JOURNAL OF BACTERIOLOGY, Mar. 1981, p. 1154-1166
0021-9193/81/031154-13$02.00/0

Vol. 145, No. 3

In Vivo Intermembrane Transfer of Phospholipids in the Photosynthetic Bacterium *Rhodopseudomonas sphaeroides*

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Received 26 September 1980/Accepted 4 December 1980

The kinetics of accumulation of phospholipids into the intracytoplasmic membrane of *Rhodopseudomonas sphaeroides* have been examined. We have previously demonstrated that accumulation of phospholipids in the intracytoplasmic membrane is discontinuous with respect to the cell cycle. In this study we demonstrated a sevenfold increase in the rate of phospholipid incorporation into the intracytoplasmic membrane concurrent with the onset of cell division. Pulse-chase labeling studies revealed that the increase in the rate of phospholipid accumulation into the intracytoplasmic membrane results from the transfer of phospholipid from a site other than the intracytoplasmic membrane, and that the transfer of phospholipid, rather than synthesis of phospholipid, is most likely subject to cell cycle-specific regulation. The rates of synthesis of the individual phospholipid species (phosphatidylethanolamine, phosphatidyglycerol, and an unknown phospholipid) remained constant with respect to one another throughout the cell cycle. Similarly, each of these phospholipid species appeared to be transferred simultaneously to the intracytoplasmic membrane. We also present preliminary kinetic evidence which suggested that phosphatidylethanolamine may be converted to phosphatidycholine within the intracytoplasmic membrane.

The fluid mosaic model of Singer and Nicholson (54) proposes that a phospholipid bilayer constitutes the matrix of biological membrane structure and membrane-associated proteins are considered to be either integral or peripheral with respect to the phospholipid matrix. Membrane phospholipids are known to be involved with the numerous functional and structural activities (23, 28, 37, 38, 44). The physical properties of the bilayer, such as lateral phase separation (20, 30), lateral diffusion (52, 55), and transmembrane asymmetry (48, 51) have been described.

With this developing view of biological membrane function and architecture, progress has been made towards an understanding of the roles of phospholipids in membrane biogenesis in eucaryotic systems. Nozawa and Thompson (40–42), working with *Tetrahymena pyriformis*, demonstrated the transfer of radioactively labeled phospholipids from their site of synthesis, the endomembranes, to the cell surface membranes. Recently, Eggens et al. (10) reported the transfer of labeled phospholipids from the endoplasmic reticulum to the mitochondria in rat hepatic tissue. There are also numerous examples of ultrastructural studies which imply membrane transfer from the endoplasmic reticulum, through the Golgi (26) apparatus, to other cellular organelles and the plasmalemma (for a review, see 36). These investigations and others have led to the proposal of a dynamic membrane flow hypothesis (26) which calls for the interconversion of membrane, including the phospholipid bilayer, between different cellular membrane systems and organelles. In procaryotes, phenomena of the kind described for eucaryotes are more difficult to demonstrate due to a general inability to localize the site(s) of phospholipid synthesis and assembly, beyond assignment of these activities to the cell envelope in general and to the cytoplasmic membrane in particular (9).

In contrast to earlier studies (2, 5, 43), recent reports on the rate of incorporation of radioactive precursors into phospholipids indicate that synchronous cultures of *Escherichia coli* accumulate phospholipids in a stepwise manner (22, 45). However, these data remain inconclusive. Our lack of understanding of cell membrane phospholipid synthesis is further apparent when consideration is given to the mechanism of phospholipid transport to the outer membrane (27) as well as the asymmetric incorporation of phospholipids into the cytoplasmic membrane (49).

Previous investigations in this laboratory using steady-state synchronously dividing popula-

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tions of *Rhodopseudomonas sphaeroides* revealed the following cell cycle-related changes in the inducible intracytoplasmic membrane (ICM): (i) a stepwise decrease in ICM specific density after a shift from D_2O- to H_2O-based media, (ii) cyclical oscillations of the ICM protein-to-phospholipid ratio, (iii) cyclical oscillations of the intrinsic specific density of the ICM, and (iv) cyclical oscillations of the fluorescence polarization of α-parinaric acid used as an intrinsic probe of ICM fluidity (14, 15, 34). The protein-to-phospholipid ratio, intrinsic density, and α-parinaric acid fluorescence polarization of isolated ICM (chromatophores) increased throughout the cell cycle until the time of cell division, and then each parameter abruptly returned to its starting level. The process was repeated during the ensuing cell division cycle. Kosakowski and Kaplan (31) had previously demonstrated by combining the density shift technique with a shift from [^{3}H]acetate to [^{14}C]acetate in asynchronously dividing cultures that neither "new" nor "old" ICM phospholipid regions are conserved. These data coupled with the continuous accumulation of protein (13) and photopigments (61) into the ICM of synchronously growing cells of *R. sphaeroides*, together with essentially no phospholipid turnover (14), strongly support the conclusions of Lueking et al., who proposed that phospholipids are only accumulated into the ICM at the time of cell division (34).

In the present communication we investigated cellular phospholipid synthesis and demonstrated the apparent cell cycle-specific transfer of phosphatidylethanolamine, phosphatidylglycerol, and an unknown phospholipid into the ICM of *R. sphaeroides*. We further revealed that the kinetics of synthesis of phospholipid species are identical. Phosphatidylcholine synthesis may take place at more than one site within the cell. We also showed that the intermembrane transfer phenomenon does not display specificity for the individual phospholipid species.

(A preliminary report of this work was presented, Abstr. Annu. Meet. Am. Soc. Microbiol., 1980; K216, p. 219.)

MATERIALS AND METHODS

Organism, media, and growth conditions. *R. sphaeroides* M29-5 (Leu$^-$ Met$^-$) was grown photoheterotrophically as described by Leuking et al. (34). For phospholipid-labeling experiments, a low-phosphate medium was employed in which the phosphate concentration was reduced to 2 mM from 20 mM and 18 mM Tris was included. No alteration in growth rate was apparent on the low-phosphate medium.

