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Phospholipid transfer activity in synchronous populations of *Rhodobacter sphaeroides*

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Studies of intracytoplasmic membrane biogenesis employing steady-state synchronously dividing populations of *Rhodobacter sphaeroides* reveal that the translocation of pre-existing phospholipid into the growing membrane is concurrent with cell division (Cain, B.D., Deal, C.D., Fraley, R.T. and Kaplan, S. (1981) J. Bacteriol. 145, 1154–1166), yet the mechanism of phospholipid movement is unknown. However, the discovery of phospholipid transfer protein activity in *R. sphaeroides* (Cohen, L.K., Lueking, D.R. and Kaplan, S. (1979) J. Biol. Chem. 254, 721–728) provides one possible mechanism for phospholipid movement. Therefore the level of phospholipid transfer activity in cell lysates of synchronized cultures was measured and was shown to increase stepwise coinciding precisely with the increase in cell number of the culture. Although the amount of transfer activity per cell remained constant throughout the cell cycle, the specific activity of the phospholipid transfer activity showed a cyclical oscillation with its highest value coincident with the completion of cell division. Purified intracytoplasmic membrane can be used as phospholipid acceptor in the developed phospholipid transfer assay by employing either cytoplasmic membrane or liposomes as the phospholipid donor. Intracytoplasmic membrane isolated from the cells prior to division (high protein to phospholipid ratio) served as a better phospholipid acceptor in the phospholipid transfer system when compared with membranes derived from the cells following cell division (low protein to phospholipid ratio).

Introduction

Rhodobacter sphaeroides is a Gram-negative facultative photoheterotrophic bacterium. It is capable of growing under a variety of environmental conditions by varying the structure and function of its intracellular membrane system. When cells are grown chemoheterotrophically, they are surrounded by a typical Gram-negative outer membrane as well as a cytoplasmic membrane. However, when cells are subject to anaerobic conditions, a new membrane, referred to as the intracytoplasmic membrane, where the photosynthetic apparatus is localized is induced resulting in invaginations from the cytoplasmic membrane. In addition to the regulation of the intracytoplasmic membrane by the presence or absence of oxygen, the amount of intracytoplasmic membrane and the relative proportion of the various intracytoplasmic membrane components are further regulated by the incident light intensity. Because of its versatility, *R. sphaeroides* provides a unique and attractive system to study membrane biogenesis [1–5].

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The assembly of the intracytoplasmic membrane has been studied employing steady-state synchronously dividing populations of *R. sphaeroides* and reveals a cell-cycle specific translocation of phospholipid into the intracytoplasmic membrane. Proteins and photopigments, on the other hand, are continuously incorporated, with respect to the cell cycle, into the intracytoplasmic membrane [6,7]. Therefore the protein to phospholipid ratio of the intracytoplasmic membrane shows a cyclical oscillation, with the highest values recorded just prior to cell division and which then abruptly return to the starting level immediately following cell division [8]. These observations have been correlated to the change of chromatophore (purified intracytoplasmic membrane) intrinsic specific density, and to chromatophore microviscosity as determined by the fluorescent probe, α-parinaric acid [9,10]. Results from pulse-chase experiments have shown that the oscillation of the chromatophore protein to phospholipid ratio results from the fact that phospholipids are transferred from a discrete site, outside of the intracytoplasmic membrane, to the intracytoplasmic membrane, concurrent with cell division [11]. The mechanism of phospholipid translocation to the intracytoplasmic membrane is unknown.

Several models which need not be mutually exclusive have been proposed and which pertain to the mechanism of intermembrane phospholipid translocation. These are, (1) vesicle transport of membrane flow [12], (2) diffusion of phospholipid [13], and (3) protein mediated phospholipid translocation [14]. Vesicle transport is based on observations in eukaryotic cells and suggests that both proteins and lipids move between membranes via small vesicles which bud off from one membrane and fuse with another. Such a hypothesis would predict the presence of a class of lipid-rich vesicles and by performing pulse-chase experiments, a precursor-product relationship should be established between these vesicles and the membrane lacking the phospholipid biosynthetic enzymes [15,16]. However, the presence of lipid-rich vesicles has not been demonstrated in *R. sphaeroides*. Further, because of the discontinuity between lipid and protein synthesis in *R. sphaeroides*, we could not imagine such vesicles, if they exist, to contain proteins. Alternatively, diffusion of phospholipid between two membranes has been proposed as a means of phospholipid translocation between the site of phospholipid biosynthesis and other subcellular membranes. Simple diffusion of hydrophobic phospholipid molecules between two isolated membranes has been demonstrated in vitro [18,19]. Lateral diffusion, which is rapid and does not require enzymatic catalysis [20,21], may be involved in phospholipid translocation between two physically connected membranes. This mechanism has been proposed for phospholipid translocation between the cytoplasmic membrane and outer membrane of Gram-negative bacteria [22,23]. A similar diffusion mechanism of phospholipid transfer from the cytoplasmic membrane to intracytoplasmic membrane of *R. sphaeroides* has also been proposed as one of several alternatives [11]. However, the junction between the cytoplasmic membrane and intracytoplasmic membrane has not been isolated and the nature of this region is unknown, since this region should be critical to the cell-cycle specific movement of phospholipid. A third model was proposed by Wirtz [14] in which intermembrane phospholipid translocation was facilitated by phospholipid transfer protein. Phospholipid transfer proteins have been demonstrated in the cytosol of numerous eukaryotic tissues (for recent reviews, see Refs. 24 and 25] as well as some prokaryotes [26,27]. However, their physiological functions remain unknown. Because of the relative ease of manipulation of bacteria and lower eukaryotes, a study of the phospholipid transfer protein in these organisms may be helpful in elucidating their biological function. Prokaryotic phospholipid transfer protein activity was demonstrated for the first time in *R. sphaeroides* [26] and subsequently found in *Bacillus subtilis* [27]. The phospholipid transfer protein in *R. sphaeroides* was purified and characterized as a nonspecific phospholipid transfer protein [28].

