Intracytoplasmic Membrane Synthesis in Synchronous Cell Populations of *Rhodopseudomonas sphaeroides*

**FATE OF "OLD" AND "NEW" MEMBRANE***

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A nonspecific density labeling technique has been employed to monitor the synthesis of intracytoplasmic membrane in synchronously dividing populations of *Rhodopseudomonas sphaeroides*. The intracytoplasmic membranes of cells synchronized in D$_2$O-based medium were found to undergo discontinuous decreases in specific density during synchronous cell growth following transfer to H$_2$O-based medium. These abrupt decreases in membrane specific density occurred immediately prior to cell division and were not observed with intracytoplasmic membranes prepared from asynchronously dividing cells (see also Kosakowski, H., and Kaplan, S. (1974) J. Bacteriol. 118, 1144-1157).

Discontinuous increases in the net accumulation of cellular phospholipid were also observed during the synchronous growth of *R. sphaeroides*. This is to be contrasted to the continuous insertion of protein and the photopigment components of the photosynthetic apparatus into the intracytoplasmic membrane during the cell division cycle (Fraley, R. T., Lueking, D. R., and Kaplan, S. (1978) J. Biol. Chem. 253, 458-464; Wraight, C. A., Lueking, D. R., Fraley, R. T., and Kaplan, S. (1978) J. Biol. Chem. 253, 465-471). Further, examination of the protein/phospholipid ratios of purified intracytoplasmic membrane preparations revealed that this ratio undergoes cyclical changes of 35 to 40% during a normal cycle of cell division.

In contrast to the results of Ferretti and Gray ((1968) J. Bacteriol. 95, 1400-1406), DNA synthesis was found to occur in a stepwise manner in synchronously dividing cell populations of *R. sphaeroides* (1-6). These studies are normally conducted with populations of organisms undergoing balanced, asynchronous growth and the results obtained are discussed in terms of regulation at the cellular level. Extrapolations of this type, although useful, are not necessarily definitive since culture growth must be viewed cautiously due to the wide distribution of cell ages in asynchronously dividing bacterial populations. The average age of a cell in an asynchronously growing culture is represented by a cell 47% of the way through its division cycle (7).

The use of synchronously dividing cell populations offers a unique approach to the study of membrane biogenesis in that molecular events can be temporally related to the parameters of cell age and growth. Several investigators have utilized division-synchronized bacterial populations for the study of membrane biogenesis (8-13). However, the preliminary and somewhat contradictory nature of these reports precludes any generalized statements concerning the relationship of membrane synthesis to the cell division cycle.

The present paper together with the accompanying communications (14, 15) describe the results of an initial characterization of intracytoplasmic membrane synthesis in synchronously dividing cell populations of the non-sulfur purple bacterium *Rhodopseudomonas sphaeroides*. The utility of employing *R. sphaeroides* for studies dealing with the mode and regulation of membrane biogenesis has been thoroughly described earlier by Kosokowski and Kaplan (16). These investigators reported an increased polydispersity in their intracytoplasmic membrane preparations following a transition from D$_2$O-based medium to H$_2$O-based medium and proposed several interpretations for these results. One proposal, tested in the present communication, considered the possibility that the formation of the intracytoplasmic membrane was temporally regulated during the cell division cycle. Finally, this organism has been shown to be amenable to division synchronization by a comparatively mild procedure (17, 18).

In the present study the density transfer technique of Kosakowski and Kaplan (16) has been employed to monitor intracytoplasmic membrane synthesis in synchronously dividing cultures of *R. sphaeroides*. The results of these experiments are discussed in relation to an observed discontinuous pattern of cellular phospholipid accumulation as well as cell cycle-specific changes in the protein/phospholipid ratio of purified intracytoplasmic membrane fractions.

