JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 607–615
0021-9193/82/110607-09$02.00/0

Vol. 152, No. 2

Kinetic Analysis of *N*-Acylphosphatidylserine Accumulation and Implications for Membrane Assembly in *Rhodopseudomonas sphaeroides*

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Received 7 May 1982/Accepted 2 August 1982

The accumulation of *N*-acylphosphatidylserine (NAPS) in response to the inclusion of Tris in the growth medium of *Rhodopseudomonas sphaeroides* strain M29-5 has been examined. In the accompanying paper (Donohue et al., J. Bacteriol. **152:000–000**, 1982), we show that in response to Tris, NAPS accumulated to as much as 40% of the total cellular phospholipid content. NAPS accumulation began immediately upon addition of Tris and was reflected as an abrupt 12-fold increase in the apparent rate of NAPS accumulation. We suggest that Tris altered the flow of metabolites through a preexisting and previously unknown metabolic pathway. NAPS accumulation ceased immediately upon the removal of Tris; however, accumulated NAPS remained largely metabolically stable. Importantly, under conditions in which NAPS was not accumulated, the intracytoplasmic membrane was shown to be virtually devoid of newly synthesized NAPS. The significance of this observation is discussed in terms of its physiological implications on phospholipid transfer and membrane biogenesis in *R. sphaeroides*.

The phospholipid bilayer constitutes the structural matrix of biological membranes, and other membrane components are either integral or peripheral to the bilayer (42). Phospholipids have been implicated in such membrane functions as constitution of a barrier to permeability (28, 29) and activation of membrane-bound enzymes (24, 44). The physical properties of phospholipids in the bilayer (9, 43), their spatial arrangement (17, 31), and interactions with membrane proteins (32, 34) remain the subject of intense ongoing investigation. The structural and functional importance of phospholipids in biological membranes has generated considerable interest in the regulation and metabolism of the various phospholipid species.

Kennedy and co-workers elucidated the biosynthetic pathway for phospholipids of *Escherichia coli* by developing in vitro enzyme assays for these activities (8, 38). These investigations established that phosphatidylethanolamine (PE) is synthesized from CDP-diglyceride through the sequential activities of phosphatidylserine synthetase (21) and phosphatidylserine decarboxylase (39). Phosphatidylglycerol (PG) is the product of a separate pathway, which diverges at the level of CDP-diglyceride (5, 21). The study of phospholipid metabolism has been further facilitated by selection of temperature-sensitive mutant strains that are defective in phospholipid synthesis (8, 38), with alterations (if any) manifested in the accumulation of known pathway products and intermediates. For example, in a phosphatidylserine synthetase-deficient mutant, the amount of cardiolipin increased and the amount of PE was reduced (37), whereas phosphatidylserine (PS) accumulated in a phosphatidylserine decarboxylase-defective strain (19). However, Nishijima and Raetz (30) recently reported that a strain virtually devoid of PG had elevated levels of glycolipids, indicating that there was an adjustment in cellular metabolism in response to the membrane phospholipid imbalance. The application of recombinant DNA techniques has allowed the construction of *E. coli* strains which overproduce phospholipid biosynthetic enzymes, but these strains do not possess altered cellular phospholipid compositions (25).

Physiological investigations of phospholipid enzymology have shown that these enzymes are restricted to the inner membranes of gram-negative bacteria (12). Unfortunately, little has been reported on the localization of the phospholipid biosynthetic enzymes in the photosynthetic bacteria which possess an extensive intracytoplasmic membrane (ICM) system.

Phospholipid accumulation occurs in a cell cycle-related stepwise manner in *Rhodopseudomonas sphaeroides* (22, 26), and a similar sug-

gestion has been made for *E. coli* (4, 35). Investigations in our laboratory of the *R. sphaeroides* ICM assembly have demonstrated oscillations in the ratio of protein to phospholipid, intrinsic specific density, and fluorescence polarization of the intrinsic membrane fluidity probe α-parinaric acid as a function of the cell cycle (15, 16, 26). These oscillatory phenomena result from a rapid net transfer of bulk phospholipid to the ICM from a site external to the ICM concurrent with cell division (3, 23). Recently, Cottrell et al. (7) reported an oscillation in the *Saccharomyces cerevisiae* ratio of mitochondrial protein to phospholipid with respect to the cell cycle. This observation may be particularly significant in view of the proposed phylogenetic relationship between the purple photosynthetic bacteria and mitochrondria (13).

