glycerol stock-culture suspensions were stored no longer than 4 to 6 months. Viable counts were made after serial dilution and spread plating of 0.1 ml of the final culture dilution on glycerol medium supplemented with 1.5% (wt/vol) agar.

Light microscopy. Unstained cells were immobilized and observed with normal bright-field optics as previously described (6). Field- and condenser-iris diaphragm settings were standardized to prevent optically induced variations in contrast. Illumination was adjusted according to the principles of Köhler, and Kodak High-Contrast Copy film was exposed so that negatives printed were all of a similar density. Film development was standardized in Kodak D-76, and all bright-field images were printed on Agfa-Gevaert no. 6 paper.

The Zeiss WL microscope was adapted for Nomarski differential interference microscopy according to the recommendations of Allen et al. (2). Cells were immobilized and photographed as described above, except that photographic images were printed on Kodabromide F-2 paper.

Measurement of dihydroxyacetone. The ability of exponentially growing and early stationary-phase cells to excrete dihydroxyacetone (DHA) was determined as follows. Cultures were grown under standard conditions, removed from the growth medium by centrifugation, and washed three times with a salts solution containing 0.05 M KH_2PO_4 and 0.01 $MgCl_2$ adjusted to pH 4.5. Cells were then suspended to an OD_{620} of 0.5 in the salts solution containing 0.3% (wt/vol) glycerol. Fifty milliliters of cell suspension was incubated in 500-ml Nephelo culture flasks under the same conditions used for growth, and samples were periodically removed to determine the amount of DHA excreted. The concentration of DHA was assayed according to the resorcinol method of Asnis and Brodie (4) except that a wave length of 490 nm (10) was used with a Bausch and Lomb Spectronic-20 spectrophotometer. The presence of glycerol was found not to interfere with the DHA assay.

Whole cell respiration. Exponential-phase cells grown in the glycerol medium were harvested after the culture reached 0.3 OD_{620}. Maximum stationary-phase cells grown in this medium were harvested 1 h after reaching their maximum turbidity. Fifty milliliters of culture was centrifuged at $2,500 \times g$ for 15 min at 4 C, and the sedimented cells were washed twice with 25 ml of 0.1 M phosphate-buffered saline adjusted to pH 6.0. Washed cells were suspended in buffered saline so as to achieve a 0.3 OD_{620} (using a cuvette, 14 by 100 mm). This suspension was then shaken for 60 min at 28 C to allow for depletion of endogenous substrates. Rates of glycerol oxidation were determined with a Gilson differential respirometer (Gilson Medical Electronics, Middletown, Wis.). The center well of the Warburg flasks contained 0.2 ml of 1% KOH and a filter paper wick; its main chamber held 0.2 ml of cell suspension (0.8 to 1.0 mg, dry weight), 30 μmol of phosphate buffer at pH 6.0, and distilled water to a total volume of 3.0 ml; the side arm contained either 800 μmol of glycerol to start the reaction or distilled water as a control. Flasks were equilibrated at 28 C for 15 min before starting the reactions.

Dry weight of suspended cells was determined by placing 1.0 ml of the final suspension into tared Corex tubes (Corning Glass Co., Corning, N.Y.) previously dried at 105 C. The cells were dried at 105 C and cooled over desiccant until identical readings were obtained on 3 successive days.

Electron microscopy of whole cells. For thin sections, exponentially growing cultures were taken at

0.5 OD_{620}, and stationary-phase cultures were selected 6 h after reaching maximum turbidity. Osmium tetroxide was added to these cultures to a final concentration of 0.1%. Cells were immediately concentrated by centrifugation and then fixed according to the procedure of Kellenberger et al. (19). Dehydration in a graded acetone series and embedding in Spurr vinylcyclohexane dioxide-based resin (36) was accomplished as previously described (6). Embedded cells were sectioned with an LKB Ultrotome III, mounted on uncoated grids, and stained with lead citrate (31).

For freeze-fracture-etching (FFE) studies, exponentially growing cultures and 6-h stationary-phase cells were selected as above. Ten milliliters of glycerol was added to each 50-ml culture, and cells were removed by centrifugation at 5,000 × *g* for 15 min at room temperature. One drop of a concentrated suspension was placed on a specimen holder and rapidly frozen in Freon 22 and liquid nitrogen. The frozen suspension was transferred to a specimen table previously cooled to −150 C in a Baltzers High Vacuum Plant (model BA360M; Baltzers AG, Baltzers, Liechtenstein). The vacuum chamber was then evacuated to 10^{-6} torr, and the specimen-table temperature was raised to −100 C. The frozen specimen was fractured, and the broken surface was allowed to sublime for 90 s. This "etched" surface was carbon coated and then shadowed with platinum. The replica was removed, cleaned on 70% sulfuric acid for 1 h, and then rinsed twice on distilled water for 5 min. The replica was then floated on a commercially prepared 5.25% sodium hypochlorite solution (Chlorox) for 30 min, rinsed on three changes of distilled water for 10 min each, then picked up with a carbon-coated grid and allowed to air-dry.

All specimens were examined with a Philips EM-300 electron microscope at an accelerating voltage of 60 kV. Images were photographed on Kodak electron microscope film.

Electron microscopy of broken cells. To prepare broken-cell envelopes for negative staining, cells were taken either during mid-exponential growth (0.5 OD_{620}) or 6 h after cultures reached maximum turbidity. Cells were concentrated by centrifuging 50 ml at 1.300 × *g* for 10 min at 4 C and then resuspending the pellet in 10 ml of the supernatant fluid. The resulting cell suspension was extruded from a cold Aminco-French pressure cell (model 4-3398) maintained at constant 2,500 lb/in^2 pressure with a Aminco motor drive laboratory press (model 5-590; American Instrument Co., Silver Spring, Md.). The extruded material was collected in an ice bath and negatively stained either immediately after treatment or after 30 min incubation at 28 C.

For thin sections, cells were broken as above, except that 1.2 mg each of deoxyribonuclease type I and ribonuclease type I-A (Sigma Chemical Co.) were added to the collection vessel for each 40 ml extruded from the pressure cell. Forty milliliters of the treated suspension was centrifuged at 10,400 × *g* for 5 min at 4 C to sediment most of the unbroken cells, and then the supernatant fluid was centrifuged at 10,400 × *g* for 30 min at 4 C to concentrate the broken-cell envelopes. The envelopes were resuspended in 0.1 ml of 1% tryptone (Difco Laboratories, Detroit, Mich.). and 1 ml of 1% OsO_4 was added to the suspension. Broken-cell envelopes were fixed, embedded, sectioned, and examined in the same manner as used with whole cells.

RESULTS

Growth, viability, and differentiation. Our preliminary study (6) demonstrated that cells formed regions of polar density at the end of exponential growth. The medium used in the preliminary study was essentially minimal (7) compared with the environment in which these cells are normally found (3). Therefore, we initially questioned if cells would differentiate when grown in a nutritionally rich medium and if this differentiation would be accompanied by a change in cell viability.