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ExPERIMENTAL PROCEDURES

Organisms, Media, and Conditions of Growth—Rhodopseudomonas sphaeroides strain 2.4.1 (wild type) and strain M29-5 (leu-), derived from strain 2.4.7, were kindly supplied by W. R. Sistrom, University of Oregon. Both organisms were routinely grown in a succinic acid minimal medium (initial pH 7.0), containing, per liter: 2.0 g of succinic acid, 0.8 g of (NH₄)₂SO₄, 0.10 g of sodium glutamate, 0.04 g of aspartic acid, 1.0 g of nicotinic acid, 0.50 mg of thiamin/HC1, 0.010 mg of biotin, and additional inorganic salts as specified by Cohen-Bazire et al. (26). Media employed for the growth of strain M29-5 was additionally supplemented with 40 \( \mu \)g/ml each, of L-leucine and L-methionine.

Stock cultures were maintained at -20°C in the above medium adjusted to 3.0% (w/v) glycerol. Cells previously adapted to the logarithmic phase of growth were the inoculum source for studies with asynchronous cell populations. Inocula for studies with synchronous cell populations were adapted as described below. Inoculations were to 0.60 with continuous saturating illumination (600 foot-candles) provided by a bank of Lumiline lamps (Sylvania). Culture growth was followed turbidimetrically using a Klett-Summerson colorimeter equipped with a No. 66 filter. A value of 270 \( \mu \)g dry weight/ml corresponds to a culture turbidity of 100 photometer units.

Synchronization Procedures—Populations of synchronously dividing cells were obtained by the stationary phase cycling technique of Cutler and Evans (7). Cultures adapted to logarithmic (asynchronous) growth on the succinic acid minimal medium were allowed to complete 0.75 to 1.0 cell doublings following cessation of exponential growth. The cells (16 ml) of this first stationary phase cycling were then harvested by centrifugation and resuspended in 160 ml of the same medium. Having reached a cell density, as determined by cell turbidity, identical with that of the previous culture, cells (70 ml) of this second cycle were harvested by centrifugation and used to inoculate a culture containing 700 ml of medium. Cells of this latter inoculum were found to possess a high degree of division synchrony without further cycling being required.

Cell Enumeration—The presence and degree of division synchrony was determined by monitoring the change in cell count per milliliter using a Hauser counting chamber. Samples (1 ml) of the culture were removed at 15-min intervals and transferred to a tube containing 1 ml of 5% (w/v) formaldehyde. Samples were stored at 4°C prior to determinations and at least 600 to 900 cells were counted per determination.

Preparations of Cell-free Membrane Fractions—The determination of membrane densities was conducted employing both crude membrane preparations (16) and purified (>90%) intracytoplasmic membrane (25) fractions. Membrane preparations were obtained by centrifugation of the culture samples (6 ml) at 10,000 x g for 45 min. The culture filtrates were collected at the end of a 4-h incubation period and the pellets were resuspended in 16 ml of 5% (w/v) formaldehyde. Samples (1 ml) of the culture were removed at 15-min intervals and the cells were harvested by centrifugation. The pellets were resuspended in phosphate buffer (pH 7.0) by brief sonication (30 s) as described above.

Density Labeling and Analysis of Membrane Fractions—Stock cultures of R. sphaeroides strain M29-5 extensively adapted (100% synchronous growth on D.O. 80%)-based medium were synchronized by two stationary phase cyclings in D.O.-based succinic acid minimal medium. The cells were then harvested by centrifugation and resuspended in O.H-O-containing medium. Samples of this latter culture were collected at 15- to 30-min intervals for the determination of membrane densities. Each sample contained a total cellular mass equivalent to 3.0 x 10⁹ cells.

The densities of the intracytoplasmic membranes in the crude and purified membrane fractions were determined essentially as described by Kosakowski and Kaplan (16). Membrane fractions were centrifuged at 105,000 x g for 60 min. The supernatant was decanted, the pellets were resuspended in 16 ml of 5% (w/v) formaldehyde, and the pellets were resuspended in phosphate buffer (pH 7.0) by brief sonication (30 s) as described above.

RESULTS

Previous studies (17, 18) have demonstrated the applicability of the stationary phase-cycling technique of Cutler and Evans (7) for the induction of division synchrony in photoheterotrophically growing cultures of Rhodopseudomonas sphaeroides. By employing this technique, an adequate degree of division synchrony was reproducibly obtained following two successive stationary phase cyclings of photosynthetically growing cells. The results of a representative synchrony experiment are presented in Fig. 1. In agreement with Ferretti and Gray (15), exponential increases in total cellular mass (Fig. 1a) and cellular protein (Fig. 1c) were observed during the synchronous growth of R. sphaeroides. As is shown (Fig. 1), both cell mass and total cellular protein exhibited comparable rates of increase.

