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Light-Induced Division and Genomic Synchrony in Phototrophically Growing Cultures of *Rhodopseudomonas sphaeroides*

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An experimental procedure for rapidly obtaining cell populations of phototrophically growing *Rhodopseudomonas sphaeroides* which display division and genomic synchrony has been developed. The basis of the procedure resides with the normal physiological response displayed by cells of *R. sphaeroides* that have been subjected to an immediate decrease in incident light intensity. After an abrupt high- to low-light transition of an asynchronously dividing cell population, an immediate cessation of increases in culture turbidity, total cell number, and net accumulations of culture deoxyribonucleic acid and phospholipid occurs. Total cell number remains constant for 2.5 h after the transition to low light, after which time, it undergoes a sharp increase. Reinitiation of high-light conditions of growth 1 h subsequent to this increase in total cell number results in a cell population possessing a high degree of division and genomic synchrony. A characterization of this procedure, together with a demonstration of its utility for studies on intracytoplasmic membrane assembly, is presented.

The nonsulfur, purple, facultatively phototrophic bacterium *Rhodopseudomonas sphaeroides* has been widely employed for investigations of the mode and regulation of membrane assembly (13, 14, 18, 19, 23). When grown phototrophically (anaerobic light), *R. sphaeroides* produces an extensive intracytoplasmic membrane (ICM) system that occurs in conjunction with the cytoplasmic and outer membranes typical of its gram-negative envelope. The ICM has been well characterized chemically and is known to harbor the photopigments and electron transport components required for photosynthetic growth (13). The utility of *R. sphaeroides* for studies on membrane assembly historically resides with the ease and predictability with which the formation and quantity of its ICM can be experimentally controlled. In this regard, a number of studies have appeared concerning the influence of light intensity and oxygen partial pressure upon the elaboration of the ICM of *R. sphaeroides* (7, 13, 15, 20-22, 24).

Recently, information on the mode and regulation of ICM assembly has been greatly augmented by studies employing synchronously dividing cell populations of *R. sphaeroides* (10, 11, 18, 25). These investigations have provided information concerning the relationships between the formation and assembly of ICM constituents and, further, have clearly demonstrated that the continued use of division-synchronized cell populations for studies on ICM assembly is highly warranted.

Unfortunately, the benefits potentially available from studies with synchronously dividing cell populations are not being fully realized by investigators in the general area of membrane biogenesis. This situation, in part, stems from the lack of rapid synchronization procedures suitable for accommodating the quantities of cell material required for the isolation of cellular membranes.

In their studies on ICM assembly in *R. sphaeroides,* Lueking et al. (18) employed the stationary-phase cycling technique of Cutler and Evans (8) to obtain division-synchronized cell populations. Although this technique provides the flexibility in culture volumes required for membrane studies, there are several problems associated with its use: (i) the required stationary-phase cyclings and intermittent cell harvesting and washing procedures are time consuming; (ii) the observed patterns of division synchrony are qualitatively and quantitatively variable; (iii) culture cycling procedures must be empirically reestablished after changes in growth medium composition or when different organisms, or different strains of the same organism, are employed; (iv) the contributions of cellular changes resulting from stationary to logarithmic phase adaptation of the culture are constantly superimposed upon the experimental data; (v) a per-

turbing effect of the technique is indicated by reductions (15 to 20%) in culture generation times.

The present report describes a procedure alternative to that of stationary-phase cycling for achieving division and genomic synchrony of phototrophically growing cultures of *R. sphaeroides*. The procedure is rapid, applicable to large and small culture volumes, and capable of accommodating cell populations in the early logarithmic phase of growth. A characterization of this technique, together with a demonstration of its utility for studies on membrane assembly in *R. sphaeroides*, is presented.

MATERIALS AND METHODS

Organisms, medium, and conditions of growth. *R. sphaeroides* M29-5 (Leu^- Met^-), derived from strain 2.4.7, was provided by Samuel Kaplan, University of Illinois, Urbana. This organism was routinely grown on a succinic acid minimal medium supplemented with 50 μg each of L-leucine and L-methionine per ml (18). Stock cultures were maintained at −20°C in this same medium adjusted to 10% (wt/vol) glycerol.