In the course of our investigations on the cell cycle-specific insertion of phospholipids into the ICM, we observed a previously unidentified phospholipid, designated P(x) (3), which was found to be transferred to the ICM in consort with the other known phospholipid species PG, PE, and phosphatidylcholine (PC). P(x) was later identified as *N*-acylphosphatidylserine (NAPS) (10). Possible explanations for the absence of NAPS in recent studies (1, 6, 27, 40) detailing the phospholipid composition of *R. sphaeroides* and the parameters regulating NAPS accumulation are the subjects of the accompanying paper (11).

In the present work we investigated the kinetic parameters governing NAPS accumulation and metabolism in order to facilitate the use of this compound as a physiological probe, as well as to define more clearly the relationship between the metabolism of NAPS and the conventional phospholipid species normally found in *R. sphaeroides*. The perturbation of phospholipid metabolism resulting in the accumulation of NAPS was observed immediately upon addition of Tris to the medium. The apparent rates of NAPS accumulation both before and after induction by Tris were determined. We found that once accumulated, NAPS remained largely metabolically stable. Finally, under conditions in which NAPS was not accumulated, the ICM was shown to be virtually devoid of newly synthesized NAPS.

(A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, Atlanta, Ga., March 1982).

MATERIALS AND METHODS

Organism, media, and growth conditions. *R. sphaeroides* strain M29-5 (Leu^- Met^-) was grown under both photoheterotrophic (26) and chemoheterotrophic conditions (31). In all studies we used Sistrom medium A (26) supplemented with leucine and methionine (final concentration, 50 μg/ml each). Since the presence of Tris causes the accumulation of NAPS (11), any addition of Tris to culture media is indicated below.

Synchronously dividing cell populations were obtained by using the stationary phase cycling technique described for *R. sphaeroides* by Lueking et al. (26).

Preparation of membrane fractions. ICM fractions were prepared from photoheterotrophically grown cells as described by Fraley et al. (14). Cell membrane contamination was measured by the penicillin-binding protein assay, using the method of Shepherd et al. (41).

Radioactive labeling of phospholipids. The kinetics of [^{32}P]orthophosphoric acid labeling in *R. sphaeroides* have been described elsewhere (3, 15). [^{3}H]glycerol was employed only as a long-term label for at least six generations. The specific activities of the radioactive labels varied and are given below each experiment.

Phospholipid extraction. Phospholipids were extracted from cells and membrane fractions by the method of Ames (2). Chloroform phases were washed once with 3 ml of 1% NaCl. Carrier cells (200 μg of phospholipid per ml of chloroform) were included. For studies in which [^{3}H]glycerol was used the carrier cells were replaced by bulk *R. sphaeroides* phospholipids which had been extensively depigmented by repeated acetone precipitations in order to minimize quenching due to photopigments.

Resolution of individual phospholipid species. The phospholipid species were resolved by the two-dimensional thin-layer chromatography system of Poorthius et al. (36), as applied to *R. sphaeroides* (3, 10).

Phospholipids containing only ^{32}P were scraped directly from the plates into scintillation vials. Silica gel was removed from ^{3}H-labeled phospholipids by elution from a Silica Gel H column (2 by 1 cm) with 15 ml of chloroform-methanol-water-formic acid (97:97:4:2). The labeled phospholipids were reduced to dryness in scintillation vials by heating (40°C) or under a stream of air. The radioactivity of all samples was determined in a toluene-based scintillant (14).

Materials. All solvents were of reagent grade and were used without further distillation. Silica Gel G was purchased from Supelco Inc., Bellefonte, Pa. Silica Gel H was purchased from Brinkmann Instruments, Inc., Des Plains, Ill. The following radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.: [2-^{3}H]glycerol (10 Ci/mmol) and [^{32}P]orthophosphoric acid (carrier-free).