Previous studies on the cellular localization of phospholipid transfer activity in *R. sphaeroides* have revealed that the level of transfer activity is related to the growth rate of the cell [29] and it was suggested that this activity may be involved in phospholipid translocation during membrane biogenesis. In this communication we studied the phospholipid transfer activity in synchronously

dividing populations of *R. sphaeroides*. Such studies are aimed at determining whether or not the cellular level of phospholipid transfer activity is subject to cell-cycle specific regulation and to relate this to phospholipid mobilization into the intracytoplasmic membrane at the onset of cell division. Furthermore, we developed an assay system in order to demonstrate the ability of the intracytoplasmic membrane to serve as phospholipid acceptor in the phospholipid transfer reaction.

Portions of this work were presented at the 69th Annual Meeting of Biological Chemistry, Anaheim, CA, U.S.A.

Materials and Methods

Organism, media and growth conditions. Rhodobacter sphaeroides 2.4.1 was used throughout. Cells were grown in the succinic acid minimal medium A [6]. Chemoheterotrophic cells were grown on a gyratory shaker at 33°C. Photoheterotrophic cells were grown in media sparged with 95% nitrogen gas and 5% carbon dioxide at 33°C employing a light intensity of 10 W/m^2.

Radioisotopically labeled intracytoplasmic membrane and cytoplasmic membrane fractions. [^{3}H]Acetate-labeled cells were prepared as described [26]. [^{14}C]Leucine-labeled chemoheterotrophic cells were prepared as described by Shepherd et al. [17]. Chromatophore vesicles were purified by discontinuous sucrose centrifugation as described by Fraley et al. [8]. Cytoplasmic membrane fractions from chemoheterotrophic cells were prepared essentially as described by Ding and Kaplan [30].

Cell synchronization. Synchronously dividing populations of *R. sphaeroides* were obtained by using the stationary phase cycling techniques of Cutler and Evans [31] as modified by Lueking et al. [6]. Asynchronously dividing cells were used as a control. Samples were withdrawn from the cultures at 20 min intervals and the cell number of the culture was determined by Petroff-Hausser counting under a phase contrast microscope. 400–1000 cells were counted per determination.

Preparation of cell lysates. Cell lysates containing periplasmic and cytoplasmic fractions of *R. sphaeroides* for the assay of phospholipid transfer activity were prepared as described previously [29].

Extraction of phospholipid. Large scale extraction of phospholipid from whole cells of *R. sphaeroides* was accomplished by the method described by Folch et al. [32]. The extracted phospholipid was separated from the photopigments by repeated precipitation in cold acetone [33] and followed by preparative two-dimensional thin-layer chromatography [34]. Radioactive phospholipids were prepared from [^{3}H]acetate-labeled photoheterotrophically grown cells of *R. sphaeroides*.

Preparation of liposomes. Phospholipid and trace amounts of β-hydroxybutyltoluene (0.3%) and [^{14}C]triolein were combined and unilamellar liposomes were prepared by sonication as described [26]. Following sonication, the suspension was subjected to centrifugation at $150\,000 \times g$ for 1 h or at 22 lb/in^2 in a Beckman Airfuge A-100 rotor for 15 min. The supernatant was collected and stored at 4°C before use. Liposomes were used in the phospholipid transfer reactions and their phospholipid compositions are indicated below.