Incubations were conducted anaerobically in the light (photoheterotrophically) both in completely filled screw-cap vessels and in flat-walled vessels (1 to 5 liters) under an atmosphere of 95% N_2–5% CO_2. Cells adapted to the logarithmic phase of growth were the inoculum source for all studies. Incubations were at 32°C with 50 to 500 ft-c illumination (538 to 5,380 lx) provided by a bank of Lumiline lamps (Sylvania). Culture growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter. A turbidity value of 100 photometer units corresponds to a cell concentration of 10^9 cells per ml.

Cell enumeration. The response of phototrophically growing cultures to varying light regimens and confirmation of the presence of division synchrony were directly determined by monitoring total cell number with a Petroff-Hausser counting chamber. Culture samples (1 ml) employed for determinations were first transferred to tubes containing 1 ml of 5% (wt/vol) formaldehyde, and 600 to 900 cells were counted per determination.

Purification of ICM. Purified ICM preparations employed for the determination of ICM protein-phospholipid ratios were obtained essentially as described by Fraley et al. (10). Culture samples, adjusted for uniform cell mass, were removed at 15-min intervals, and the cells were harvested by centrifugation at 12,000 × *g* for 10 min. The pelleted cells were washed once in 10 mM phosphate buffer (pH 7.0), the resulting cell pellets were resuspended in 4 ml of the same buffer, and the cells were disrupted by sonication for 10 min (35% efficiency; 40% pulse regimen) with a Branson model W-350 sonicator. After treatment with DNase and RNase (5 μg/ml, each), the preparation was freed of whole cells and debris by centrifugation at 12,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 106,000 × *g* for 1 h. The pellet obtained from this latter centrifugation was suspended in a minimal volume of phosphate buffer, layered onto a discontinuous sucrose gradient (20 to 40 to 60%, wt/vol), and centrifuged at 75,000 × *g* for 8 h. The material banding at the 20%–40% gradient interface was removed, diluted with phosphate buffer, and pelleted by centrifugation at 106,000 × *g* for 1 h. This material, consisting of purified (≳93%) ICM vesicles (10), was then used directly for the determination of ICM protein and phospholipid contents.

Phospholipid extraction. The phospholipids present in whole cells and purified membrane fractions were extracted by the method of Bligh and Dyer (3) as described by Ames (1). A solution of 2 N KCl containing 0.01 N HCl (6) was used to replace the water additions during phase partitioning and washing of the chloroform extracts. All extractions were performed in duplicate.

DNA, protein, and lipid phosphorus determinations. DNA determinations were conducted employing the Burton modification (4) of the diphenylamine assay exactly as described by Lueking et al. (18). All analyses were performed in duplicate.

Whole-cell and membrane protein determinations were conducted by the method of Lowry et al. (17), employing bovine serum albumin as a standard. Protein assays utilized samples that had been treated for 15 min at 60°C in 1 N NaOH.

Samples employed for lipid phosphorus determinations were digested as described by Goldfine et al. (12), and phosphorus was determined by the method of Bartlett (2). Lipid phosphorus values were multiplied by a factor of 25 to obtain micrograms of phospholipid.

Bacteriochlorophyll and carotenoid determinations. The quantitation of bacteriochlorophyll and carotenoids present in whole-cell acetone-methanol (7:2, vol/vol) extracts was conducted as described by Wraight et al. (25). Extinction coefficients of $\epsilon_{775} = 75$ mM^{-1} (5) and $\epsilon_{484} = 128$ mM^{-1} (7) were employed for calculation of the concentrations of bacteriochlorophyll and carotenoids, respectively.

Freeze-fracture electron microscopy. Cell samples employed for freeze-fracture were processed both with and without prior fixation. Sample fixation consisted of a 15-min treatment in 0.08 M sodium cacodylate buffer (pH 7.2) containing 2% paraformaldehyde and 3% glutaraldehyde. Fixed cell preparations were thoroughly washed in 0.1 M cacodylate buffer and incubated (1 h) in this same buffer containing 25% glycerol. Fixed and unfixed cell preparations were collected by centrifugation, and cell pellets (0.5 μl) were mounted on gold alloy stubs, frozen in liquid nitrogen-cooled Freon 22, and stored in liquid nitrogen.

Samples were fractured at 115°C and shadowed with platinum-carbon in a Balzers 301 high-vacuum freeze-etch unit. Replicas were cleaned in Clorox containing 1% NaOH, washed in distilled water, and mounted on Formvar-coated 200-mesh grids. Micrographs were obtained using a Hitachi HU-11E electron microscope. No morphological differences were observed between fixed and unfixed cell samples. The micrographs included in the present report were obtained using unfixed cell preparations.