Cell synchronization. Synchronously dividing cell populations were obtained using the stationary-phase cycling technique of Cutler and Evans (7) as adapted to *R. sphaeroides* by Lueking et al. (34). For radioactive labeling experiments (see below), Sistrom medium A was used during the synchronization procedure followed by suspension of the cells to a density of approximately 30 Klett units in low-phosphate medium. Before sampling, the cells were allowed to grow for 1 h in the low-phosphate medium. Asynchronously growing cells were treated similarly, but were never allowed to achieve stationary phase. Pulse-chase labeling studies were facilitated by employing the low-phosphate medium throughout the synchronization procedure.

Radioactive labeling of phospholipids. The method of [^{32}P]orthophosphoric acid labeling of phospholipids was as described by Fraley et al. (14). Upon conclusion of the labeling period (usually 15 min), samples were taken from the subcultures for whole cell phospholipid determination, quantitative resolution of whole cell phospholipid species, and chromatophore preparation (20 ml of culture, see below).

Radioactive pulse-chase labeling of phospholipids. One liter of low-phosphate medium was inoculated to 40 Klett units with either asynchronously or synchronously dividing cells. [^{32}P]orthophosphoric acid (25 μCi/ml final concentration) was added to either an asynchronous cell culture when the turbidity reached 55 Klett units or to a synchronous cell culture just as the cells entered interphase. A 5 M phosphate-chase solution (K^+:Na^+, 2.4:1.0, pH 7.0) was added after 15 min to increase the phosphate concentration in the medium 200-fold to a final level of 400 mM. Samples were removed at 10-min intervals commencing 5 min after addition of the phosphate-chase solution. Culture samples were either extracted for bulk phospholipid determination or for resolution and quantitation of the individual phospholipid species from both whole cells and chromatophores after breakage and isolation. Control experiments demonstrated that the high phosphate level had no effect upon cell growth.

Quantitative chromatophore preparation. Isolation of chromatophores was essentially as described by Fraley et al. (13). To obtain accurate values for labeled phospholipids expressed in terms of counts per minute per milliliter of culture, each 20-ml culture sample was independently monitored for chromatophore recovery during preparation. Cells were harvested by centrifugation (12,000 $\times$ *g*, 10 min) and stored frozen until all samples were collected. After suspension of the cell sample in 2 ml of buffer (0.1 M NaH_2PO_4-5 mM EDTA [pH 7.0], used throughout), duplicate "prebreakage" volumes were drawn and extracted for bulk phospholipid determination. Cells were disrupted on ice by sonication (Branson W-350, 70% efficiency, 40% duty cycle, 7.5 min). Each sample was treated with crude DNase (5 μg/ml, room temperature, 1 h) and clarified by centrifugation (12,000 $\times$ *g*, 10 min). The crude membrane fractions were pelleted by centrifugation (80,000 $\times$ *g*, 2 h) from the clarified supernant using a Spinco Type 40 rotor. The membranes were suspended in a measured volume (approximately 0.4 ml) of buffer before taking "postbreakage" volumes for bulk phospholipid determination. Internal marker chromatophores containing approximately 5 $\times$ 10^4 cpm of [^{3}H]leucine, prepared from *R.*

sphaeroides M29-5 grown for eight generations on Sistrom medium A containing 1 μCi of [^{3}H]leucine per ml, were added to each of the crude membrane fractions. "Pregradient" aliquots were removed, and the level of ^{3}H was determined in a Triton-toluene-based scintillant (13). The membrane fractions were applied to discontinuous (20:40:60, wt/vol) sucrose gradients and centrifuged in a Beckman SW 50.1 rotor (75,000 × *g*, 12 h). The ICM recovered from the 20:40 interface was washed with buffer (as above) and pelleted by centrifugation (80,000 × *g*, 2 h). After suspension in 0.5 ml of buffer, "postgradient" volumes were taken and ^{3}H was determined. The remainder of the chromatophores were extracted for bulk phospholipid determination as well as resolution of the individual phospholipid species (see below). Unless otherwise stated all steps were performed at 4°C.

The ratios of postbreakage ^{32}P-phospholipid to prebreakage ^{32}P-phospholipid and postgradient ^{3}H-chromatophores to pregradient ^{3}H-chromatophores generate values for the fraction of cells broken (average of 50%) and fraction of chromatophores recovered from the sucrose gradients (average of 65%), respectively. Application of the fractional values for cell breakage and chromatophore recovery together with accurate volume determinations for these fractions yields a true value for the amount of ^{32}P incorporated into the phospholipid of the chromatophore fraction. This value was routinely between 30 and 35% of the total cellular phospholipid during radioactive labeling experiments using asynchronously dividing cells.

Phospholipid extraction and radioactivity determination. Phospholipid extractions of both whole cells and chromatophores were conducted by the method of Bligh and Dyer as described by Ames (1). However, carrier cells (20 μg of phospholipid per ml of $CHCl_3$, final concentration) were included, and the extracts were washed with 3 ml of 1% NaCl. For all bulk ^{32}P-phospholipid determinations, the extracted phospholipids were dried at 60°C in scintillation vials. Radioactivity was measured in a toluene-based scintillant (13).

Individual phospholipid species were resolved using the two-dimensional thin-layer chromatography system of Poorthius et al. (46). Phospholipids were detected by iodine vapor staining or autoradiography. The phospholipids were scraped from the thin-layer chromatography plates directly into scintillation vials. Radioactivity was determined as before.

Materials. [^{32}P]orthophosphoric acid and [^{3}H]leucine were purchased from New England Nuclear Corp., Boston, Mass. DNase was obtained from Worthington Diagnostics, Freehold, N.J. Silca Gel G Type 60 was purchased from Brinkmann Instruments, Inc., Westbury, N.Y. All solvents and chemicals were of reagent grade and were used without further purification.

RESULTS

Rate of ICM phospholipid incorporation. From studies previously cited we have concluded that in *R. sphaeroides* undergoing steady-state photoheterotrophic growth, replication of the ICM phospholipid bilayer can be accounted for by a rapid cell cycle-specific accumulation of phospholipid into the ICM at the time of cell division as opposed to continuous phospholipid incorporation into the ICM. Therefore, it was essential to define the kinetic parameters governing the cell cycle-specific incorporation of phospholipid into the ICM. The kinetics of cellular phospholipid synthesis and ICM phospholipid incorporation were studied in pulse-labeling experiments employing asynchronously and synchronously dividing cell populations. As described in Materials and Methods, 22-ml subcultures were removed at 20-min intervals from a 1-liter master culture and labeled for 15 min with [^{32}P]orthophosphoric acid.