Cytoplasmic membrane cross-absorbed chromatophore specific γ-immunoglobulin (IgG). 18 mg of chromatophore specific IgG was incubated with 0.7 mg of cytoplasmic membrane at 30°C for 30 min. The antigen-antibody precipitate was removed by centrifugation at $8700 \times g$ in a Beckman microfuge B. This process was repeated twice and the excess cytoplasmic membrane in the supernatant was removed by centrifugation for 15 min in a Beckman Airfuge A-100 rotor at 21 lb/in^2. The resultant supernatant was collected and used as the cytoplasmic membrane cross-absorbed chromatophore specific IgG.

Assay of phospholipid transfer activity. Two assay systems were used in measuring phospholipid transfer activity. The system developed by Cohen et al. [26] was routinely used and measured the transfer of [^{3}H]acetate-labeled phospholipid from donor chromatophores to [^{14}C]triolein-labeled liposomes (70% phosphatidylcholine and 30% phosphatidylethanolamine). The donor and acceptor membranes were separated by centrifugation following the addition of chromatophore specific antibody. The transferred phospholipids in the liposomes were extracted by the method of Bligh and Dyer [35] as described by Ames [36] and radioactivities were measured in a toluene based

scintillant [37]. One unit of activity is defined as the amount of protein required to transfer one pmol of phospholipid per min.

The other assay system was developed specifically for the measurement of phospholipid transfer employing chromatophores as phospholipid acceptor. Two phospholipid donors, cytoplasmic membrane fraction and liposomes derived from the whole cell phospholipids of *R. sphaeroides*, were tested respectively. Both donor membranes were double labeled containing [^{3}H]acetate-labeled phospholipids and a ^{14}C-labeled non-transferable marker. The non-transferable marker in the cytoplasmic membrane fraction was [^{14}C]leucine-labeled membrane protein and in the liposomes was [^{14}C]triolein. The assay mixture contained 3.8 nmol of donor phospholipid, 20.6 nmol of chromatophore phospholipid (the ratio of donor and chromatophore phospholipid was 6) and partially purified phospholipid transfer protein and was incubated at 30°C. Partially purified phospholipid transfer protein was prepared by 40–70% ammonium sulfate fractionation of the cell lysate [28]. The reaction was terminated at different time points by the addition of chromatophore specific IgG or the IgG fraction after cross-absorption with the cytoplasmic membrane. Incubation continued for 10 min after termination of the reaction, the antigen-antibody complexes were pelleted by centrifugation at $13200 \times g$ in a 13.2 rotor of the Beckman microfuge 11. Phospholipid in the pellet was extracted, quantitated and the radioactivity determined. The specific radioactivity of the phospholipid (PL) was calculated as follows:

$$\begin{aligned}&(\text{measured } ^3\text{H-PL} - \\ &\quad \text{liposomal } ^3\text{H-PL}(\text{measured } ^{14}\text{C}/\text{liposomal } ^{14}\text{C})) \\ &\quad \times (\text{measured PL} - \\ &\quad \text{liposomal PL}(\text{measured } ^{14}\text{C}/\text{liposomal } ^{14}\text{C}))^{-1}\end{aligned}$$

Protein concentration. The amount of protein was quantitated by the method described by Lowry et al. [38] employing bovine serum albumin as standard.

Chemicals. Dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine were purchased from Avanti Polar Lipids, Inc. Lysozyme was obtained from Worthington Biochemicals. [^{3}H]Acetic acid (3.8 Ci/mmol), [^{14}C]triolein (99.8 mCi/mmol) and [^{14}C]leucine (343 mCi/mmol) were purchased from New England Nuclear Corp.

Results

I. Phospholipid transfer activity in cell lysates of synchronously dividing cultures of R. sphaeroides

Previous results have shown that 90% of the phospholipid transfer activity in *R. sphaeroides* is found in the cytosol which consists of the periplasmic and cytoplasmic fractions [29]. Cell lysates containing these two fractions were prepared by treating cells with lysozyme and EDTA to release the periplasmic fraction followed by sonication to disrupt the spheroplasts.

In synchronously dividing populations of *R. sphaeroides*, the total cell mass as well as the soluble protein of the cell lysate increases exponentially, while the cell number increases stepwise during the course of the cell cycle [6]. Phospholipid transfer activity in the cell lysate was observed to increase stepwise coinciding precisely with the increase in cell number (Fig. 1). The level of phospholipid transfer activity per cell remained constant at $3.0 \cdot 10^{-9}$ units $\pm$ 10% throughout the cell cycle. Fig. 1 also shows that the rate of increase of phospholipid transfer activity during cell division is somewhat faster than the increase noted for bulk soluble protein. Results for asynchronously growing culture are shown in Fig. 2, cell number, bulk soluble protein and phospholipid transfer activity increase exponentially with the phospholipid transfer activity per cell remaining constant and similar to that found in the synchronous culture. However, the specific activity of the phospholipid transfer activity in the synchronous culture was different from that in the asynchronous culture, which remained constant (Fig. 2f). In the synchronous culture the specific activity showed a cyclical oscillation (Fig. 3) with the highest value coincident with the completion of cell division and dropping thereafter. Fig. 3 also shows a correlation between the change in the specific activity of the phospholipid transfer activ-