RESULTS

Effects of a high- to low-light transition. The growth rate and internal content of ICM-associated photopigments of phototrophically growing cells of *R. sphaeroides* are determined by the incident light intensity employed for growth (13). Cells adapted to steady-state, phototrophic growth under high-intensity light display rapid rates of growth and relatively reduced photopigment contents. Conversely, cells undergoing balanced growth under low-intensity light exhibit correspondingly reduced rates of growth and possess markedly elevated photopigment contents. Thus, the total cellular photopigment content and, presumably, the quantity of ICM possessed by *R. sphaeroides* are inversely related to the incident light intensity utilized for growth.

An abrupt high(500 ft-c) to low (50 ft-c) light transition of a high-light-adapted culture of *R. sphaeroides* results in a transient (1.5-h, approximately) cessation of culture growth (Fig. 1a) accompanied by a period of preferential synthesis of cellular photopigments (see Fig. 3c and d). The cells, having in this manner satisfied the increased cellular demand for photopigments, then resume growth at a rate commensurate with the posttransition light intensity (Fig. 1a).

The influence of a low-light transition upon the net accumulation of total cellular protein (Fig. 2b) and phospholipid (Fig. 2c) qualitatively mimics the effect observed upon culture turbidity (Fig. 1a). Thus, the net production of these cellular constituents is transiently inhibited immediately after the shift to low light, and the resumption of their formation is at a rate proportional to the increase in culture mass. In contrast, the posttransition changes in total cell mass indicated by the values for culture turbidity (Fig. 1a) do not simply reflect corresponding changes in total cell number. As is shown by the data presented in Fig. 1b and 2a, total cell number becomes fixed at its pretransition value for a period of 2.5 h after the shift to low light, whereupon it undergoes a sharp increase. Since this increase (38%, average) in total cell number occurs in the absence of a measurable increase in the net accumulation of culture DNA (Fig. 1c), the cumulative effect of these adaptive responses is a reduction in the average number of genome equivalents per cell. Importantly, this reduction in the average cellular content of DNA, presumably resulting from the partitioning of existing completed genomes to newly formed daughter cells, was interpreted as indicating that a cell population more aligned with respect to the cell division cycle had been generated.

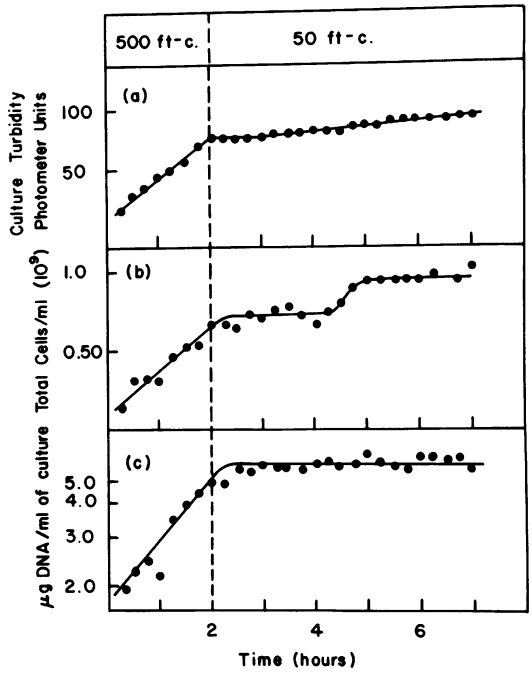

FIG. 1. *Response of a high-light-adapted, phototrophically growing culture of R. sphaeroides to an immediate low-light transition. Cells undergoing logarithmic-phase growth (8×10^8 cells per ml) at a high light intensity (500 ft-c) were immediately subjected to a transition to low levels of incident light (50 ft-c). (a) Culture turbidity; (b) total cells per milliliter; (c) micrograms of DNA per milliliter. Total cell number and DNA determinations were conducted as described in the text.*