Employing an asynchronous culture of *R. sphaeroides*, the increase in the rate of cellular phospholipid synthesis as well as incorporation of phospholipids into the ICM paralleled growth (Fig. 1). The fraction of the ^{32}P-labeled phospholipid found in the chromatophores was 0.31 ± 0.06 of the total labeled cellular phospholipid.

The pattern of whole cell phospholipid synthesis as well as ICM phospholipid incorporation for synchronously dividing cell cultures of *R. sphaeroides* differed substantially from that observed in asynchronous cultures. In agreement with Fraley et al. (14), the rate of cellular phospholipid synthesis increased exponentially throughout interphase, but became constant with the onset of cell division (Fig. 2c). Lueking et al. (34) also reported a lag in the net accumulation of cellular phospholipid at the time of cell division. In contrast to the stepwise increase in the rate of cellular phospholipid synthesis, the rate of phospholipid incorporation into the ICM oscillated in a cell cycle-specific manner (Fig. 2d). The rate of ICM phospholipid incorporation rose dramatically, with 0.7 ± 0.1 of the total cellular phospholipid label incorporated during the 15 min coincident with the onset of cell division becoming resident within the chromatophore membrane fraction; in late interphase, as little as 0.1 ± 0.1 of the total cellular phospholipid label was incorporated into the chromatophores. After accounting for a degree of asynchrony in the culture, we conclude that virtually all ICM phospholipid accumulation occurs at or near the time of cell division.

Intracellular transfer of phospholipid. Measurement of the rate of ICM phospholipid accumulation represented direct confirmation of the discontinuous incorporation of phospholipid into the ICM, but did not provide evidence regarding the mechanism responsible for this accumulation. However, analysis of the data presented in Fig. 2d suggested an experimental approach to distinguish between the accumulation of phospholipid into the ICM resulting from either cell cycle-specific regulation of phospho-

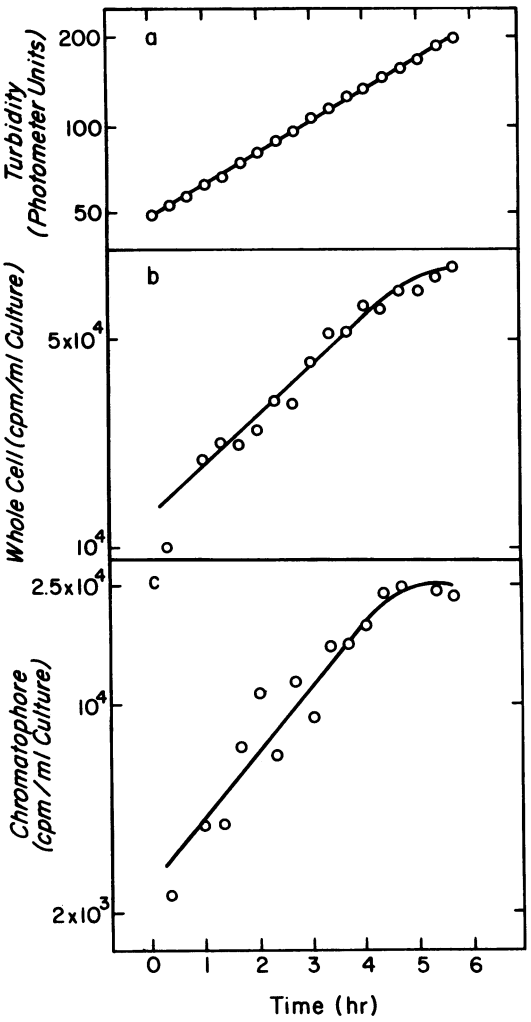

FIG. 1. *Rates of cellular phospholipid synthesis and intracytoplasmic membrane phospholipid accumulation in an asynchronously dividing culture of R. sphaeroides. At each point cells were radioactively labeled for 15 min and chromatophores were prepared as described in the text. (a) Culture turbidity; (b) total cellular phospholipid; (c) total chromatophore phospholipid. 100 Klett units = 0.2 optical density = 1 × 10⁹ cells per ml.*

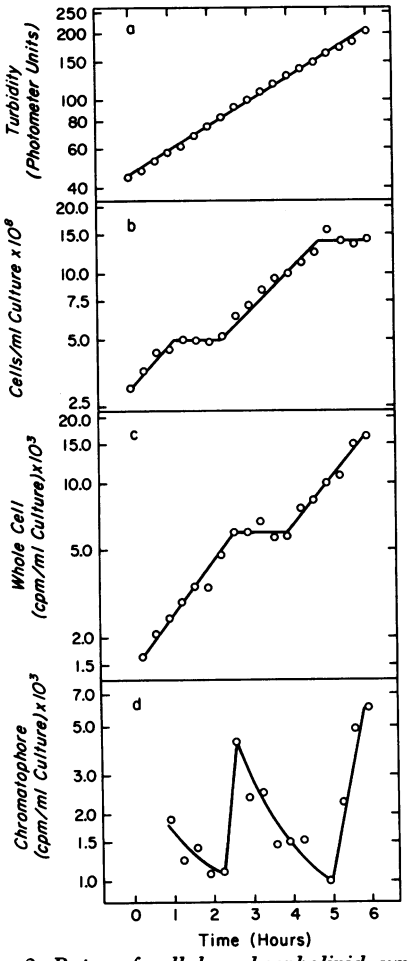

FIG. 2. *Rates of cellular phospholipid synthesis and intracytoplasmic membrane phospholipid accumulation in a synchronously dividing culture of R. sphaeroides. At each point cells were radioactively labeled for 15 min and chromatophores were prepared as described in the text. (a) Culture turbidity; (b) total cells per milliliter of culture; (c) total cellular phospholipid; (d) total chromatophore phospholipid.*

lipid synthesis in the ICM or cell cycle-specific transfer of phospholipid synthesized elsewhere throughout the division cycle. Application of a pulse of ^{32}P followed by an unlabeled phosphate-chase solution, to dilute the pulse of radioactivity, to a synchronously dividing culture just after a period of phospholipid accumulation would result in a low but constant level of labeled phospholipid in the chromatophore membrane fraction for the remainder of the division cycle. If phospholipid synthesis associated within the ICM was being regulated, then the level of labeled phospholipid in the chromatophore membrane fraction would continue to remain low during the following cell cycle, since phospholipids are not accumulated into the ICM until a subsequent round of cell division. However, if the transfer of phospholipids previously synthesized and stored elsewhere in the cell was being regulated, then the level of labeled phospholipid in the chromatophores would rise abruptly concomitant with cell division, since some of the phospholipids incorporated into the ICM during the subsequent round of cell division would have been synthesized during the earlier labeling pe-

riod. To test these alternatives we performed pulse-chase experiments as described (see Materials and Methods).