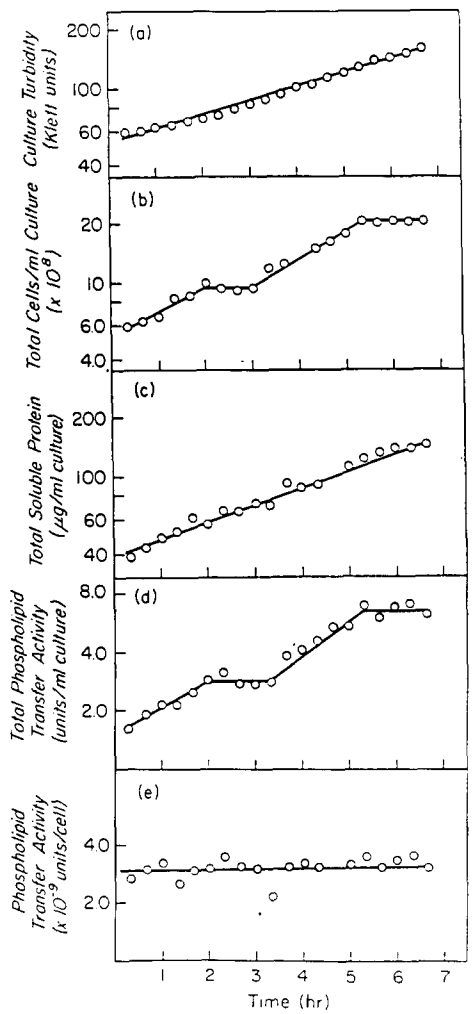

Fig. 1. Changes in the phospholipid transfer activity during synchronous growth of *R. sphaeroides*. Division synchrony and cell lysates were prepared and phospholipid transfer activity was determined as described in Materials and Methods. (a) culture turbidity; (b) total cell number per ml of culture; (c) total soluble protein per ml of culture; (d) phospholipid transfer activity per ml of culture; and (e) phospholipid transfer activity per cell.

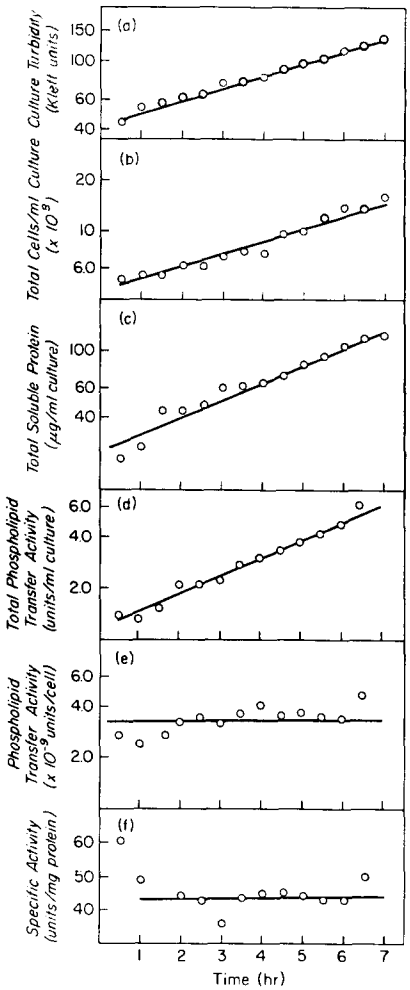

Fig. 2. Phospholipid transfer activity in an asynchronously dividing population of *R. sphaeroides*. For an explanation of panels (a) to (e), see the legend to Fig. 1. (f) specific activity of the phospholipid transfer activity.

ity and the protein to phospholipid ratio of the purified chromatophores derived from the same synchronous cell culture.

II. Ability of chromatophores to serve as phospholipid acceptor in the transfer reaction

Most of phospholipid biosynthetic activities in *R. sphaeroides* are associated with the cytoplasmic membrane with the exception of phosphatidylserine synthetase and phosphatidylethanolamine *N*-methyltransferase which are found in the cytosol following cell disruption [39]. No phospholipid biosynthetic activity has been uniquely associated with the intracytoplasmic membrane. Further, the rate of whole cell phospholipid synthesis changes in a stepwise fashion with the plateau in the rate of phospholipid biosynthesis coinciding with cell division [11]. Therefore, phospholipids which reside in the intracytoplasmic membrane must be derived from their sites of biosynthesis, outside the intracytoplasmic membrane, and their movement into the intracytop-