Evaluation of light-mediated culture alignment. To determine the validity of the above proposal, the response of a putatively aligned cell population to the reinitiation of high-light conditions of growth was examined. The inherent assumption was that an aligned cell population should exhibit a discontinuous increase in total cell number when the individual cells are simultaneously exposed to conditions supporting rapid growth. For this study, a high (500 ft-c)-light-adapted, asynchronously dividing cell population was initially subjected to a low (50 ft-c)-light transition for a period (3.5 h) of sufficient duration to allow the presumed alignment process to be completed. Upon reexposure of this cell population to high-intensity light (Fig. 3), immediate and exponential increases in culture turbidity (Fig. 3a) and the net accumulations of the cellular photopigments bacteriochlorophyll (Fig. 3c) and carotenoids (Fig. 3d) were observed. In marked contrast, however, increases in total cell number (Fig. 3b)

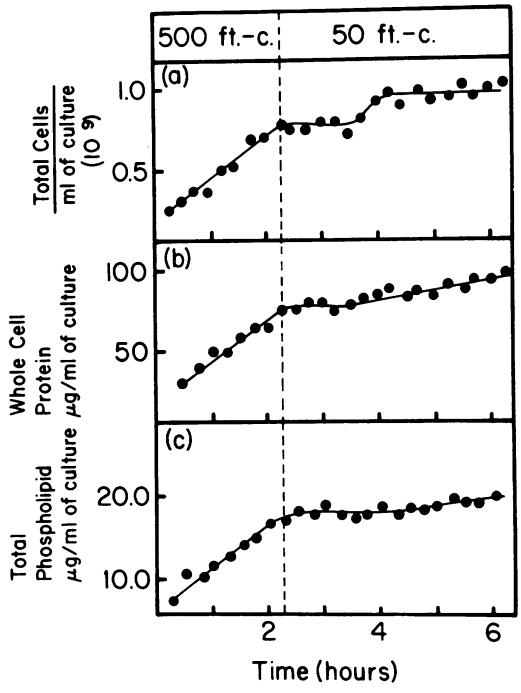

FIG. 2. *Response of a high-light-adapted culture of R. sphaeroides to an immediate low-light transition. Conditions were exactly as described in the legend to Fig. 1. (a) Total cells per milliliter; (b) whole cell protein; (c) micrograms of phospholipid per milliliter. The procedures employed for the measurements of whole cell protein and cellular phospholipids are described in the text.*

occurred discontinuously. These features have previously been shown to be characteristic of synchronously dividing cell populations of *R. sphaeroides* (18).

The efficacy of the procedure outlined above for producing synchronously dividing cell populations of *R. sphaeroides* is best evidenced by the results presented in Fig. 4. The cells utilized for this study were subjected to the same high-low-high light regimen as the cells employed to obtain the results shown in Fig. 3; however, in this instance the entire light-induced synchronization protocol was conducted with cells at an earlier stage of logarithmic growth. Also, culture samples for the determinations of total cell number, culture turbidity, and DNA were not removed until the completion of the light cycling regimen. These modifications allowed a more thorough evaluation of the degree of synchrony produced by the present procedure. In this regard, the results shown in Fig. 4 clearly demonstrate the efficacy of the present procedure for obtaining division and genomic synchrony in phototrophically growing cultures of *R. sphaeroides*. The division synchrony achieved by this technique is typically maintained for 2.5 cycles of cell division.

Utility for studies on ICM assembly. Previous studies on ICM assembly in division-synchronized cell populations of *R. sphaeroides* have shown that cell cycle-dependent fluctuations in the ICM protein-to-phospholipid ratio constitute a dominant feature of the assembly of this specialized membrane (11, 18). The data presented in Fig. 5 provide an independent confirmation of this observation and, further, demonstrate the utility of the light-induced synchronization procedure for studies on the mode and regulation of ICM assembly. The cells utilized for this study (Fig. 5) were synchronized during early logarithmic phase growth by the light cycling procedure described above. Purified ICM preparations were obtained from cells at representative stages of the cell division cycle by the procedures described in Materials and Methods. The magnitudes of the fluctuations in the ICM protein-to-phospholipid ratio shown in Fig. 5 are comparable to those previously reported for ICM preparations obtained from cells synchronized by the technique of stationary-phase cycling (18).

Mechanism of cell division. It is noteworthy that the abrupt 38% increase in total cell number occurring subsequent to the transition of a culture to low-intensity light was not accompanied by a measurable increase in the net accumulation of culture phospholipid (Fig. 2c). This observation suggests that either preexisting cellular phospholipids are being utilized for the production of a division septum, or, possibly, de novo septum formation per se is not a requisite for division by cells of *R. sphaeroides*. In this regard, qualitative evidence supporting the latter proposal is provided by the freeze-fracture electron micrographs presented in Fig. 6. These micrographs provide convincing evidence that cell division in *R. sphaeroides* occurs via a uniform constriction of its gram-negative envelope.