In an asynchronously dividing control culture after a pulse of [^{32}P]orthophosphoric acid, whole cell phospholipid label reached a plateau within 30 min after addition of the chase solution and rose only gradually thereafter (Fig. 3b). The total phospholipid label in the chromatophore fraction remained essentially constant at 4.26 × 10^3 cpm/ml of culture ± 14%, from the outset of and throughout the experiment (Fig. 3c). Importantly, no perturbation in culture growth was observed during the experiment (Fig. 3a).

In synchronously dividing cells, the ^{32}P label was added as the cells entered interphase (Fig. 4b), at which time the rate of ICM phospholipid accumulation was approaching the low point in its oscillation (i.e., immediately after completion of the accumulation of phospholipid into the ICM, see Fig. 2d). As was the case for the asynchronous culture, 60% of the post-chase phospholipid label into whole cells was completed within 30 min after addition of the chase and rose only gradually for the next 120 min (Fig. 4c). In the chromatophore fractions, the total phospholipid label remained constant from the first sample until cell division; concurrent with division, the label in the chromatophore fractions abruptly increased and established a new steady value (Fig. 4d). Before cell division, 5.3 × 10^3 cpm/ml of culture ±16% (95% confidence limits) was in the chromatophore membrane fraction; in contrast, immediately after cell division, 9.0 × 10^3 cpm/ml of culture ±11% (95% confidence limits) was in the chromatophores. Therefore, approximately 3.7 × 10^3 cpm/ml of culture was transferred into the ICM. Since we have shown by quantitative chromatophore preparation that 30 to 35% of the cellular phospholipid of *R. sphaeroides* is recovered in the chromatophore membrane fraction, we were able to calculate an expected value for the amount of labeled phospholipid transferred to the ICM from the data presented in Fig. 3. These calculations generated a range of 3.7 × 10^3 to 4.3 × 10^3 cpm/ml of culture for the expected amount of labeled phospholipid transferred. Clearly the value obtained experimentally and the calculated values are in agreement. Further, if the data depicted in Fig. 4c and d are expressed as a ratio of ^{32}P in the chromatophore phospholipid to total cellular phospholipid ^{32}P, a line with a negative slope is generated throughout the interphase period followed by a dramatic discontinuity coincident with cell division (Fig. 4e). This dramatically illustrates that no label entered the ICM during interphase despite a measurable amount of ^{32}P leakage from the cellular phosphate pools into phospholipid. This is in agreement with previous findings (13–15, 34) as well as other results reported here (Fig. 2d and see 6b).

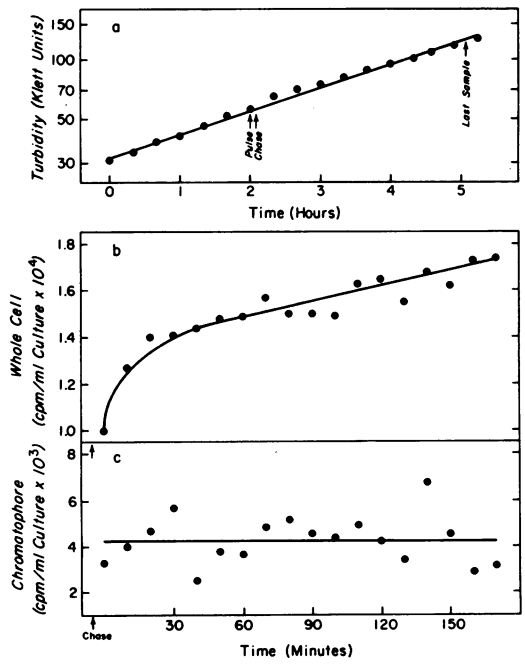

FIG. 3. *Kinetics of pulse-chase [^{32}P]orthophosphoric acid labeling of an asynchronously dividing culture of R. sphaeroides. Radioactive pulse labeling, application of the unlabeled chase solution, and chromatophore preparation were as described in the text. Arrows indicate addition of the chase solution. (a) Culture turbidity; (b) total cellular phospholipid label; (c) total chromatophore phospholipid label.*

In view of the results reported above and the previous demonstration that phospholipid turnover in *R. sphaeroides* is negligible (14), we could only conclude that phospholipids are synthesized at a site other than the ICM and are then transferred to the ICM in response to some form of cell cycle-specific regulation. The fact that there is leakage of the label into whole cell phospholipid after the chase does not alter the basic interpretation of the experimental results since none of the new phospholipid enters the ICM during interphase (13–15, 34; Fig. 2d and see 6b; also see Discussion).