Cells grown in the complex glycerol medium showed a 3-h doubling time (Fig. 1), and this was about three times faster than that found with the minimal medium used in our preliminary study. In the glycerol medium, cells grew exponentially for about seven generations before reaching a stationary-phase cell concentration of 4 × 10^9 viable cells per ml. The rate of turbidity increase closely parallelled the increase in viable numbers during exponential growth, and culture viability was fully maintained for at least 55 h after entrance into the stationary phase (Fig. 1).

Throughout growth, unstained cells were periodically observed with the bright-field microscope. Exponentially growing cells were short, frequently arranged in pairs, and essentially homogeneous in density, with only an occasional cell appearing slightly darker at one pole (Fig. 1a). Regions of slight polar differentiation were first observed in a large number of cells shortly after their growth rate began to decrease. By the time the culture reached maximum turbidity, distinct polar regions and an occasional peripheral region were easily detected (Fig. 1b). During the first 6 h of stationary phase, differentiated regions increased in density, and cells increased in length. Six hours after reaching maximum turbidity, the cells were fully viable (Fig. 1), and most cells were two to four times longer than exponentially growing cells. At this time, all cells exhibited regions of extreme density at both poles, and most cells showed other regions of lesser density (Fig. 1c).

This differentiation was also easily detected in live cells using Nomarksi differential interfer-

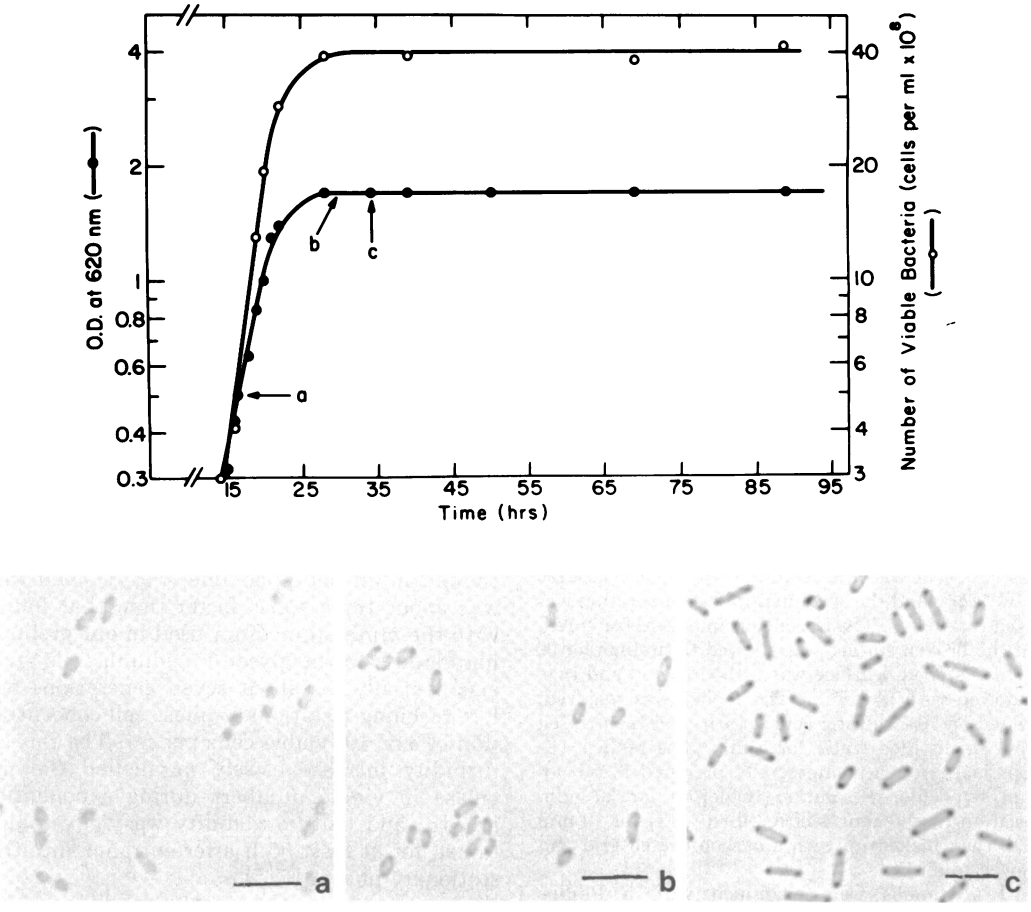


FIG. 1. *Changes in bright-field microscopic appearance of unstained G. oxydans during growth. Cellular turbidity increase (closed circles) is closely paralleled by the increasing number of viable cells per milliliter (open circles). Cells were sampled for microscopic observation at the points marked with arrows on the growth curve. Unstained, exponentially growing cells (a) appeared short, predominately in pairs, and comparatively homogenous in density. Cells sampled during the first hour of maximum culture turbidity (b) were generally longer, unpaired, and contained definite polar and occasional peripheral regions of density. Cells sampled 6 h after cultures obtained maximum turbidity (c) were much longer, contained polar regions of extreme density, and usually exhibited additional regions of lesser density. Cells remained fully viable long after developing regions of internal density. Bar markers represent 5.0 μm.*

ence contrast (DIC) microscopy (Fig. 2). This optical system demonstrates regions of density within cells by translating steep gradients in phase shift into an illusion of height. The resulting image, although it appears to be "shadow cast," is in reality an optical interpretation of abrupt internal density differences within the cell. This effect is easily seen in Fig. 2, where the same cells are observed with both optical systems. In comparison, undifferentiated cells lacking internal regions of density appeared uniformly smooth when observed with DIC optics. Because DIC optics yields a high-contrast image that is easy to record and because it readily detects differences in internal density, we found it preferable for routine detection of differentiation in *Gluconobacter*.

Differentiation and glycerol oxidation. Since differentiated cells were fully viable, it was possible that the onset of differentiation during entrance into the stationary phase might be accompanied by a change in metabolic activity. To test this possibility, polyol oxidation was chosen as a characteristic metabolic indicator.