DISCUSSION

The influence of variations in incident light intensity upon the phototrophic growth of *R. sphaeroides* has been studied by a number of investigators (7, 13, 15, 22, 24). In most instances, however, these investigations have been restricted to examinations of the differential regulatory properties of light intensity upon the formation or assembly, or both, of ICM constituents. Consequently, little information is available concerning the effects of light intensity upon the more general aspects of the metabolism and growth of *R. sphaeroides*. Information that

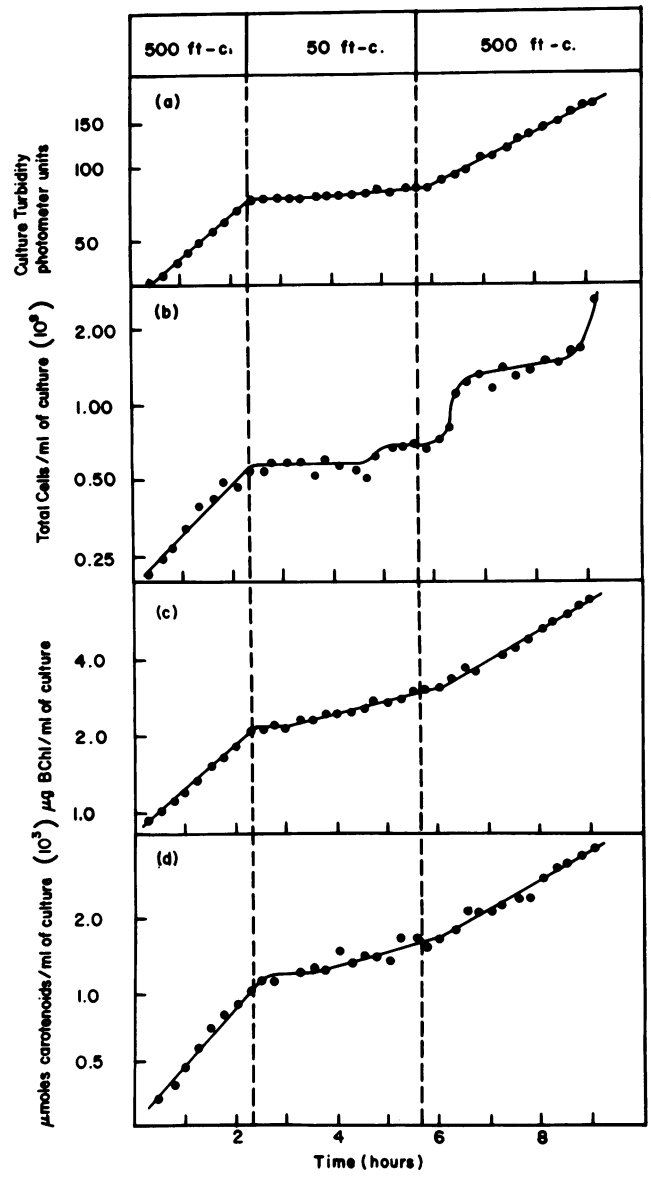

FIG. 3. *Effects of reinitiation of high-light growth upon a culture of R. sphaeroides previously subjected to a high- to low-light transition. Cells adapted to logarithmic-phase growth at high light (500 ft-c) were subjected to low levels of incident light (50 ft-c) for 3.5 h before being reexposed to high-intensity light (500 ft-c). (a) Culture turbidity; (b) total cells per milliliter; (c) micrograms of bacteriochlorophyll per milliliter; (d) micromoles of total carotenoids per milliliter. All measurements were conducted as described in the text. During the 3.5-h period before reinitiation of high-light growth, the specific cellular level of bacteriochlorophyll increased from 2.59 to 3.31 μg of bacteriochlorophyll per 100 μg of cellular protein.*

has appeared relating to this topic has recently been reviewed by Kaplan (13).

The molecular basis for the observed abrupt inhibition of total cellular protein accumulation is unknown. Similar results have been reported for the effect of a down-shift in light intensity upon the rates of accumulation of cellular RNA (9, 16) and DNA (16); however, the degree of inhibition of DNA accumulation observed in the present study was more pronounced than that previously reported (16).