Kinetics of synthesis of phospholipid species. The demonstration of transfer of bulk phospholipid from one cellular location to another raised the question as to the coincidence of transfer of the individual phospholipid species. Perhaps noncoordinate synthesis or accumulation of the different phospholipid species at the whole cell level or ICM, respectively, would

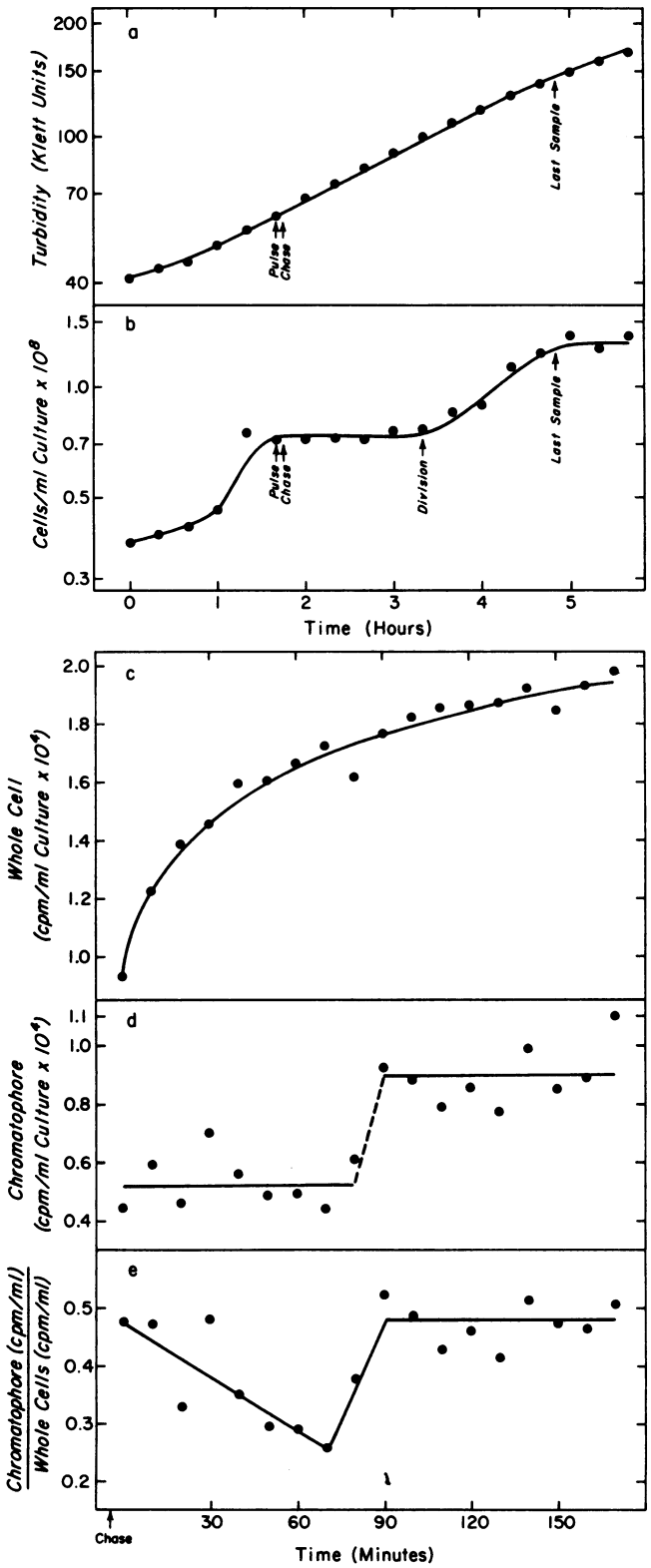

FIG. 4. *Kinetics of pulse-chase [^{32}P]orthophosphoric acid labeling of a synchronously dividing culture of R. sphaeroides. Radioactive pulse labeling, application of the unlabeled chase solution, and chromatophore preparation were as described in the text. Arrows indicate addition of the chase solution. (a) Culture turbidity; (b) total cells per milliliter of culture; (c) total cellular phospholipid label; (d) total chromatophore phospholipid label; (e) ratio of total chromatophore phospholipid label to total cellular phospholipid label.*

provide information regarding the regulation of the movement of bulk phospholipids. For example, a specific phospholipid species could be involved in the regulation of a specific cellular activity involved in the transfer of phospholipids to the ICM. Precedent for such a mechanism was established by Takai et al. (56), who observed changes in protein kinase activity in response to individual phospholipid species. Early investigations had shown that phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and trace quantities of cardiolipin (CL) constitute the major phospholipids of *R. sphaeroides* (19, 25, 32, 60; for a review see 29; B. W. Nichols and A. T. James, Biochem. J. **94**:22p). Recently, Russell and Harwood (50) reported that PG is enriched in the chromatophore membrane fraction when compared with PG distribution in whole cells. These data further stimulated our interest in investigating the specificity of phospholipid transfer. Therefore, we determined the rates of cellular synthesis and ICM accumulation of the individual phospholipid species as described above for bulk phospholipid (Fig. 1 and 2). The individual phospholipids were resolved by thin-layer chromatography.

Figure 5 depicts the results obtained for an asynchronously growing culture of *R. sphaeroides*. In addition to revealing the rates of synthesis of the individual phospholipid species in exponentially growing cells, these results demonstrated the reproducibility of the methods. The rates of synthesis of the cellular phospholipid species as well as the rates of phospholipid species accumulation into the ICM showed no significant changes from one to another as growth proceeds (i.e., the percent of the total ^{32}P label in each phospholipid did not change significantly throughout the experiment). Ninety-five percent of the total phospholipid ^{32}P label was routinely recovered in PE, PG and P(x), an unidentified lipid which accumulates in *R. sphaeroides* M29-5. In agreement with Lascelles and Szilagyi (32), PC was found to label at a very low rate.

Employing a synchronously dividing culture of *R. sphaeroides*, the kinetics of synthesis of the individual phospholipid species at the whole cell level (Fig. 6a) revealed no significant differences when compared to bulk phospholipid synthesis (Fig. 2c). The rates of accumulation of the individual phospholipids (Fig. 6b) into the ICM also reflected the pattern observed for bulk phospholipid accumulation into the ICM (Fig. 2d). Therefore, we concluded that there is no apparent cell cycle-specific selectivity in either synthesis at the whole cell level or transfer to the ICM of PE, PG and P(x) in *R. sphaeroides*.