Glycerol was chosen as a typical polyol, and initial studies employed an assay for the detection of the primary glycerol oxidation product,

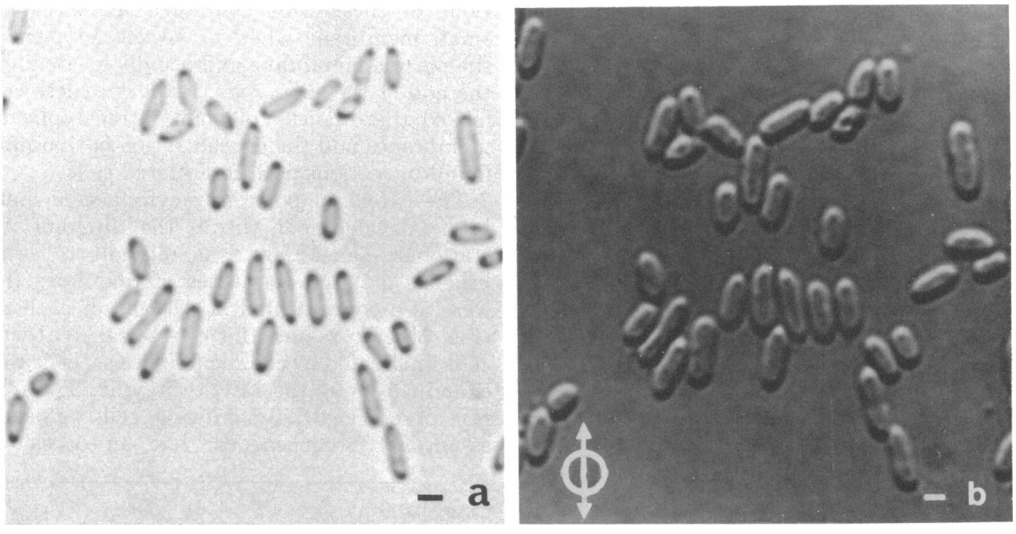


FIG. 2. *Appearance of differentiated G. oxydans as revealed with normal bright-field (a) and Nomarski differential interference contrast microscopy (b). Panel (a) is representative of viable unstained cells 6 h after entering the maximum stationary phase. Panel (b) shows the same cells viewed with the Nomarski differential interference contrast microscope. Differentiated regions in (b) are particularly evident when the cell's long axis is oriented parallel to the direction of optical shear (⇕). Bar markers represent 1.0 μm.*

DHA. Cells were grown in the glycerol medium, and undifferentiated exponential-phase cells were harvested at 0.3 OD_{620}. Early stationary-phase cells were collected from 5 to 10 h after cultures reached their maximum turbidity. Rates of DHA excretion from the same quantity of exponential- and stationary-phase cells are shown in Fig. 3. Differentiated stationary-phase cells excreted DHA at a rate of 3.5 μmol/mg per h, whereas undifferentiated exponential-phase cells excreted DHA at a rate of 2.4 μmol/mg per h. These results show a 50% greater rate of DHA excretion by differentiated stationary-phase cells, and they suggest increased glycerol oxidation by these cells. On the other hand, it was conceivable that glycerol oxidation in both cell types is the same, but that DHA is released at a more rapid rate from differentiated cells. Thus, it was necessary to more directly examine the oxidative activity of these two cell types.

Cells were again grown in the glycerol medium. Exponential-phase cells were harvested at 0.3 OD_{620}, and they appeared as shown in Fig. 1a. Stationary-phase cells were harvested 1 h after obtaining maximum culture turbidity, and they appeared as shown in Fig. 1b. Both cell types were starved for an oxidizable energy source and then respirometrically tested for rates of glycerol oxidation (Fig. 4). Differentiated stationary-phase cells routinely exhibited a 100% increase in the rate of glycerol oxidation when compared with the same quantity of undifferentiated exponential-phase cells. Thus, we concluded that cell differentiation in *G. oxydans* is accompanied by a significant increase in both oxidation and excretion of the oxidation product, and we began an investigation of the fine-structural changes that accompany differentiation in this bacterium.

Ultrastructure of intact cells. Initial studies on stationary-phase cells after growth in a minimal medium (6) showed that regions of internal density occurred at the same location and with the same frequency as localized masses of intracytoplasmic membranes and ribosomes. It seemed likely that similar structures were responsible for differentiation after growth in the nutritionally rich medium. On the other hand, it was equally possible that differentiation of cells in this medium was caused only by accumulation of ribosomes or by concentration of other cellular components. In order to determine if intracytoplasmic membranes were being formed during differentiation on the nutritionally rich medium, both differentiated and undifferentiated cells were sectioned and observed with the electron microscope.

Differentiated cells were sampled 6 h after cultures reached their maximum turbidity (point c, Fig. 1), and, when viewed at low magnification (Fig. 5a), longitudinal sections revealed polar regions that were opaque to the

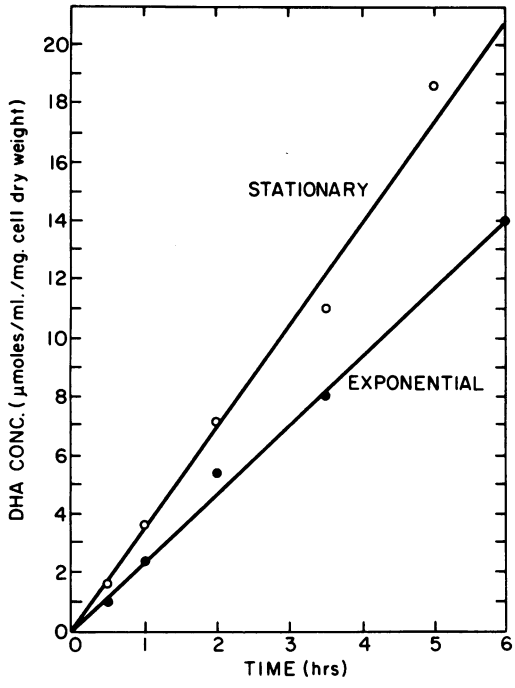


FIG. 3. *Rates of dihydroxyacetone excretion from stationary-phase cells (open circles) and from undifferentiated exponential-phase cells (closed circles) incubated with glycerol. Exponential-phase cells were harvested at 0.50 OD_{620}, and stationary-phase cells were harvested 5 to 10 h after reaching maximum turbidity in the glycerol medium. Cells were washed three times, suspended in a solution containing 0.3% (wt/vol) glycerol, 50 mmol of KH_2PO_4, 10 mmol of $MgCl_2$, and 14 mg (dry weight) of cells in a total of 50 ml, and then this suspension was incubated under the same conditions used for growth. Samples were periodically withdrawn and analyzed for dihydroxyacetone.*

electron beam. When viewed at higher magnifications (Fig. 5b), these electron-opaque regions were found to contain concentrations of intracytoplasmic membrane and ribosomes. The appearance of intracytoplasmic membranes was largely masked by ribosomes; however, these membranes appeared predominately convoluted and whorled rather than organized into discrete vesicles or lamellar masses. These sections appeared essentially identical to sections of early stationary-phase cells observed after relatively slow growth in a chemically defined medium (6).

In comparison, undifferentiated exponentially growing cells viewed at low magnification lacked the dense polar regions that characterized differentiated cells (Fig. 6a). When observed at higher magnification (Fig. 6b), sections of these cells contained an occasional small membrane whorl or vesicle adjacent to the plasma membrane and usually located near the end of the short rods. However, these cells lacked the quantity of polar intracytoplasmic membranes and the concentration of ribosomes found in sections of differentiated cells.