To our knowledge, the effect of a low-light transition upon the production of phospholipids by cells of *R. sphaeroides* has not been previ-

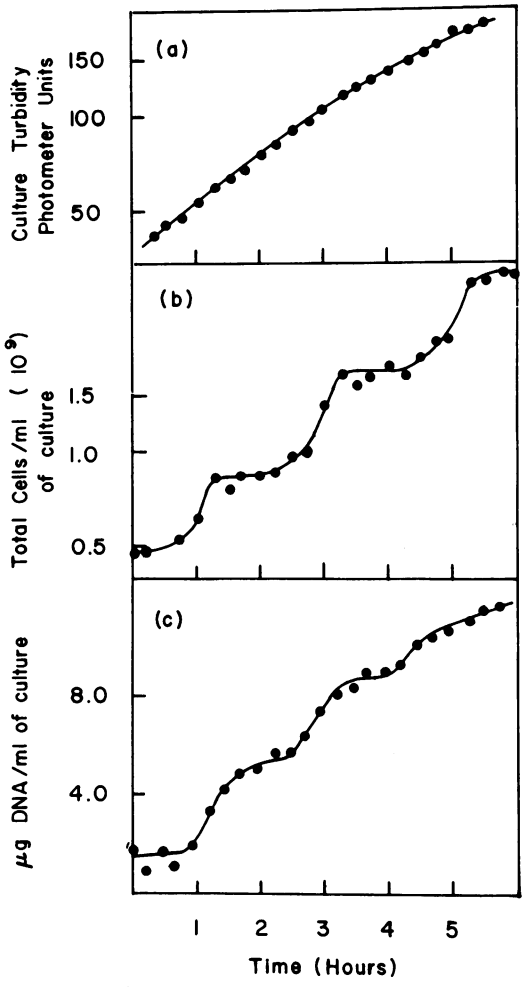

FIG. 4. *Efficacy of a high-low regimen of incident light for producing division and genomic synchrony in phototrophically growing cultures of R. sphaeroides. High-light-adapted cells in the early logarithmic stage of growth were initially subjected to a low-light transition (500 to 50 ft-c) for a period of 3.5 h. After this period, the cells were reexposed to high levels of incident light (500 ft-c), and samples were removed for the determination of culture growth characteristics. (a) Culture turbidity; (b) total cells per milliliter; (c) micrograms of DNA per milliliter. Determinations were conducted as described in the text.*

ously investigated. In this regard, our observation that a net increase in the accumulation of culture phospholipids did not occur during the period of preferential photopigment production is consistent with a proposal by Kaplan (13) and suggests that the adaptive response of *R. sphaeroides* to low-light growth is characterized, in part, by the enrichment of preexisting ICM with ICM-specific photopigments and their associated proteins.

At present, the molecular basis for the observed influence of a high- to low-light transition upon total cell number is unknown. However, the fact that the abrupt increase in total cell number 2.5 h after the low-light transition results in a cell population aligned with respect to the cell division cycle is well evidenced by the discontinuous increases in total cell number observed after the reinitiation of high-light conditions of growth. Furthermore, a specific requirement for this limited increase in total cell number for generating an aligned cell population has been demonstrated by studies in which the reexposure to high-intensity light was conducted during the period before this increase (unpublished data). These studies were unsuccessful in producing synchronously dividing cell populations.

The information presented on the mechanism of cell division by *R. sphaeroides* should significantly aid future interpretations of data concerning cell cycle-related events. In many instances, the question of septation, especially as regards phospholipid synthesis, is a pivotal issue for evaluations of this type. It should be emphasized that the apparent absence of septum formation per se during division is not considered a feature