The enumeration of viable cells in cultures growing anaerobically in the light was precluded by the fluctuation in cell doublet formation accompanying the transition of cells from stationary phase to the logarithmic phase of growth. Thus, the presence and degree of division synchrony was determined by directly monitoring the total number of cells. However, the potentially subjective nature of direct cell-counting techniques warranted the verification of synchrony by an additional independent criterion. In this regard, increases in the amount of culture DNA were monitored in cultures of synchronously dividing cells. In contrast to the results of Ferretti and Gray (15), the net accumulation of DNA was found to be discontinuous in synchronously dividing populations of R. sphaeroides (Fig. 2). Furthermore, it is evident...
from the present results (Fig. 2) that culture DNA accumulation precedes cellular division. This observation supports the previous conclusion of Cutler and Evans (7) that the stationary phase-cycling technique is unique in inducing both division and genomic synchrony. At present, the reason for the discrepancy between the present results on DNA synthesis and those reported by Ferretti and Gray (17) is obscure.

Several workers (16, 26, 27) have employed density-labeling techniques to study the mode of synthesis and partitioning of bacterial membranes. A previous study from this laboratory (16) employed growth in deuterated medium to label the intracytoplasmic membrane system of \textit{R. sphaeroides}. It was shown that deuterated membranes (specific density 1.22 to 1.23 g/cm$^3$) were readily separable from intracytoplasmic membranes of normal density (1.175 to 1.180 g/cm$^3$) and that apparently no structural alterations of the membranes resulted from the presence of deuterium. This study, conducted with steady state, asynchronously dividing cell populations, demonstrated that, following transfer of cells from D$_2$O to H$_2$O-based medium, the median intracytoplasmic membrane density underwent a continuous decrease which was in direct accordance with a theoretically determined profile assuming random, homogeneous insertion of new membrane material (i.e. directly proportional to culture growth). With time, following the density transfer, the density of the intracytoplasmic membrane became indistinguishable from normal, “light” membrane, suggesting the orderly dilution of all components of the “old” membrane with “new” materials. The result of an identical experiment conducted with a synchronously dividing cell population is presented in Fig. 3. The cells were synchronized by two stationary phase cyclings in D$_2$O-based medium. They were then harvested by centrifugation, washed, and resuspended in H$_2$O-based medium and the specific density of the intracytoplasmic membranes determined during synchronous growth as described under “Experimental Procedures.” In contrast to the results previously observed with asynchronously growing cell populations (16), the median specific density of the intracytoplasmic mem-
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Membranes decreased in a discontinuous manner, ostensibly reflecting increases in total cell number rather than cell mass. During the initial 2.5 h of culture growth the median membrane specific density remained virtually constant (1.225 g/cm$^3$) (Fig. 3c), while total culture mass (turbidity) increased 1.8-fold (Fig. 3o). As is shown (Fig. 3c), membrane specific density abruptly decreased immediately prior to cell division to a value of 1.208 g/cm$^3$, which defined a second plateau lasting for 1.5 h and which also preceded a final, sharp decrease in membrane density. Essentially identical intracytoplasmic membrane density profiles were obtained from three separate experiments employing synchronously dividing cell populations. The accuracy with which the median intracytoplasmic membrane densities were determined in the present report is reflected by the membrane density profiles presented in Fig. 4. This figure shows the optical density profiles and CsCl gradient values from which the data in Fig. 8 were calculated.