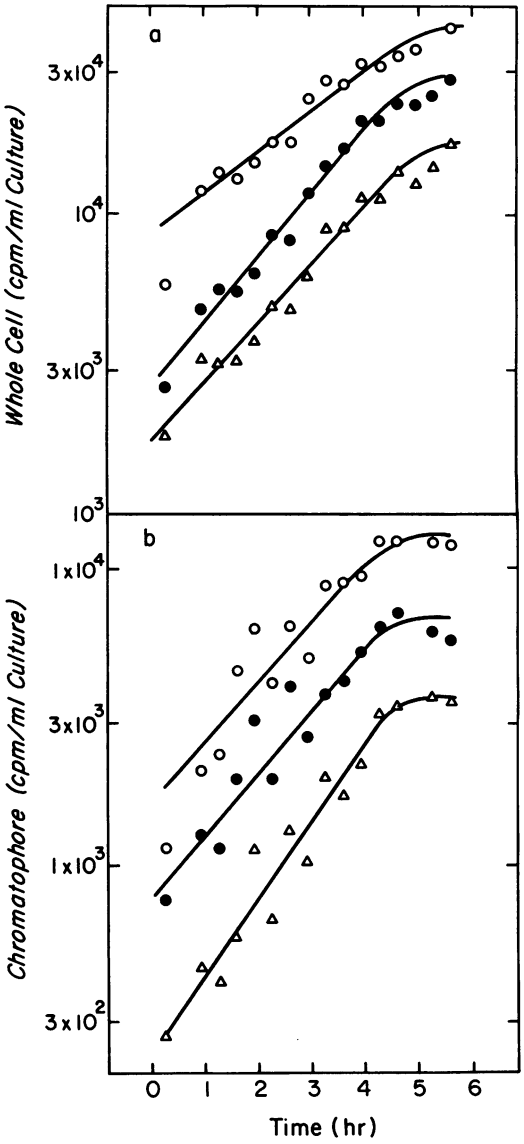

FIG. 5. *Rates of cellular synthesis and intracytoplasmic membrane accumulation of the individual phospholipid species in an asynchronously dividing culture of R. sphaeroides. Radioactive labeling, chromatophore preparation, and resolution of phospholipid species were as described in the text. (a) Whole cell phospholipid species; (b) chromatophore phospholipid species. Symbols: ○, phosphatidylethanolamine; ●, phosphatidylglycerol; △, unidentified phospholipid.*

Fate of phospholipid species. As stated above, PC could not be labeled to any significant extent during a 15-min exposure to [^{32}P]orthophosphoric acid, although PC constitutes at least 10 to 15% of the phospholipid from whole

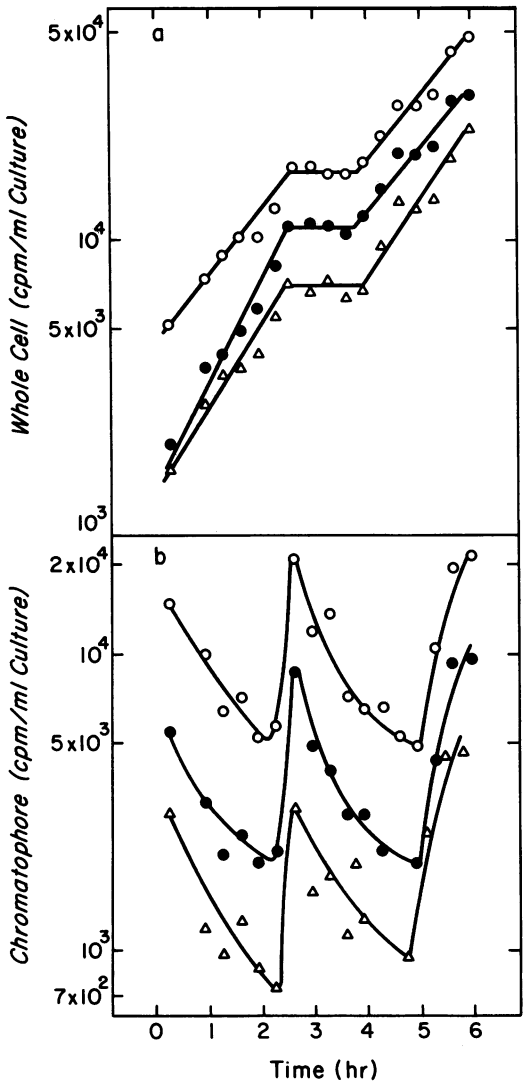

FIG. 6. *Rates of cellular synthesis and intracytoplasmic membrane accumulation of the individual phospholipid species in a synchronously dividing culture of R. sphaeroides. Radioactive labeling, chromatophore preparation, and resolution of phospholipid species were as described in the text. (a) Whole cell phospholipid species; (b) chromatophore phospholipid species. Symbols: ○, phosphatidylethanolamine; ●, phosphatidylglycerol; △, unidentified phospholipid.*

cells and chromatophores. In view of the slow kinetics of PC ^{32}P-labeling and the presence of P(x), we felt compelled to follow the extent of labeling of individual phospholipid species with time as well as the flow of label into the various phospholipid species. Since we demonstrated that all phospholipid species behave identically with regard to their synthesis and transfer during synchronous and asynchronous growth, only asynchronous cultures were employed for studies.

To follow the time course of labeling, a series of subcultures of *R. sphaeroides* was labeled with ^{32}P for increasing lengths of time and the fraction of the total phospholipid label present for each of the species of phospholipids was determined (Fig. 7). The fractions of ^{32}P in both PG and P(x) attained their steady-state values within 15 min. The fraction of PE remained constant throughout most of the experiment, declining somewhat at later times as the fraction of label in PC became significant. The appearance of label in PC was apparently at the expense of PE, as might be expected in view of PC metabolism (29).