RESULTS

Induction of NAPS accumulation. In the accompanying paper (11), we report that inclusion of Tris in the growth medium of *R. sphaeroides* strain M29-5 caused the accumulation of NAPS to as much as 40% of the total cellular phospholipid content. The data in Fig. 1 define the kinetic parameters governing the induction of NAPS accumulation. Photoheterotrophically growing cells were prelabeled with [^{32}P]orthophosphoric acid for a full culture doubling to insure label equilibration with the extensive intracellular phosphate pool (3) before the direct

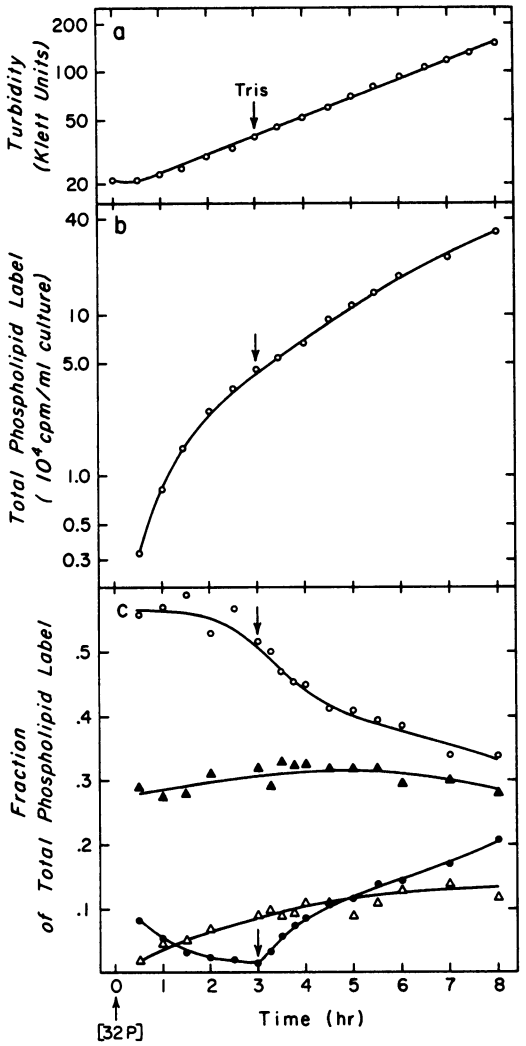

FIG. 1. Effect of addition of Tris to an exponentially growing photoheterotrophic culture of *R. sphaeroides*. [^{32}P]orthophosphoric acid (100 μCi/ml of culture) was added to the cells at zero time. After 3 h, 1 M Tris (pH 7.0) was added to a final concentration of 20 mM (arrows). At different times culture samples were removed for determinations of culture density, bulk phospholipid radioactivity, and radioactivity of the individual phospholipid species. (a) Culture turbidity. (b) Total cellular phospholipid radioactivity. (c) Fraction of the total phospholipid label in NAPS (●), PC (△), PE (○), and PG (▲).

addition of Tris to the culture medium. Tris addition did not alter the growth of the culture (Fig. 1a) or the rate of incorporation of label into bulk phospholipids (Fig. 1b). However, the addition of Tris had a profound effect on the distribution of label among the individual phospholipid species (Fig. 1c). At the time of Tris addition, less than 2% of the phospholipid label was found in NAPS. Immediately after the inclusion of Tris, the fraction of the phospholipid label in NAPS began a steady increase, which continued throughout the remainder of the experiment (more than two culture doublings). In agreement with our investigations of steady-state-labeled cells (11), NAPS accumulation appeared to be at the expense of PE. PC biosynthesis also contributed to the relative decline in PE, but Tris addition did not significantly affect the conversion of PE to PC. Importantly, no change was detected in the fraction of the label in PG. Therefore, the effect of Tris on phospholipid metabolism was specific to the PS branch of the phospholipid biosynthetic pathway.