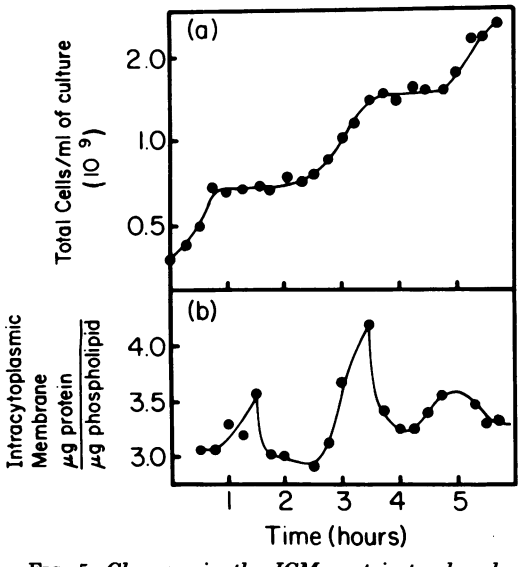

FIG. 5. *Changes in the ICM protein-to-phospholipid ratio in synchronously dividing cell populations of R. sphaeroides. Division synchrony was obtained by employing the regimen of high-low-high light cycling described in the legend to Fig. 4. Purified ICM preparations were obtained as described by Fraley et al. (10). Protein and phospholipid determinations were conducted as described in the text. (a) Total cells per milliliter; (b) ICM protein-to-phospholipid ratio.*

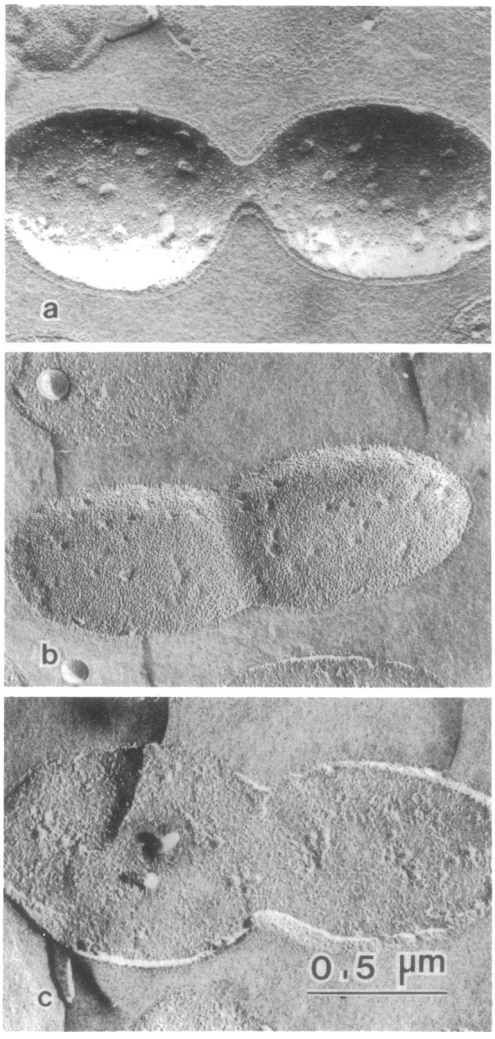

FIG. 6. *Freeze-fracture replicas of unfixed preparations of phototrophically growing cells of R. sphaeroides. (a) E-face view of cytoplasmic membrane showing uniform constriction of the cellular envelope of a cell undergoing division; (b) P-face view of the cytoplasmic membrane of a cell undergoing division; (c) cytoplasmic fracture through a cell at an early stage of division. Note that both membrane fracture faces (a and b) reveal an absence of discontinuities in the distribution of intramembraneous particles in the area of envelope constriction. Similarly, no evidence of septum formation was apparent in cytoplasmic fractures through the cellular region of envelope constriction. Samples were obtained during the period of abrupt increase in total cell number of a culture that had been subjected to a high- to low-light transition. Magnification, ×35,000.*

peculiar to the adaptive response of *R. sphaeroides* to low-light growth. Electron microscopic examination of freeze-fractured preparations of cells obtained from normal, phototrophically growing cultures also failed to reveal any evidence of septation in those cells undergoing division.

In addition to furthering existing knowledge of the adaptive response of cells of *R. sphaeroides* to variations in incident light intensity, the present study directly demonstrates how cellular changes resulting from an immediate high- to low-light transition can be effectively utilized to obtain cell populations exhibiting division and genomic synchrony. The described synchronization procedure obviates the problems associated with the technique of stationary-phase cycling (8) (see Introduction), and we, accordingly, prefer it for obtaining division-synchronized cell populations of *R. sphaeroides*. Although not shown in the present study, the described procedure is also directly applicable for obtaining division synchrony in *R. sphaeroides* 2.4.1.

The utility of the present procedure for studies on ICM assembly is clearly shown by the results presented in Fig. 5. These data confirm the results of previous investigations (11, 18) and, most importantly, show that the observed cell cycle-dependent fluctuations in the ICM protein-to-phospholipid ratio are independent of the method employed to obtain division-synchronized cell populations of *R. sphaeroides*.

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