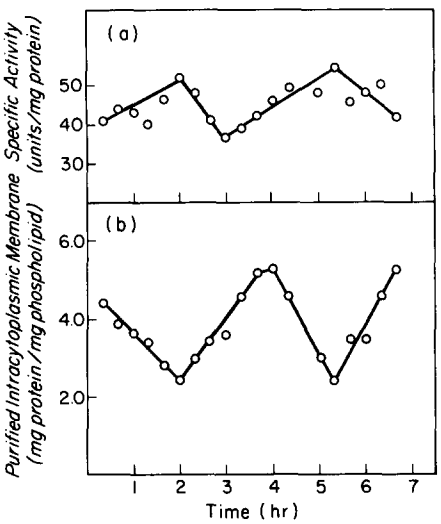

Fig. 3. Correlation of the specific activity of phospholipid transfer activity and the protein to phospholipid ratio of purified intracytoplasmic membranes from synchronously dividing cells of *R. sphaeroides*. Panel (a), the specific activity of the phospholipid transfer activity in the cell lysates from *R. sphaeroides* was derived from the results presented in panels (c) and (d) in Fig. 1. Panel (b), the protein to phospholipid ratio of the intracytoplasmic membranes purified from *R. sphaeroides* depicted in Fig. 1, was measured as described in Materials and Methods.

lasmic membrane is cell-cycle specific. If the phospholipid transfer protein is responsible for phospholipid transfer from outside the intracytoplasmic membrane to the intracytoplasmic membrane in vivo, it is essential to demonstrate that chromatophores are capable of serving as phospholipid acceptors in vitro and further, chromatophores of low phospholipid content should be better acceptors than those of high phospholipid content. Secondly, it is important to show that the cytoplasmic membrane, the site of phospholipid synthesis in photosynthetically grown *R. sphaeroides* [39] can serve as phospholipid donor. Two phospholipid transfer assay systems were developed employing chromatophores as phospholipid acceptor and either cytoplasmic membrane or liposomes derived from the phospholipids of *R. sphaeroides* as phospholipid donor.

When the cytoplasmic membrane fraction was employed as the phospholipid donor and chromatophores as the acceptor, the separation of donor and acceptor membranes was difficult. Since the specific intrinsic densities of these two membranes show only small differences [40], it is difficult to separate these two membranes by simple centrifugation under standard conditions. Further, both membrane fractions share many common membrane proteins [41–43], and therefore antibody against chromatophores also precipitates substantial amounts of the cytoplasmic membrane (Fig. 4a). However, if the IgG against these common antigenic components is removed by cross-absorbing the chromatophore-specific polyclonal IgG with a purified cytoplasmic membrane fraction, the resultant antibody should be specific for chromatophores. After two treatments of cross-absorption, the resultant IgG still possessed the ability to precipitate $87 \pm 2\%$ of chromatophores but did not cross-react with the cytoplasmic membrane when treated separately with each these two membrane fractions (Fig. 4b). However, when a mixture of cytoplasmic membrane and intracytoplasmic membrane was employed, $50 \pm 2\%$ of cytoplasmic membrane fraction was also precipitated by the cross-absorbed IgG fraction (Fig. 4c) and co-precipitation of cytoplasmic membrane and chromatophores increased as the time of incubation of these two membrane fractions increased. This indicated that the cross-absorbed chromatophore specific IgG contained antibody which might be monovalent for cytoplasmic membrane antigenic sites, some intrinsic factor(s) residing on the membranes caused the aggregation of cytoplasmic membrane and chromatophores, and/or cytoplasmic membrane was trapped non-specifically in the lattice structure of the chromatophore-IgG complex. The latter possibility was confirmed by using IgG against bovine serum albumin in the presence of cytoplasmic membrane. Over 20% of the cytoplasmic membrane fraction was co-precipitated with bovine serum albumin. Therefore, by employing the specifically cross-absorbed IgG against intracytoplasmic membrane and a predetermined correction for non-specific cytoplasmic membrane trapping in the immune complex we could demonstrate the unequivocal transfer of phospholipid from the cytoplasmic membrane to the intracytoplasmic membrane in vitro.