Figure 2 shows a kinetic analysis of NAPS induction generated from the data shown in Fig. 1. After introduction of Tris, the quantity of [^{32}P]NAPS per milligram of cell mass increased linearly throughout the course of the experiment (Fig. 2a). Even after two culture doublings, a new steady-state level of NAPS had not been

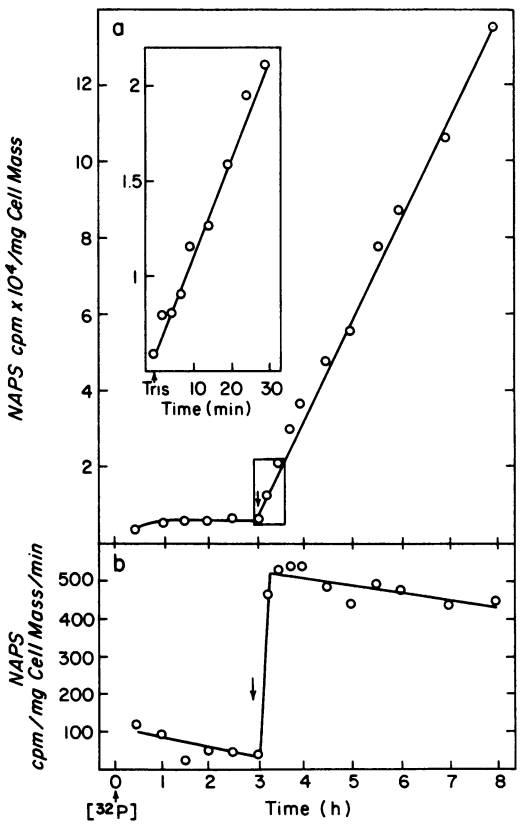

FIG. 2. Induction of NAPS accumulation after addition of Tris to an exponentially growing culture of *R. sphaeroides*. The experimental details were as described in the legend to Fig. 1. (a) Accumulation of NAPS. (b) Apparent rate of NAPS accumulation.

achieved, so that the fractions of label present in the individual phospholipid species (Fig. 1c) did not reflect the absolute cellular levels of these lipids. The onset of NAPS accumulation occurred essentially immediately (within no more than 2.5 min) after Tris addition (Fig. 2a). Indeed, addition of Tris caused an abrupt 12-fold increase in the apparent rate of NAPS accumulation (Fig. 2b). The absence of even the slightest detectable lag between the addition of Tris and the onset of NAPS accumulation suggested that the induction of a new enzyme responsible for the synthesis of NAPS was unlikely (3); rather, Tris either stimulated or inhibited an existing activity which resulted in the accumulation of NAPS.

Turnover of accumulated NAPS. Since Tris induced the accumulation of NAPS, we attempted to follow the fate of accumulated NAPS by removing Tris from the medium. This experiment required that phospholipids synthesized before the removal of Tris be distinguishable from those synthesized in the absence of Tris. Therefore, cells were first labeled with [^{3}H]glycerol and [^{32}P]orthophosphoric acid in medium containing Tris, harvested, and suspended in medium without Tris and [^{3}H]glycerol but retaining the ^{32}P label. If withdrawal of Tris stopped NAPS accumulation, then little ^{32}P would enter NAPS, and assuming no [^{3}H]glycerol was being incorporated into NAPS as well, the NAPS ratio of ^{3}H to ^{32}P would remain stable. A decrease in the ratio of ^{3}H to ^{32}P would indicate continued ^{32}P incorporation into a phospholipid species despite the removal of Tris.

The culture remained in exponential growth after transfer to fresh medium (Fig. 3a). Simply withdrawing the cells from [^{3}H]glycerol effectively halted incorporation of ^{3}H into chloroform-extractable material (Fig. 3b); the amount of chloroform-extractable ^{3}H actually decreased at a rate of approximately 7% per culture doubling in the fresh medium. ^{32}P labeling of phospholipids paralelled culture growth (Fig. 3b). This labeling pattern was clearly reflected in the sharp decreases in the PE, PC, and PG ratios of ^{3}H to ^{32}P (Fig. 3d). The apparent high ratio of ^{3}H to ^{32}P for PG was due to the labeling of the two glycerol moieties of this phospholipid with ^{3}H (i.e., no correction was made in the data presented in Fig. 3d). In contrast to the other phospholipids, the NAPS ratio of ^{3}H to ^{32}P declined very little (Fig. 3d). An examination of the data in terms of absolute incorporation of both ^{3}H and ^{32}P into NAPS confirmed that neither label was incorporated into NAPS to any extent after the removal of Tris (Fig. 3c). Both the magnitude and the rate of the decreases in the levels of NAPS labels (Fig. 3c) were insufficient to implicate a direct precursor-product relationship between NAPS and the other phospholipid species. However, knowing that conversion of PE to PC occurs (3), the PE and PC ratios of ^{3}H to ^{32}P were significantly higher than those predicted, indicating some continued ^{3}H incorporation into these phospholipids (Table 1), whereas the PG ratio decreased essentially as predicted after accounting for two glycerol moieties per molecule of PG. Since the [^{3}H]glycerol chase was effective and no perturbation was observed in total ^{32}P incorporation into phospholipids, the source of the additional ^{3}H in PE and PC seems to be within the PS branch of the