The extent of polar intracytoplasmic membrane development within the differentiated stationary-phase cells was difficult to assess because of large quantities of ribosomes that were also concentrated at the end of each rod (Fig. 5b). To reduce this effect and to observe cells unaffected by chemical fixatives, cells were examined using the FFE technique. The interior of exponentially growing cells appeared essentially homogeneous, but an occasional

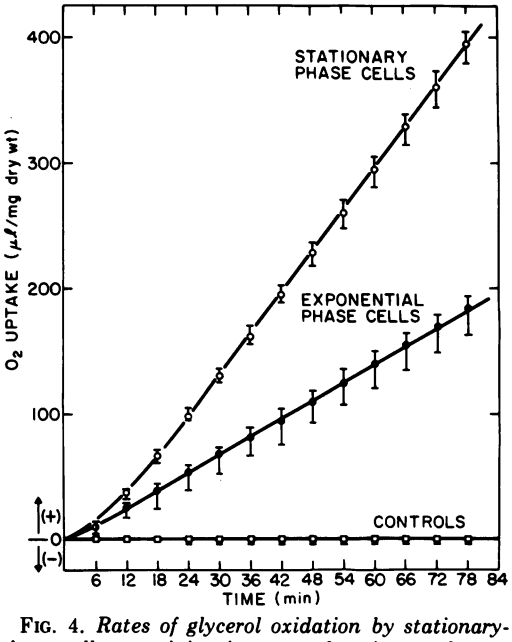


FIG. 4. *Rates of glycerol oxidation by stationary-phase cells containing intracytoplasmic membranes (open circles) compared with exponential-phase cells lacking these membrane complexes (closed circles). Each curve shows the range in values from three experiments, with open and closed circles added to represent the mean value for each set of determinations. Exponential phase cells were harvested at 0.30 OD_{620}, and stationary-phase cells were harvested 1 h after obtaining maximum culture turbidity. Cells were washed and starved as described in the Materials and Methods, and then they were immediately tested for glycerol oxidation with differential respirometry. Complete reaction mixtures contained 0.8 to 1.0 mg (dry weight) of resting cells, 30 μmol of phosphate buffer at pH 6.0, 800 μmol of glycerol, and distilled water to a total volume of 3.0 ml. Distilled water was substituted for glycerol in the control flasks.*

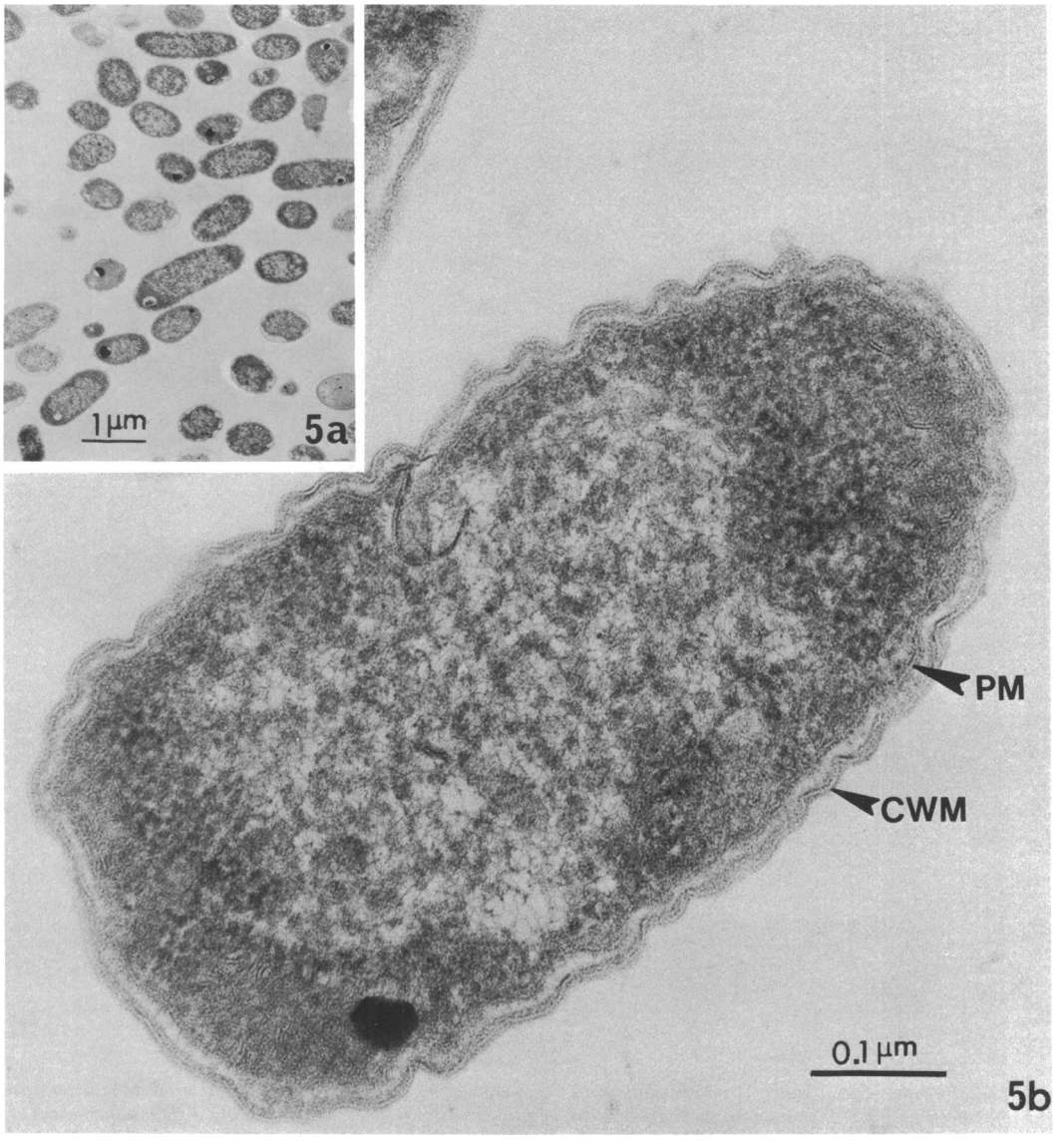


FIG. 5. *Representative silver-grey sections of G. oxydans sampled 6 h after the culture obtained maximum turbidity (see point c, Fig. 1). Longitudinal sections viewed at low magnification demonstrate predominately polar regions of density (a) similar in location and frequency to the differentiated regions observed with the light microscope (Fig. 1c). When these sections were viewed at higher magnification (b) both the cell wall membrane (CWM) and the plasma membrane (PM) were readily apparent. The dense polar regions appeared to be predominately composed of ribosomes and convoluted membrane masses that were adjacent to the plasma membrane. Small black inclusions are probably polymetaphosphate, because of their extreme density and their frequent volatilization in the electron beam.*

membrane vesicle was noted at the cell periphery (Fig. 7). The location and infrequent occurrence of these vesicle-like structures within FFE preparations of exponentially growing cells appeared very similar to observations made with thin sections of the same populations (Fig. 6). On the other hand, replicas of FFE cells sampled 6 h into the stationary phase exhibited a large number of intracytoplasmic membranes, and most of these appeared concentrated at each end of the cell (Fig. 8). Although these polar areas were typically filled with small