The developed cytoplasmic membrane-chromatophore specific assay system measured the transfer of [^{3}H]acetate-labeled phospholipid from

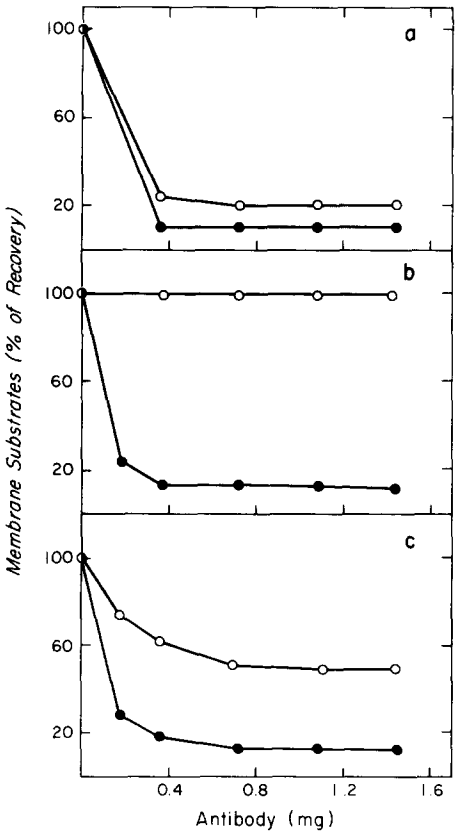

Fig. 4. Properties of chromatophore-specific immunoglobulin before and after cross-absorption with cytoplasmic membranes. Chromatophores and cytoplasmic membranes and chromatophore specific immunoglobulin (IgG) before and after cross-absorption were prepared as described in Materials and Methods. Panel (a) chromatophore specific IgG before cross-absorption was incubated with 46.8 μg of ^{3}H-labeled chromatophore or 9.7 μg of ^{14}C-labeled cytoplasmic membrane, respectively, at 30°C for 10 min. The antibody-antigen complex was removed by centrifugation at 8700 × g in a Beckman Microfuge B for 4 min. The percentage of radioactivity of intracytoplasmic (●) and cytoplasmic (○) membranes remaining in the supernatants were measured. Cross-absorbed chromatophore-specific IgG was incubated with cytoplasmic and chromatophore membranes, respectively, (panel b) or with a mixture of these two membranes (panel c) as described in panel (a). The radioactivities remaining in the supernatant were measured.

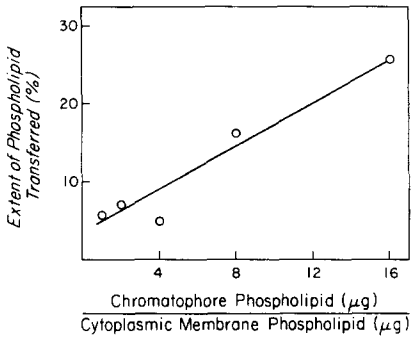

Fig. 5. Effects of the ratio of donor and acceptor membranes on the extent of the phospholipid transfer reaction. ^{3}H-labeled cytoplasmic and non-radioactive chromatophore membranes, prepared from asynchronous cultures of *R. sphaeroides*, were used as the phospholipid donor and acceptor, respectively. The phospholipid contents were determined by the method of Bartlet [63]. The phospholipid transfer reaction mixture contained 0.2 mg of partially purified phospholipid transfer protein and varying ratios of chromatophore and cytoplasmic membrane phospholipid. Reactions were performed as described in Materials and Methods.

the cytoplasmic membrane, containing [^{14}C]leucine-labeled protein as a non-transferable marker, to the chromatophore. After correcting for cross-contamination between these two membranes as described above and in Materials and Methods, the specific radioactivity of chromatophore phospholipid increased linearly during the course of the 2 h reaction. The assay system could not differentiate as to whether the transfer was a net transfer or an exchange process. Either the extent or efficiency of the phospholipid transferred was increased by increasing the ratio of acceptor to donor membranes in the reaction mixture (Fig. 5). The ability of chromatophores to serve as phospholipid acceptor was also demonstrated in the assay employing liposomes as the phospholipid donor in place of the cytoplasmic membrane (unpublished results). The transfer reaction was linear during the 2 h incubation. The advantage of employing liposomes as phospholipid donor over the cytoplasmic membrane fractions was that the co-precipitation was lower, approximately 20%, and did not increase with time of incubation.

The experiments depicted in Figs. 1–3, reveal a possible role for phospholipid transfer activity in the movement of lipid to the photosynthetic membrane at the time of cell division. Therefore, the results presented in Figs. 4 and 5 are important in demonstrating both that, intracytoplasmic membrane can serve as a phospholipid acceptor and that cytoplasmic membrane can serve as a phospholipid donor.