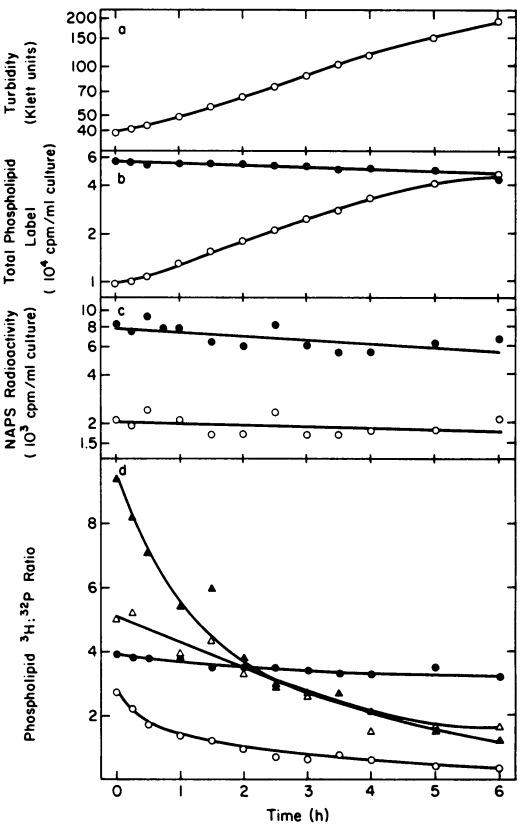

FIG. 3. Effect of Tris removal on phospholipid synthesis in *R. sphaeroides*. An exponentially growing photoheterotrophic culture was labeled for six generations in medium containing 20 mM Tris with [^{3}H]glycerol (1 μCi/ml) and [^{32}P]orthophosphoric acid (30 μCi/ml). The cells were harvested by centrifugation at 12,000 × *g* for 10 min at 25°C and then suspended in fresh medium containing the ^{32}P label but no Tris or [^{3}H]glycerol. Samples were removed at different times for determinations of culture turbidity, bulk phospholipid radioactivity, and radioactivity in the individual phospholipid species. (a) Culture turbidity. (b) Total ^{3}H (●) and ^{32}P (○) in bulk phospholipid species. (c) Total ^{3}H (●) and ^{32}P (○) in NAPS. (d) Ratio of ^{3}H to ^{32}P in each of the individual phospholipid species. Symbols: ●, NAPS; △, PC; ○, PE; ▲, PG.

TABLE 1. Stability in the ratios of ^3H to ^{32}P for the individual phospholipid species shown in Fig. 3[a]

Phospholipid	Observed ratio[b]	Predicted ratio[c]
NAPS	0.85	0.87
PG	0.22	0.20
PE	0.22	0.11[d]
PC	0.42	0.18[e]

[a] Based upon an analysis of two culture doublings.
[b] Taken from Fig. 3 between 60 and 360 min.
[c] Based upon ratios of ^3H to ^{32}P between 60 and 360 min (Fig. 3) and accounting for 7% loss of ^3H label from phospholipids and the presence of two [^3H]glycerol moieties in PG.
[d] Assuming conversion of PE to PC.
[e] Based on metabolic turnover of NAPS.

phospholipid biosynthetic pathway. Importantly, the decrease in the level of ^3H in NAPS was sufficient to alter the PE and PC ratios observed, providing that PE was produced stoichiometrically as the result of NAPS turnover. Therefore, the possibility that a precursor-product relationship exists between NAPS and PE cannot be excluded. Although NAPS accumulation ceased upon Tris removal, the bulk of the NAPS previously accumulated in the presence of Tris remained largely metabolically stable. This led to the conclusion that dilution through cell division was the primary mechanism for the reduction within the cellular membranes of abnormally high NAPS levels.