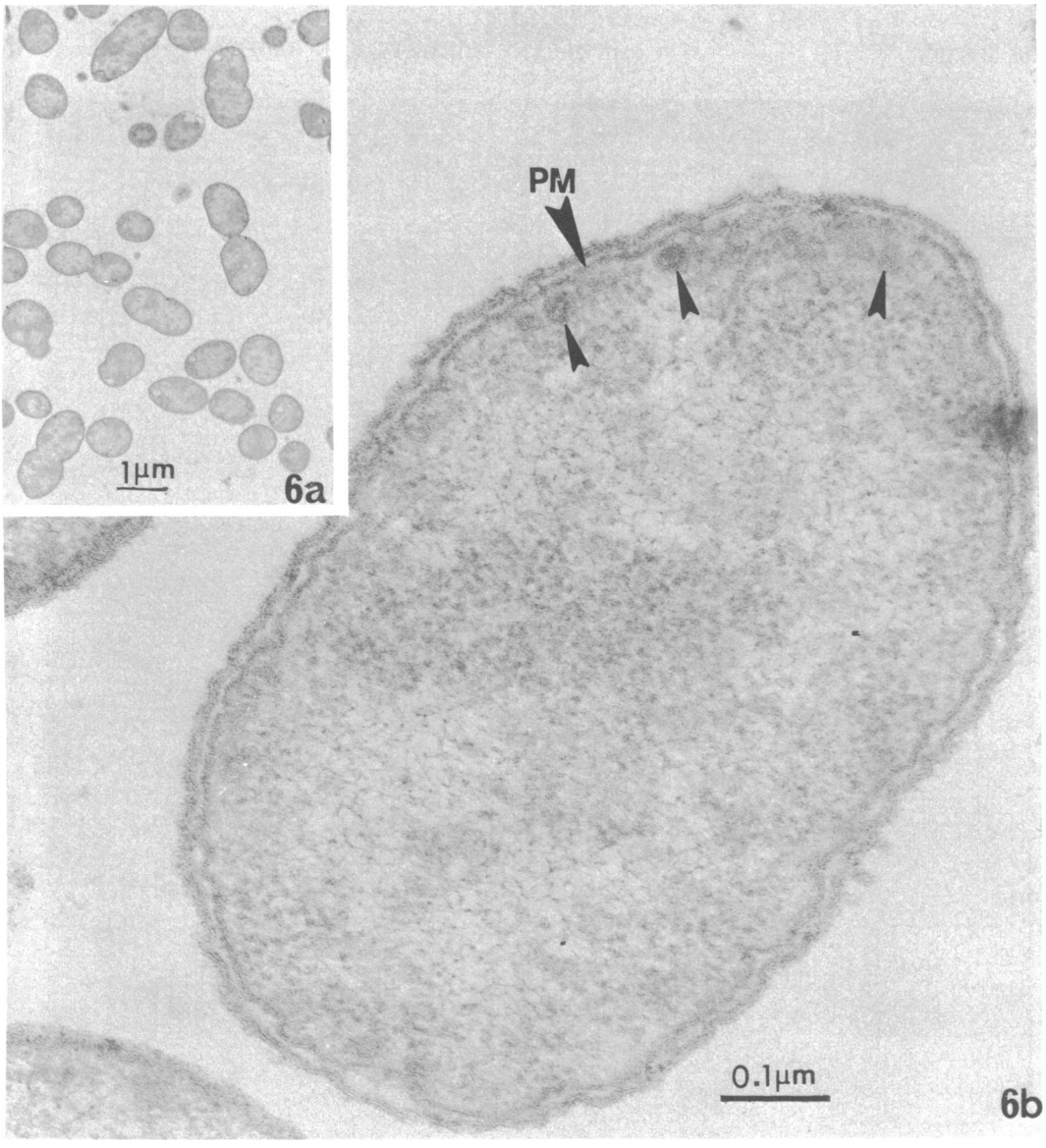
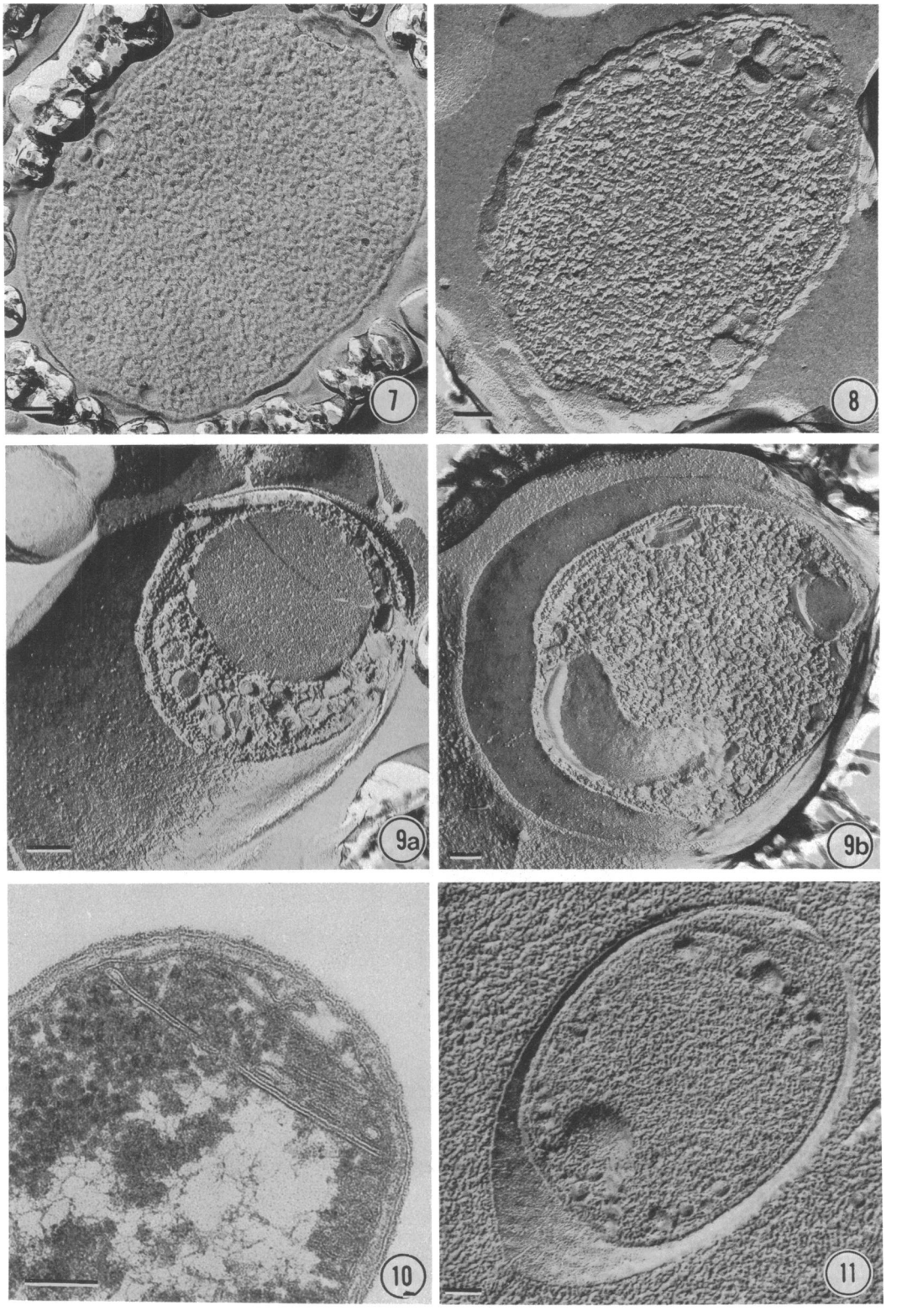