Studies previously cited [11] demonstrated that

the mobilization of phospholipid into the intracytoplasmic membrane occurs only at the onset of cell division when the intracytoplasmic membrane has the highest protein to phospholipid ratio. If the mechanism of phospholipid translocation were to involve phospholipid transfer protein activity, it is possible that differences in the protein to phospholipid ratio of the chromatophores should reflect differences in their ability to serve as phospholipid acceptor in the phospholipid transfer reaction. This is a critical test of the hypothesis that phospholipid transfer activity might be involved in the movement of phospholipids from the cytoplasmic membrane to the intracytoplasmic membrane. This possibility was tested by employing a liposome-chromatophore assay system using chromatophores of various protein to phospholipid ratio (derived from cells at different stages in the cell cycle, see Fig. 3b) and revealed that the rate of phospholipid transfer into chromatophores of high protein to phospholipid ratio purified from cells just prior to the cell division was over three-fold greater than the rate of transfer of phospholipid employing chromatophores of low protein to phospholipid ratio obtained from cells just following cell division (Table I). Although it was impossible to determine whether transfer resulted from an exchange reaction or a net transfer, the important point is that there is a distinctly measurable difference in the rate of transfer which accelerates as the protein-to-lipid ratio of the acceptor increases.

TABLE I

COMPARISON OF THE ABILITY OF CHROMATOPHORES TO SERVE AS PHOSPHOLIPID ACCEPTOR

The comparison of chromatophores of different protein to phospholipid ratios to serve as phospholipid acceptors was studied by measuring the transfer of [^{3}H]acetate-labeled phospholipid from liposomes derived from whole cell phospholipid of *R. sphaeroides* to chromatophores. Chromatophores having a protein/phospholipid ratio of 5.2 and 2.4 were purified from the cells immediately prior to and after cell division. The phospholipid transfer reactions were performed as described in Materials and Methods.

Chromatophores		Donors	Rate of phospholipid transfer (pmol/min)
Source, culture	protein to phospholipid ratio (w/w)		
Synchronous	5.2	liposome	4.72
	2.4	liposome	1.52
Asynchronous	3.4	liposome	2.07

Discussion

This work extends previous studies on intracytoplasmic membrane assembly in synchronously dividing cultures of *R. sphaeroides* and attempts to correlate the movement of phospholipid between membrane compartments in synchronously dividing cells to the cellular level of phospholipid transfer activity. These earlier studies showed that, during synchronous growth, major intracytoplasmic membrane proteins are synthesized at constant rates, while the rate of whole cell phospholipid synthesis shows a discontinuity with a plateau occurring simultaneously with cell division [6,8]. Further, the incorporation of protein and phospholipid into the intracytoplasmic membrane are not coupled, with new protein being continuously inserted into the intracytoplasmic membrane throughout the cell cycle and phospholipid being transferred to the intracytoplasmic membrane only prior to cell division [11]. The mechanism of phospholipid translocation into the intracytoplasmic membrane is unknown and one of the possibilities to be considered is protein-mediated phospholipid transfer. The present study shows that the transfer activity of the cell culture increased proportionally to cell number with the level of phospholipid transfer activity per cell remaining constant during the course of the cell cycle. However, the cellular level of phospholipid transfer activity and the specific phospholipid transfer activity do reveal distinct cell-cycle specific regulation.

Studies of the comparison of phospholipid transfer activity between normal eukaryotic tissues and developing [44,45] or transformed cells [46], and between cells of *R. sphaeroides* growing under different growth conditions [29] reveal that the level of cellular phospholipid transfer activity is related to the growth rate of the cell. Kader [25] suggested that the amount of phospholipid transfer protein within the cell depends on the intensity of membrane biogenesis. We show that the amount and specific activity of the phospholipid transfer activity is directly related to cell division. We can

not, at the present time, differentiate between a stepwise increase in phospholipid transfer activity being due to the regulation of synthesis at the level of gene expression or regulation in the activity of previously synthesized phospholipid transfer protein. However, based on the observations presented in this paper that (1) the amount phospholipid transfer activity per cell remains constant and is apparently not affected by gene copy number, (2) the rate of increase of phospholipid transfer activity during cell division is faster than the rate of increase in the total soluble protein and (3) the change of specific activity of the phospholipid transfer activity shows a cyclical oscillation with its highest value coincident with the completion of cell division, it is postulated that the biosynthesis of phospholipid transfer protein may occur only at the onset of cell division. Further experimentation is required to rigorously test this hypothesis.

A similar cell-cycle specific regulation of membrane phospholipid content has also been observed in another system. Studies employing steady-state synchronous cultures of yeast reveal that the mitochondrial phospholipid to protein ratio varies in a cyclical fashion during the cell cycle and reaches its highest value at the onset of the time of bud formation [47]. This suggests an influx of phospholipid into the mitochondria at the time of budding. Further, phospholipid transfer protein activity also has been detected in the cytosol of yeast [48,49]. Though the regulation of phospholipid synthesis has not been studied in this system to the extent that it has been documented with *R. sphaeroides*, it is possible that phospholipid transfer activity is responsible for the abrupt increase of phospholipid within the mitochondria.