To test this hypothesis, an experiment similar to the one outlined above was performed. However, in this case, ^{32}P was omitted from the medium before the removal of Tris, but it was added to the fresh medium containing no Tris. As predicted, little ^{32}P was detected in NAPS, whereas the ^3H content of NAPS remained at a high level and was essentially stable (data not shown).

Rate of NAPS turnover. The demonstration that virtually no NAPS accumulated after Tris removal allowed us to assume that incorporation of ^{32}P from the intracellular phosphate pools into NAPS after the removal of Tris was nil. Therefore, a simplified label-chase procedure was used to determine the apparent rate of turnover of accumulated NAPS. Cells were prelabeled with [^{32}P]orthophosphoric acid in the presence of Tris, harvested, and suspended in medium containing neither label nor Tris.

Figure 4 shows the results obtained from a photoheterotrophically growing culture. As expected, a small quantity of radioactivity, representing a 7% increase per culture doubling, continued to accumulate in phospholipids after withdrawal of the label (Fig. 4b). The amounts of radioactivity in PG and NAPS decreased with respect to cell mass at rates proportional to culture growth rather than metabolic turnover (Fig. 4c and e). The relatively slow incorporation of label into PC from PE accounted for the unique behavior of the PC label (Fig. 4d and f) (3).

An analysis of the data in Fig. 4, as well as the data obtained from a similar study of chemotrophically grown cells, generated values for the apparent rates of turnover of accumulated NAPS (Table 2). The NAPS accumulated during Tris treatment was not entirely metabolically inert. However, inclusion of Tris in the fresh chase medium completely masked NAPS turnover (Table 2). The apparent rates of turnover of PG and PE are included in Table 2 for compari-

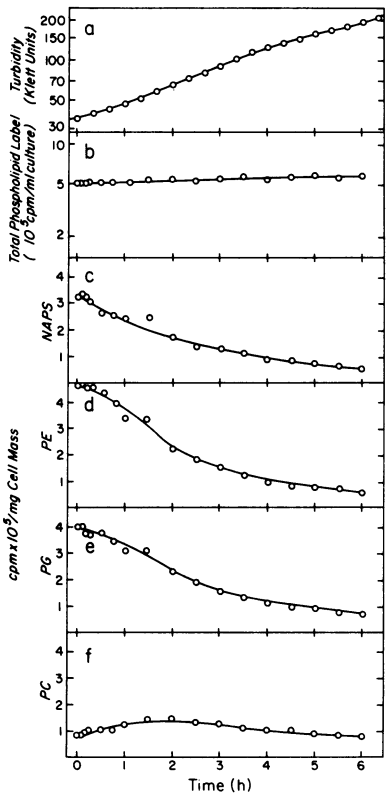

FIG. 4. Turnover of preexisting NAPS after removal of Tris from *R. sphaeroides*. A photoheterotrophic culture was prelabeled for eight generations with [^{32}P]orthophosphoric acid (20 μCi/ml) in medium containing 20 mM Tris. The cells were harvested by centrifugation at 12,000 × *g* for 10 min at 25°C and then inoculated into fresh medium containing neither Tris nor isotope. Samples were removed for determinations of culture tubidity, bulk phospholipid radioactivity, and radioactivity of the individual phospholipid species. (a) Culture turbidity. (b) Total phospholipid label. (c through f) Specific radioactivities of the indicated phospholipids.

son; however, the very slight continued incorporation of ^{32}P after conclusion of the labeling period depressed these rates to a small degree. Nevertheless, PE conversion to PC was clearly reflected in the relatively rapid rate of PE turnover.

Donohue et al. (11) reported what might be interpreted as a much more rapid rate of turnover for newly synthesized NAPS. The nature of the mechanisms responsible for rapid metabolism of newly synthesized NAPS coupled with slow turnover of NAPS accumulated in excess of normal cellular levels remains obscure. (see below).