FIG. 6. *Representative silver-grey sections of G. oxydans sampled during exponential growth (see point a, Fig. 1). Sections viewed at low magnification show many dividing pairs of short rods having a comparatively random distribution of ribosomal material (a). The number of dividing pairs, the average cell shape, and the lack of localized regions of density in (a) closely parallels the appearance of unstained cells viewed with the light microsocope (Fig. 1a). Sections viewed at higher magnification (b) demonstrate random ribosome and chromatin distribution, and they lacked masses of intracytoplasmic membrane that were characteristic of stationary-phase cells. Exponentially growing cells usually exhibited several membrane vesicles (arrows) adjacent to the plasma membrane (PM).*

FIG. 7. *Longitudinal fracture through a typical exponential-phase cell. FFE cells usually contained several peripheral membrane-like structures, and they appeared very similar to thin sections of undifferentiated cells (Fig. 6b). Bar marker represents 0.1 μm.*

FIG. 8 to 11. *Cells sampled 6 h after cultures obtained maximum turbidity. Although longitudinal fractures were rarely seen, cells fractured as in Fig. 8 were frequently observed, and these usually contained localized accumulations of membrane-like structures. Fractured cell tips characteristically exhibited many small membranes (Fig. 9a), and they also occasionally contained larger flat or curved membrane "plates" (Fig. 9a and 9b). Since large membrane folds were occasionally observed in thin-sectioned cell tips (Fig. 10), it is believed that the plates observed after freeze fracturing are membrane folds lying in the plane of fracture. Plasmolyzed cells (Fig. 11) showed vesicle-like intracytoplasmic membranes closely associated with the inside surface of the plasma membrane. Although infrequently observed, plasmolysis occurred most often with stationary-phase cells. All bar markers represent 0.1 μm.*



FIGS. 7 to 11.

membrane vesicles (Fig. 9a), larger flat or curved membrane "plates" were also observed in these regions (Fig. 9a and 9b). Since large membrane folds were also occasionally observed at the end of thin-sectioned cells (Fig. 10), these "plates" were interpreted as membrane folds lying in the plane of fracture. Fractures through the occasionally observed plasmolyzed cells (Fig. 11) showed the vesicle-like intracytoplasmic membranes closely associated with the inside surface of the plasma membrane.

Ultrastructure of broken cells. Invagination of the plasma membrane is thought to be the usual mechanism by which procaryotic cells form intracytoplasmic membranes, and the membranes so formed usually remain attached to the broken-cell envelope (29). To determine if this generalization could also be applied to *G. oxydans*, cells were broken with the French press, and the resulting cell wall-membrane (envelope) fraction was observed after negative staining and thin sectioning. If cells were broken with a cold pressure cell, extruded into an ice bath, and immediately stained and examined, broken stationary-phase envelopes appeared as shown in Fig. 12a. These envelopes showed no signs of internal elaboration, but, instead, their undulated surface appeared similar to that observed with negatively stained whole cells. It was presumed that much of the wall peptidoglycan remained intact with this treatment, and the presence of this rigid material was preventing the amount of collapse necessary to reveal complex internal membrane.

To stimulate autolytic destruction of the peptidoglycan, broken-cell suspensions were warmed to 28 C for 30 min and then negatively stained and observed with the electron microscope. These envelopes appeared as shown in Fig. 12b. Each pole appeared very different from the rest of the collapsed envelope, and a sharp demarcation frequently separated the differentiated pole from the remainder of the envelope. These polar regions were not observed in envelopes from undifferentiated exponential-phase cells. It seemed likely that large quantities of intracytoplasmic membranes remained attached to envelopes of stationary-phase cells and caused this appearance. To determine this, envelopes from both cell types were sectioned and examined.

Sections of undifferentiated exponential-phase cell envelopes demonstrated only a few

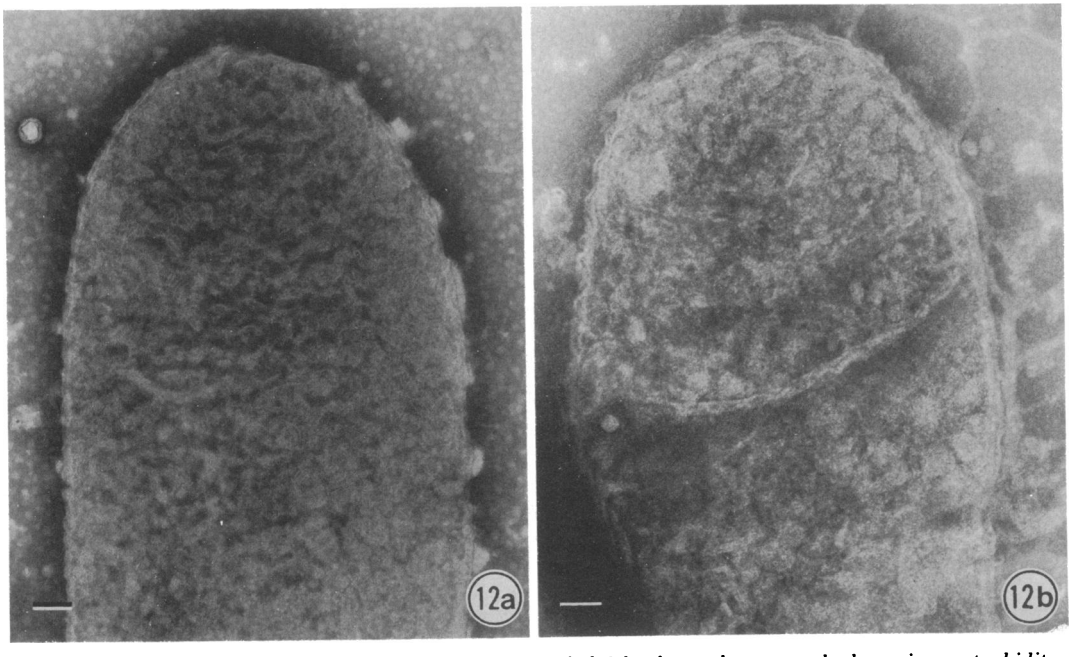


FIG. 12. *Negatively stained envelopes from cells sampled 6 h after cultures reached maximum turbidity. Panel (a) is representative of envelopes kept at 4 C and observed immediately after breakage. Panel (b) represents envelopes that were broken at 4 C, incubated at 28 C for 30 min to allow autolytic destruction of the peptidoglycan, and then observed. The polar appearance shown in (b) was observed only after the envelopes were warmed or stored overnight at 4 C prior to their examination. Essentially all envelopes appeared this way after treatment. Bar markers represent 0.1 μm.*