The ability of the purple bacteria to adjust their membrane content in response to changes in light intensity, although experimentally advantageous, could influence the results of the above type of experiment. Since the synchronization procedure employed necessitates the use of inocula of high cell densities, decreases in the effective incident light intensity as a consequence of self-shading (28, 29) would tend to increase the total amount of cellular membrane. Membrane synthesis in such self-shaded cultures would then be expected to be temporarily inhibited due to the increase in effective light intensity.
intensity upon dilution of the culture at the start of the experiment. Thus, this latter situation would be roughly analogous to a low to high light shift (19). In order to determine the effect, if any, of self-shading on the initial plateau in membrane density observed above (Fig. 3c), the density shift experiment was repeated with cells that had undergone only one stationary phase cycling (Fig. 5). Although the inoculum for this experiment was at the same cell density utilized previously (Fig. 3), and, thus, exposed to the same extent of self-shading, an immediate and continuous decline in the intracytoplasmic membrane density was observed following transfer of the cells to H$_2$O-based medium (Fig. 5b). This observation strongly suggests that the influence of self-shading on the results of the density transfer experiments are negligible and, most importantly, shows (Fig. 5a) that two stationary phase cyclings are required to obtain division synchrony. The absence of a significant self-shading effect on the synthesis of intracytoplasmic membrane is also indicated by the patterns of bacteriochlorophyll, carotenoid, and cytochrome synthesis reported by Wraith et al. (15) and the observations on particulate protein synthesis reported by Friley et al. (14) in similarly adapted cultures. For purposes of comparison, the decrease in intracytoplasmic membrane density observed when asynchronously dividing cells are employed in the density shift experiment is shown in Fig. 9c.

At present, the mode of deuterium entry and the quantitative distribution of deuterium in the membrane system of *R. sphaeroides* is unknown. However, the highly reduced state of the acyl moieties of the membrane phospholipids suggests that a major portion of the increased density of membranes from cells grown in deuterated medium is attributable to these membrane components. Accordingly, the net accumulation of total cellular phospholipid was monitored during the synchronous growth of *R. sphaeroides*. Inspection of the differential plot (Fig. 6b) of micrograms of lipid phosphorus versus culture dry weight reveals the presence of two regions of discontinuity which coincide with the two periods of cell division (Fig. 6a) in a synchronously dividing culture. In contrast, the accumulation of phospholipid in asynchronously dividing cultures (Fig. 7) is clearly exponential (i.e. directly proportional to increases in cell mass). Importantly, throughout the range of growth studied, culture turbidity was directly proportional to total culture dry weight.

Since it is known (14) that the synthesis of particulate protein is continuous in synchronous cultures of *R. sphaeroides*, it would be predicted from the observed cell cycle-specific pattern of phospholipid synthesis (Fig. 6b) that the membrane protein/phospholipid ratio should steadily increase until, at a specific period in the division cycle, an increased accumulation of phospholipid into the intracytoplasmic membrane would restore the ratio to its original value prior to division. The validity of this assumption was determined by measuring the protein and phospholipid content of purified (>90%) intracytoplasmic membranes prepared from cells at representative stages of the cell division cycle. As predicted (Fig. 8b), the value (3.2) for the protein/phospholipid ratio of purified intracytoplasmic membranes from newly formed cells increased to a peak value (4.2) in membranes from cells poised for division and then decreased to a "basal" value (2.6) concomitant with cell division. The decrease in the intracytoplasmic membrane protein/phospholipid ratio (Fig. 8b) which occurs coincident with cell division (Fig. 8a) and the decrease in intracytoplasmic membrane density (Fig. 8c) is presumably a direct consequence of the observed increase in phospholipid accumulation occurring at (or immediately prior to) the time of cell division (Fig. 6b). The results of an identical experiment conducted with asynchronously dividing cells are presented in Fig. 9. As is shown, the protein/phospholipid ratio (Fig. 9b) of purified intracytoplasmic membranes from asynchronously dividing cells remains constant (3.7 average) throughout the course of the experiment, while the density of the purified membranes (Fig. 9c) decreases as a direct function of
brane; c, intracytoplasmic membrane specific density. Membrane densities were determined employing the method of Lowry (24). Membrane densities were determined employing the method of Lowry (24). Membrane densities were determined employing the method of Lowry (24).