A pulse-chase experiment was performed as described in Materials and Methods, except that culture samples were removed at various time intervals after the chase. The fraction of the

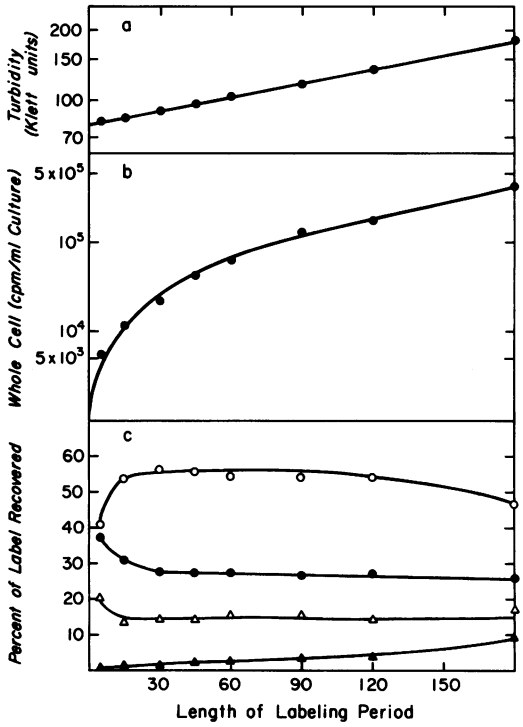

FIG. 7. *Effect of increasing time exposure to label on the fractions of label in the phospholipid species of R. sphaeroides. Radioactive labeling and resolution of the individual phospholipid species were as described in the text. (a) Culture turbidity; (b) total cellular phospholipid label; (c) percent of cellular phospholipid label in individual phospholipid species. Symbols: ○, phosphatidylethanolamine; ▲, phosphatidylcholine; ●, phosphatidylglycerol; △, unidentified phospholipid.*

total phospholipid label was determined for each of the individual phospholipid species in whole cells and chromatophores (Fig. 8). Within 30 min after the chase, the fraction of label in PG and P(x) had stabilized in both whole cells and chromatophores. In contrast, the fraction of ^{32}P in PE and PC continued to change well beyond 30 min. In whole cells, the fraction of PE decreased for 90 min after the chase and the fraction of ^{32}P in PC increased in a reciprocal manner. In the ICM, the decrease in the fraction of PE continued throughout the experiment (180 min) whereas the fraction of PC increased steadily.

We interpret these data to mean that the conversion of PE to PC can occur in the ICM, although not exclusively within the ICM. On the other hand, the synthesis of PG, P(x), and PE appeared not to take place in the ICM, and accumulation of these phospholipids into the ICM was the result of transfer described earlier.

DISCUSSION

This work constituted a partial characterization of cellular phospholipid synthesis and accumulation of phospholipid into the ICM of *R. sphaeroides*. In synchronously dividing cells, the rate of cellular phospholipid synthesis increased in a stepwise manner. The plateau in the rate of whole cell phospholipid synthesis occurred simultaneously with cell division. In marked contrast, the rate of incorporation of phospholipid into the ICM oscillated with respect to the cell cycle. The rate of ICM accumulation of phospholipid peaked coincident with the onset of cell division. During interphase, when the rate of cellular phospholipid synthesis was increasing exponentially, the rate of ICM accumulation of phospholipid was essentially nonexistent. Therefore, we concluded that, in contrast to accumulation of phospholipid at the whole cell level by synthesis, accumulation of phospholipid into the

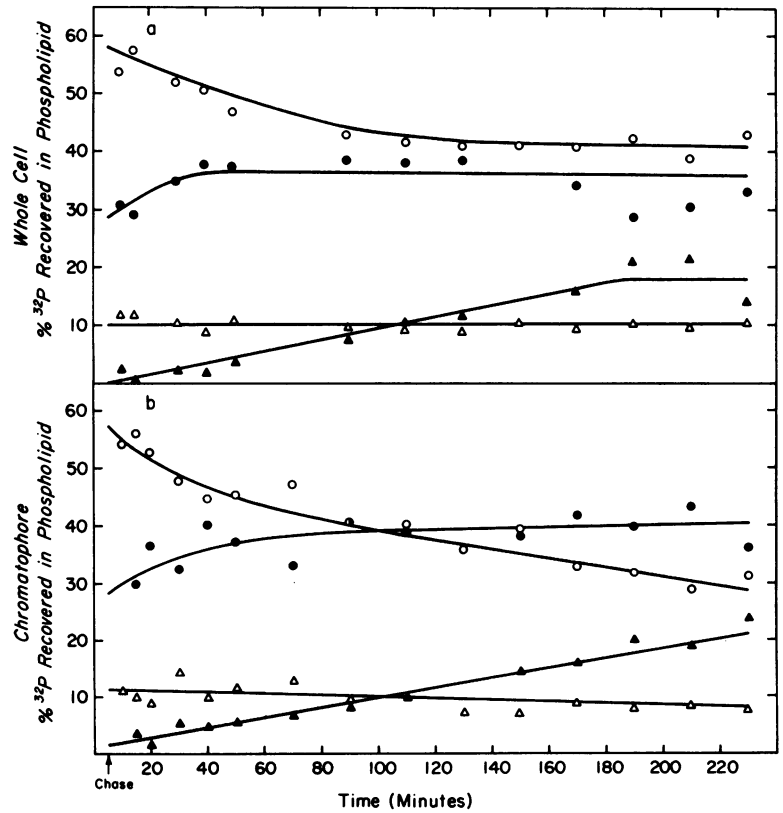

FIG. 8. *Fate of phospholipid species after pulse-chase ^{32}P-labeling in R. sphaeroides. Pulse-chase radioactive labeling, chromatophore preparation, and resolution of phospholipid species were as described in the text. (a) Cellular phospholipid species fraction of label; (b) chromatophore phospholipid species fraction of label. Symbols: ○, phosphatidylethanolamine; ▲, phosphatidylcholine; ●, phosphatidylglycerol; △, unidentified phospholipid.*

ICM is in either an "on" or "off" state. Further, it was clear that the phospholipids of the cell envelope do not freely equilibrate with the phospholipids of the ICM. We assumed that phospholipid is accumulated into either the cytoplasmic membrane or outer membrane or both whereas ICM phospholipid incorporation is "off" during interphase. Most significantly, these data represented a demonstration of the transfer of phospholipids to the ICM. Although there was leakage of label into the whole cell phospholipid after the chase, as shown in the experiment demonstrating transfer of phospholipid after the chase (Fig. 4C), this in itself did not compromise the interpretation of the data since we have repeatedly demonstrated that phospholipid is not incorporated into the ICM during interphase. It is likely that our inability to completely prevent residual label from entering the cells after the addition of a 200-fold excess of unlabeled phosphate (to a final concentration of 0.4 M) stemmed from the extremely high intracellular pools of organophosphates resulting from photosynthetic growth (16). Under photosynthetic growth, large pools of organophosphate carriers were recycled as a consequence of CO_2 fixation via the reductive Calvin cycle.