membrane vesicles, and each of these appeared adjacent to the plasma membrane (Fig. 13). The number and location of these vesicle-like membranes within the envelopes appeared very similar to that observed when exponentially growing whole cells were freeze fractured (Fig. 7) or thin sectioned (Fig. 6b).

In contrast to exponential-phase cell envelopes, differentiated stationary-phase envelopes exhibited many membrane vesicles and folds located primarily at each end of the cell (Fig. 14). Their presence in virtually every longitudinally sectioned envelope further demonstrated the extent of intracytoplasmic membrane development as cells entered this phase of growth. Intracytoplasmic membranes retained their polar position and their vesicular or tubular appearance within the broken-cell envelopes, and this strongly supports the idea that intracytoplasmic membranes within *G. oxydans* remain tightly attached to the envelope after their formation. In some longitudinal sections, the plasma membrane was deeply invaginated at the end of the cell envelope (Fig. 15). The frequency of this latter observation supports the proposal that intracytoplasmic membranes of *G. oxydans* arise from invaginations or folds of the plasma membrane within the cell's polar region.

DISCUSSION

Intracytoplasmic membrane formation in *G. oxydans* was first shown to occur (6) after exponential growth in essentially a minimal medium (7). Since nutritional stress is known to stimulate intracytoplasmic membrane formation in other gram-negative procaryotes (12, 25, 26, 28), it seemed likely that formation of these membranes in *G. oxydans* might be stimulated by low levels of nutrients present during exponential growth. The present study demonstrates intracytoplasmic membrane formation after growth in a nutritionally rich medium; thus low levels of nutrients during growth do not seem to be the cause of this differentiation. Recent work in our laboratory (M. A. Kugatow, unpublished data) demonstrates that, if differentiated cells are removed at the early stationary phase from this rich medium and this "spent" medium is inoculated with actively growing cells, the spent medium will support their continued active growth, and cells will not differentiate until the end of exponential growth. Thus, we presently conclude that differentiation is not caused by nutrient limitation or depletion.

Evidence obtained in this study demonstrates that differentiated *G. oxydans* contains large quantities of intracytoplasmic membrane vesicles, and that these membranes are located predominately at the poles after their formation. Several other gram-negative procaryotes are known to produce intracytoplasmic membrane vesicles, but the vesicles of these bacteria occur either as a parallel arrangement of single vesicles (12, 24) or as a series of constricted tubules (24, 25) both located around the entire periphery of the cell. At present, the accumulation of these inner membranes at the end of exponential growth and the predominately polar distribution of these membranes within *G. oxydans* appears unique among the bacteria.

In addition to the presence of polar membrane vesicles, differentiated *G. oxydans* also contain heavy polar concentrations of ribosomes. We have found that differentiation of these cells is accompanied by preferential uptake of basic dyes at the end of each cell. We now presume that this bipolar basophilic characteristic of stationary-phase cultures is caused by the extensive accumulation of ribosomes at the site of intracytoplasmic membrane accumulation. Our observations to date suggest that a local accumulation of ribosomes parallels intracytoplasmic membrane synthesis in *G. oxydans*. One might expect ribosomes to accumulate in an area where intracytoplasmic membranes are being rapidly synthesized. At least in this regard, this bipolar staining might support Bisset's view (8) that the pole of rod-shaped cells is a site of active biosynthesis. The appearance of bipolar staining in other gram-negative bacteria, such as *Pasteurella* (9), *Sphaerophorous* (32), *Escherichia*, and *Enterobacter* (G. W. Claus, unpublished observation) causes us to wonder if polar intracytoplasmic membrane formation may occur more commonly than is now realized.

Although we do not yet know if accumulated membranes or ribosomes or a combination of both structures causes the density observed with the light microscope, our findings demonstrate that light microscopy can be effectively used to assay for internal differentiation with *G. oxydans*. Phase-contrast microscopy has been previously used to detect accumulation of intracytoplasmic membranes within gram-negative photosynthetic (15, 30) and nitrifying (23) bacteria. However, phase microscopy of viable *G. oxydans* shows much less difference in contrast between differentiated regions and the remainder of the cell than that demonstrated with the bright-filed microscope. The DIC optics produces a high-contrast image without losing the appearance of internal differentiation, and this image is much easier to photographically record than that produced with