Studies on phospholipid biosynthesis in Gram-negative bacteria reveal that most phospholipid biosynthetic enzymes are localized in the cytoplasmic membrane [39,50,51], and the rate of whole cell phospholipid synthesis in synchronous cultures show a stepwise increase during the cell cycle [11,52]. It is not clear whether these newly-synthesized phospholipids are accumulated in the cytoplasmic membrane or immediately equilibrated with the outer membrane. The translocation of phospholipid between cytoplasmic membrane and outer membrane in synchronous cultures has not been studied and its mode of transfer throughout the cell cycle is not known. However, the mobilization of phospholipid into the intracytoplasmic membrane of *R. sphaeroides* is cell-cycle specific. Though the distribution of phospholipid transfer activity between the membrane-associated and free forms as well as between the periplasmic and cytoplasmic fractions of synchronous populations of *R. sphaeroides* have not been investigated, the present study shows that the increase in the specific activity of the phospholipid transfer activity is coincident with the transfer of phospholipid into the intracytoplasmic membrane. From previous studies we have shown that the protein to phospholipid ratio and membrane viscosity of the intracytoplasmic membrane are closely related [10]. Therefore we hypothesize, when the cellular membrane fluidity decreases to a certain threshold level, the activity of the phospholipid transfer protein will be increased which, in turn, will facilitate the translocation of phospholipid into the photosynthetic membrane to increase its fluidity.

By employing the liposome-chromatophore assay system we showed that chromatophores having a protein to phospholipid ratio of 5.2 to 1.0 are a much better substrate, i.e. acceptor, than those possessing a protein to phospholipid ratio of 2.4, in the phospholipid transfer reaction. The difference in the rate of transfer is more than three-fold. The reason causing this difference is not clear. It is possible that some unique interaction(s) between chromatophores of high protein to phospholipid ratio and the phospholipid transfer protein are favorable for the transfer reaction. At the present time, it is unknown whether or not this transfer in vitro is an exchange process or net transfer. However, the intracytoplasmic membrane of low protein to phospholipid ratio possess a higher fluidity [10] and phospholipid molecules derived from membrane with less structural lipids are preferable for transfer [53–58]. Therefore, the amount of net phospholipid transferred into chromatophores of high protein to phospholipid ratio would be expected to be much higher than that into chromatophores of low protein to phospholipid ratio.

Under the conditions of cell growth reported here, we can calculate that between $5 \cdot 10^6$ and $1 \cdot 10^7$ phospholipid molecules must be trans-

located to the intracytoplasmic membrane at the time of cell division in order to double the intracytoplasmic membrane phospholipid content and to restore the low protein to phospholipid ratio which is observed. Given the phospholipid protein transfer activity per cell (approximately $(3–5) \cdot 10^{-9}$ units) we can calculate that it would take less than one min to effect the net transfer of phospholipid from outside the intracytoplasmic membrane to the intracytoplasmic membrane at the time of cell division. Our results, reveal that the movement of phospholipid to the intracytoplasmic membrane is completed within at least 10 min. Therefore, there is ample cellular phospholipid transfer activity to accommodate the movement of cellular phospholipid.

Taken together these data display a strong correlation between membrane fluidity, expression of phospholipid transfer activity, phospholipid translocation and cell division and that these activities are well coordinated. Though the interrelationship between these events remains to be elucidated, the mobilization of phospholipid in and/or out of a membrane system may influence the activity of the membrane protein via the effect of lipid-dependency or the change in the microviscosity of the membrane. Kagawa et al. [59] have shown that transfer of exogenous phosphatidylcholine into the reconstituted ATPase-containing vesicles lacking such phospholipid will activate phosphorylation. It also has been demonstrated that the activities of monoamine oxidase on the microsomal and glucose-6-phosphatase on the mitochondrial membranes of hepatoma cells [60,61] and cytochrome *P*-450 in the rat liver microsomal membrane [62] will increase after phosphatidylcholine transfer into these membranes. These phenomena can be correlated with observations in vivo. Studies on synchronous cultures of *R. sphaeroides* reveal that the activities of succinate dehydrogenase and NADH oxidase undergo a cyclical change throughout the cell cycle [7]. Furthermore, the intracytoplasmic membrane-associated ATPase activity is dependent on the protein to phospholipid ratio of the membrane (manuscript in preparation). Similar results have been demonstrated in synchronous cells of yeast, i.e. the stepwise increase of cytochrome c oxidase activity coincides with the increase in the peak of cardiolipin content of the cells [47]. It is possible that the transfer of phospholipid in or out of membranes in vivo may serve to modulate the activities of membrane-associated and lipid-dependent proteins. Direct evaluation of the physiological function of the phospholipid transfer protein will undoubtedly require genetic and molecular approaches. Studies on the phospholipid transfer protein in microorganisms have the advantage in this respect and hopefully will assist us in elucidating the regulation and physiological function of the activity within living cells.

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