Intracellular segregation of newly synthesized NAPS. Our earlier demonstration of the cell cycle-specific transfer of phospholipids into the ICM implies that ICM phospholipids are segregated from the phospholipids present in the other cellular membrane systems (3), and the relatively high level of label in NAPS after a short pulse-labeling period (11) suggested a possible test of this hypothesis. If phospholipids accumulate in the ICM largely as the result of intracellular transfer, the apparent rapid turnover of label present in NAPS in the absence of Tris would initially provide a high-specific-activity radioactive phospholipid species whose decay would be short relative to the observed period of phospholipid transfer. Therefore, we might expect to find high levels of radioactivity in the NAPS found in the ICM. Cells were labeled with ^{32}P for short periods of time in the absence of Tris, and then whole-cell and chromatophore samples were analyzed in terms of the fraction of the recovered radioactivity in each of the phospholipid species. The fraction of the total phospholipid label present in the individual species during short labeling periods did not reflect absolute cellular phospholipid levels (3, 11).

The first study employed a synchronously dividing cell population (Fig. 5). The label was applied at the onset of cell division (the time of transfer of phospholipids to the ICM) (3) to maximize both the relative level of label in NAPS in whole cells and the number of cells

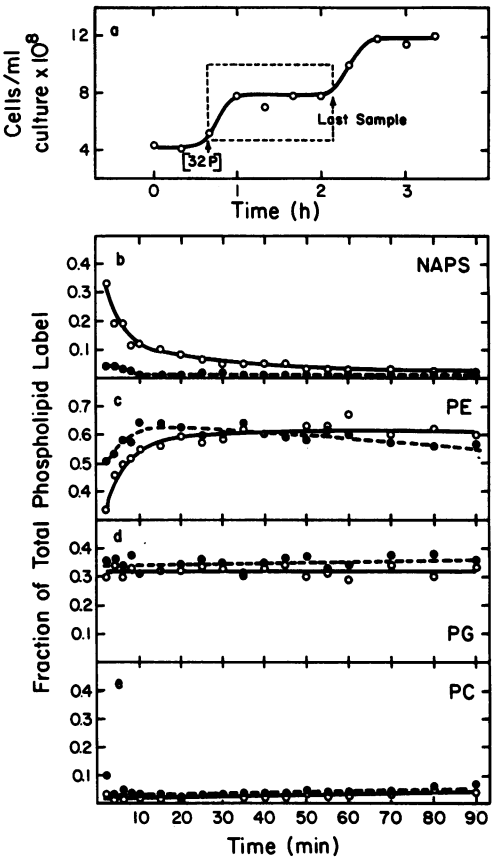

FIG. 5. Distribution of phospholipid label in cells and chromatophores in a synchronously dividing photoheterotrophic culture of *R. sphaeroides* briefly labeled with ^{32}P. A synchronously dividing cell population was obtained as described by Lueking et al. (26). [^{32}P]orthophosphoric acid (100 μCi/ml) was added near the onset of cell division (arrow). Samples (25 ml) were removed at different times. Growth was halted by treatment with 2 ml of sodium azide (1 mg/ml) and chloramphenicol (2 mg/ml) on ice. Individual phospholipids were resolved from both whole cells and ICM fractions (see text). (a) Cell enumeration. (b through e) Fractions of the total phospholipid label in whole cells (○) and chromatophores (●) for the indicated phospholipid species.

TABLE 2. Turnover of ^{32}P-labeled phospholipids in *R. sphaeroides*

Growth conditions	^{32}P pulse medium[a]	Chase medium	Half-life of turnover (culture doublings)		
			NAPS	PG	PE
Chemotrophic	20 mM Tris	No Tris	17	47	4.1
Photoheterotrophic	20 mM Tris	No Tris	32	ND[b]	3.5
Photoheterotrophic	20 mM Tris	20 mM Tris	ND	ND	4.0

[a] Cells were pulsed with [^{32}P]orthophosphoric acid for at least six culture doublings to insure steady-state labeling of phospholipids.