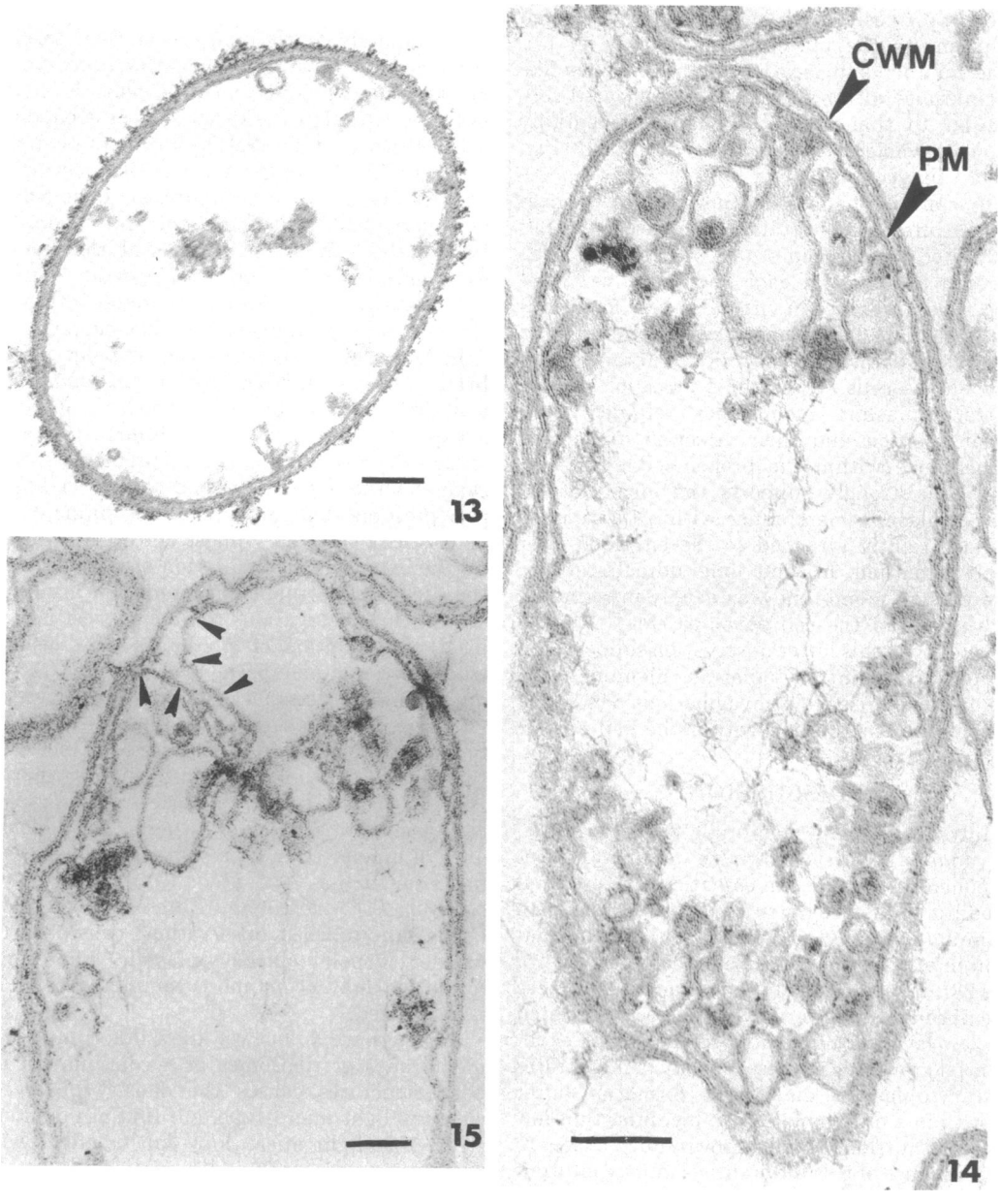


FIG. 13. *Representative longitudinal section of exponential-phase cell envelopes. Intracytoplasmic membrane vesicles were relatively infrequent and always located near the plasma membrane. Bar marker represents 0.1 μm.*

FIG. 14. *Longitudinal section representative of envelopes prepared from cultures sampled 6 h after obtaining maximum turbidity. Intracytoplasmic membranes appeared as vesicles concentrated in the poles of broken-cell envelopes. Some nucleic acid fibrils were apparent after inadequate treatment with deoxyribonuclease and ribonuclease. Bar markers represent 0.1 μm.*

FIG. 15. *Thin-sectioned stationary-phase cell envelope showing invagination of the plasma membrane (arrows). Similar observations were frequent in longitudinal sections of these envelopes. Bar marker represents 0.1 μm.*

bright-field optics. Hitchens et al. (17) also used DIC optics to detect internal changes during endospore formation in *Bacillus megaterium*, and they conclude that DIC optics is a particularly sensitive method for determining the earliest stages of differentiation.

Reavely and Burge (29) state that intracytoplasmic membranes appear to be almost universally formed by invagination of the plasma membrane, and the evidence presented in this report strongly suggests that the intracytoplasmic membranes formed by *G. oxydans* conform to that generalization. The inner membrane vesicles remain attached to broken-cell envelopes after all other cytoplasmic constituents are released, and their polar position is retained. Sections through the polar region of broken cells occasionally reveal sac-like invaginations of the plasma membrane. In addition to structural evidence for continuity between the plasma membrane and the intracytoplasmic membranes, we have shown that cells containing intracytoplasmic membranes oxidize nearly 100% more glycerol than cells lacking these membranes, and the enzyme responsible for this oxidation is known to be bound to the particulate fraction (42) thought to be the plasma membrane of *G. oxydans* (20). Recent studies in our laboratory (D. L. Heefner and G. W. Claus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P106, p. 158) demonstrate that cells containing intracytoplasmic membranes have about 40% more extractable lipid than undifferentiated cells, yet quantitative analyses show that both differentiated and undifferentiated cells contain the same type of lipids. This data strongly suggests that intracytoplasmic membrane development in *G. oxydans* results from continued synthesis and subsequent invagination of the cell's plasma membrane. The absence of appreciable quantities of intracytoplasmic membrane during times of active cell division and the occurrence of these membranes soon after active cell division slows or stops suggest that the factors which control wall synthesis and cell division are not closely linked with plasma membrane biosynthesis in *G. oxydans*.

It is frequently stated that intracytoplasmic membranes of bacteria predominately play an oxidative role, and our glycerol oxidation studies in *G. oxydans* support this generalization. However, it is difficult to understand the advantage of extra oxidation and energy production for a cell that has stopped active division. If one considers other non-photosynthetic gram-negative procaryotes, it is noted that intracytoplasmic membranes are formed predominately by the methane oxidizers (13, 27, 34, 35, 40) and the ammonia or nitrite oxidizers (39). These bacteria are obligate aerobes and they characteristically derive their energy from removal of a single electron pair from CH_4, NH_4^+, or NO_2^-. Because the potential of the electrons removed from these compounds is relatively high, it is assumed that these bacteria have a limited electron transport system, and therefore a very inefficient mechanism for oxidative phosphorylation. This idea prompted Davies and Whittenbury (13) to suggest that extensive intracytoplasmic membrane development in these bacteria increases their respiratory capacity, thus compensating for a low phosphorylation efficiency. In this way, the metabolism of the methane oxidizers and the ammonia or nitrite oxidizers appears similar to that of *Gluconobacter*. The *Gluconobacter* are also obligate aerobes that carry out rapid, single-step oxidations of their energy source (3). Although cytochromes *b*, *c*, *o*, and ubiquinone Q_{10} reportedly occur in *G. oxydans* (33, 43), it appears that cytochrome c_{554} (Cyto *o*) is the only component of the electron transport system that is routinely present in high concentration (33). This latter observation, coupled with reports of low PLO ratios (21), suggests that limited electron transport and low phosphorylation efficiency is also characteristic of *G. oxydans*. Thus, one might speculate that intracytoplasmic membranes are also formed in *G. oxydans* in order to compensate for inefficient oxidative phosphorylation. If this were true, one would expect large amounts of intracytoplasmic membranes to be a constant structural feature of *G. oxydans*, yet they only appear to be formed after active growth stops. In this respect, differentiation in *G. oxydans* is similar to endospore and cyst formation in other procaryotes. The fact that these membranes are formed at the end of exponential growth also suggests that their formation may be in response to a stressed environment. Intracytoplasmic membrane formation in response to an altered environment has been observed with other gram-negative procaryotes (1, 11, 12, 18, 22, 41). We do not yet understand the functional advantage of intracytoplasmic membrane development for *Gluconobacter*, but it is tempting to speculate that the ability to form these membranes somehow helps the cell to survive until active growth can once again take place.

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