Notwithstanding this difficulty, the critical period over which phospholipid was accumulated into the ICM (Fig. 4d, samples 8 to 10) was precisely at the time of cell division as previously demonstrated and showed only a 3.0×10^2 cpm/ml $\pm$ 15 cpm/ml (95% confidence limits) increase in labeled phospholipid at the whole cell level. During the same interval there was, on the average, greater than a 3.7×10^3 cpm/ml increase in labeled phospholipid appearing in the ICM, i.e., there was greater than 12 times as much phospholipid incorporated into the ICM than was actually synthesized. Even if we consider the worst case, i.e., the scatter depicted in Fig. 4d, which is inherent in cell breakage and subcellular fractionation, there would be a 2.0×10^3 cpm/ml increase in labeled phospholipid appearing in the ICM, i.e., approximately seven times as much phospholipid incorporated into the ICM than was actually synthesized. Despite the scatter within each set of data points, no overlap occurred between these sets of data points. Considering that the phospholipid of the ICM is approximately one-third of the total cellular phospholipid, the specific enrichment of phospholipid into the ICM, relative to whole cell synthesis during the interval in question, was between 20- (worst case), 36- (average), and 54-fold (best case). In light of these results we could only conclude that the bulk (80 to 90%, minimal level) of the phospholipid increase into the ICM must have resulted from phospholipid synthesized previously, i.e., transferred from outside the ICM to the ICM. Although the data were expressed as counts per minute per milliliter of culture, the actual amounts of radioactivity measured were 20-fold greater, reflecting the actual size of the sample (Materials and Methods). It is important to point out that this experiment has been repeated on several occasions with essentially identical results. It follows that the transfer mechanism is responsive to some form of cell cycle-specific control.

Numerous reports are available which suggest that the ICM of the *Rhodospirillaceae* exists as an invagination of the cytoplasmic membrane, whereby both leaflets of the bilayer of the two membrane systems are continuous. Thin-section ultrastructural studies (8, 58) show continuity of the ICM and cytoplasmic membrane of the cell envelope. Few chromatophores are released when *Rhodospirillum rubrum* is disrupted by osmotic shock and the ICM and cytoplasmic membrane appear as a continuum (4, 59). Prince et al. (47) demonstrated continuity of the periplasmic space and the interior of the ICM of *R. sphaeroides* by demonstrating the accessibility of cytochrome c_2, trapped within isolated chromatophores, to monospecific antibody after disruption of the integrity of the vesicles. Shimada and Murata (53), working with *R. rubrum*, showed that the inner leaflet of the chromatophore bilayer is accessible from outside intact cells to trinitrobenzene sulfonic acid, a nonpenetrating probe. We have observed similar results with *R. sphaeroides* (B. D. Cain and S. Kaplan, manuscript in preparation). More recent experiments (33, 35, 57) regarding the orientation of chromatophores and spheroplast-derived vesicles support the concept of ICM and cytoplasmic membrane continuity in *R. sphaeroides*.

In view of our demonstration that the site of phospholipid synthesis is distinct from the ICM, the most likely site for phospholipid metabolism appears to be the cytoplasmic membrane of the cell envelope. However, it is essential that in vitro enzymatic studies be undertaken. If the ICM is indeed a continuous invagination of the cytoplasmic membrane, then the data presented in this communication represent (to our knowledge) the first evidence of zonal segregation of phospholipids in a procaryotic organism. As noted by Nicholson (39), a restriction to lateral diffusion in a phospholipid bilayer implies the existence of some mechanism to account for this phenomenon. One possible mechanism might involve the aggregation of membrane particles (17, 24). In the case of *R. sphaeroides*, a further requirement for the ability to overcome any mechanism hypothesized to account for zonal

segregation of phospholipids must exist because new phospholipids are known to intercalate into all previously existing ICM in a nonconservative fashion (14, 15, 31). The nature of the mechanism (i.e., barrier) which segregates the cytoplasmic membrane phospholipids from the ICM phospholipids remains open to speculation.

Although the nature of the barrier is entirely speculative, the possible mechanism(s) of phospholipid transfer is somewhat more clearly defined. Cohen et al. (6) demonstrated phospholipid exchange protein activity in *R. sphaeroides*. Unfortunately, no net transfer of phospholipids has yet been demonstrated with this activity. Alternatively, vesicles derived at the site of phospholipid synthesis might travel to and fuse with the ICM as established in eucaryotic systems (36). The temporary relaxation of the restriction to lateral diffusion between the ICM and cytoplasmic membrane would also account for an influx of phospholipid into the ICM (53, 62).

How cell cycle-specific regulation is imposed upon the transfer of phospholipids remains for future investigation. The possibility does exist that the fluidity of the ICM may be crucial. The results of Fraley et al. (12, 15) have shown that virtually all ICM phospholipids could be associated with protein as boundary lipid. These results have received independent confirmation from Birrell et al. (3). Because the physical state of the membrane has been shown to effect enzymatic activity (11), the physical state of the ICM might also regulate phospholipid transfer. Irrespective of the actual molecular mechanism(s) of phospholipid transfer and its regulation, the phospholipid transfer between membrane systems of *R. sphaeroides* constitutes an example of dynamic membrane flow (36) in a procaryotic organism.

The preliminary kinetic evidence that PE is converted to PC in the ICM requires direct confirmation. However, this observation could be significant in view of the positive correlation between the presence of PC and procaryotic organisms containing internal membrane systems (21). The enzymatic synthesis of PC is currently under investigation.

Finally, these data together with previous studies from this laboratory demonstrating cyclical alterations in ICM density, protein-to-lipid ratio, and fluorescence polarization lead us to suggest that, in steady-state cells of *R. sphaeroides*, preexisting ICM serves as a template for the incorporation of all new ICM components. In view of this conclusion we are led to suggest that preexisting ICM invaginations are replicated at the time of cell division, thereby conserving the amount of ICM per cell as well as the number of ICM invaginations (18).

ACKNOWLEDGMENTS

This investigation was supported by a research grant from the National Institutes of Health (GM 15590) to S. Kaplan. C.D.D. is a recipient of a National Institutes of Health Predoctoral Traineeship (GM 7288).

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