The study of membrane biogenesis in synchronously dividing cell populations necessitates the adoption of a flexible and rapid method of culture synchronization that will accommodate sufficient cellular material for preparative analysis. In this regard, the stationary phase-cycling technique of Cutler and Evans (7) appears best suited for this type of research. Importantly, the feature which renders this technique most attractive is the absence of a requirement for the imposition of extreme physical or nutritional culture conditions to achieve synchrony. However, as with most induction synchrony procedures, the presence of subtle culture perturbations were indicated by moderate (15 to 20%) fluctuations in cell generation times.

As stated previously, the physiological mode of membrane deuteration and the quantitative distribution of membrane-associated deuterium is unknown. It is clear, however, that the loss or dilution of the major deuterated membrane component(s) is limited to that period immediately preceding cell division (Fig. 3c). Furthermore, in view of the increased accumulation of phospholipid (Fig. 6b) occurring coincidently, with the observed abrupt decreases in intracytoplasmic membrane specific density, it is tempting to speculate that these two phenomena are intimately related. Accordingly, the discontinuous decreases in membrane specific density observed in synchronously dividing cultures of R. sphaeroides are currently envisaged as representing a cell cycle-specific dilution of membrane-deuterated phospholipid with normally protonated phospholipid. Additional intracytoplasmic membrane constituents (i.e. protein, bacteriochlorophyll, carotenoids), although apparently contributing to overall membrane density, do not significantly alter the observed membrane density profiles, since it is known (14, 15) that these components are synthesized continuously in synchronously dividing cells of R. sphaeroides. The possibility does exist, however, that influence upon membrane density exhibited by these components are masked by the initial high density of the membranes from cells grown in deuterated medium. Membrane protein synthesis in the absence of net phospholipid accumulation would tend to increase the density of the membrane. This possibility is supported by the recent finding of McIntyre and Bell (1), that Escherichia coli membrane preparations with increased protein/phospholipid ratios display dramatic increases in membrane density.

To obviate problems associated with precursor labeling techniques the accumulation of phospholipid in the present study was followed by direct chemical analysis. Thus, the relative contributions of phospholipid synthesis and turnover to the observed discontinuous pattern of phospholipid accumulation are unknown. In E. coli, the synthesis of phospholipid presumably occurs continuously throughout the cell

**DISCUSSION**

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division cycle (10, 13) although contradictory results have been reported (9).

The data presented on membrane densities reflect only those density changes uniquely associated with the intracytoplasmic membranes of R. sphaeroides. No attempts were made to resolve the cytoplasmic or outer membrane fractions or to determine their densities. In contrast, the procedure employed for phospholipid analysis does not discriminate between the three membrane systems and, therefore, reflects the pattern of phospholipid accumulation at the whole cell level. Consequently, due to the high internal membrane content of photosynthetically growing R. sphaeroides (50 to 60% of total lipid phosphorus), alternate patterns of phospholipid synthesis specifically associated with the cytoplasmic or outer membrane fractions may have been obscured.

In a previous report (1), McIntyre and Bell found that, upon limitation of phospholipid synthesis by glycerol deprivation, the ratio of protein/phospholipid in the cytoplasmic and outer membrane fractions of E. coli increased 60%. As a result, they concluded that membrane protein and phospholipid synthesis were not tightly coupled and, also, that a cellular control mechanism exists which functions to maintain the membrane protein content below a saturating value. In the present study, the protein/phospholipid ratio of purified intracytoplasmic membranes of R. sphaeroides was shown to increase 35 to 40% during the cell division cycle. In agreement with McIntyre and Bell, this suggests that the synthesis of membrane protein and phospholipid are not tightly coupled, but, further, it strongly indicates that the observed changes in the membrane protein/phospholipid ratio is a normal feature of membrane assembly. Furthermore, this indicates that the increase in intracytoplasmic membrane polydispersity observed by Kosakowski and Kaplan (16) following a density transfer experiment employing asynchronous cultures of R. sphaeroides may reflect true compositional heterogeneity in the membrane vesicle population derived from these cells.

In view of the known effects of proteins on the physical properties of model membranes (30), it is intriguing to propose that the cell cycle specific changes in the protein/phospholipid ratio of the intracytoplasmic membranes of R. sphaeroides serve a regulatory function. Attempts to assign a physiological function to the observed membrane compositional changes are currently being initiated.

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