[b] ND, None detectable.

transferring phospholipids to the ICM (Fig. 5a). Initially, the relative level of label in chromatophore NAPS did not reflect the high proportion of the phospholipid label present in NAPS at the whole-cell level (Fig. 5d). The gradual convergence of whole-cell and chromatophore labels in NAPS fractions of the phospholipid label resulted from the decline in whole-cell NAPS label relative to that of the other phospholipids. The low level of radioactive NAPS in the chromatophore fractions could be accounted for by cell membrane contamination, as judged by the penicillin-binding capacity of membranes prepared as described above. Radioactive PG and PC levels were essentially identical in both whole cells and chromatophores throughout the course of the experiment (Fig. 5e and f), but the lack of transfer of newly synthesized NAPS was reflected in the relative enrichment of labeled PE in the chromatophores (Fig. 5c).

In the absence of Tris, newly synthesized NAPS was not transferred to the ICM, and this implies that the transfer of NAPS to the ICM found earlier (3) was the result of NAPS accumulation in Tris-supplemented medium. Furthermore, the segregation of newly synthesized NAPS away from the ICM confirmed that ICM phospholipids do not freely equilibrate with all of the phospholipids in the other cellular membrane systems (3).

An asynchronously dividing culture generated results essentially identical to those shown in Fig. 5 (data not shown). These data indicated that the segregation of NAPS observed in Fig. 5 was not an artifact of the synchronization procedure and revealed that the lack of phospholipid equilibration between ICM and other membrane systems must be physiologically significant.

DISCUSSION

In this work we partially characterize the kinetic parameters governing induction of NAPS accumulation and turnover of preexisting NAPS in *R. sphaeroides*. Addition of Tris to the culture medium caused a dramatic increase in the apparent rate of NAPS accumulation. The onset of NAPS accumulation occurs immediately upon application of Tris. In contrast, removal of Tris from the medium did not result in rapid turnover of NAPS which had accumulated in excess of normal cellular levels. Although a small quantity of NAPS is metabolized, excessive NAPS levels are largely dissipated through dilution after cell growth. On the other hand, Donohue et al. (11) showed that under conditions in which NAPS was not accumulated (i.e., cultures grown without Tris in the medium), NAPS appeared to be metabolized rapidly. Extension of these studies to the examination of chromatophores indicated a conspicuous lack of newly synthesized NAPS in the ICM when measurements were made in the absence of Tris.

Recently, Onishi and Niederman (33) have reported the accumulation of an unknown phospholipid (designated PX) in *R. sphaeroides* NCIB 8253, which we believe to be NAPS (10, 11). While measuring the initial rates of phospholipid synthesis, these authors reported a lag, which was never observed in any of our studies, between the introduction of [^{32}P]orthophosphoric acid and the first appearance of ^{32}P-labeled phospholipids. The use of a low-specific-activity label for the short labeling periods of the experiment of Onishi and Niederman probably accounts for this lag. Importantly, the presence of a lag, coupled with our observations pertaining to short labeling periods (3, 10) (Fig. 5a), in which we used up to 100-fold-higher specific activities, leads us to interpret the rate determinations of Onishi and Niederman (33) as reflecting label equilibration rather than initial rates of phospholipid synthesis. These authors also reported the rapid turnover of 30 to 40% of the steady-state-labeled PE, PC, and PG (approximately 20% of the cellular phospholipid) after a shift to unlabeled medium. After this initial decrease in the level of label, the level of ^{32}P in these phospholipids remained essentially stable, with a slight increase in radioactivity throughout the remainder of the experiment (33). The data in Fig. 4 are in agreement with the general stability of phospholipids, but no initial rapid turnover of any phospholipid species was observed. Although this difference may be attributed to the use of saturating light intensity in this study (generation time, 2.5 to 3 h), as opposed to the low light conditions of Onishi and Niederman (33) (generation time, 11.8 h), it seems unlikely that the reported rapid decrease in the levels of phospholipid label was due to phospholipid turnover because (i) the apparent turnover did not continue throughout the experiment and (ii) the low light intensity would be expected to promote maximum development of the ICM, with a coincident requirement for phospholipids. However, beyond these suggestions we are unable to provide a more satisfactory explanation for the observations of Onishi and Niederman and those which we report here.

The cell membrane is firmly established as the site of phospholipid synthesis (12) and the source of the phospholipids that constitute the outer membrane (18, 20) in other gram-negative bacteria. Indeed, flow of phospholipids between these membrane systems is bidirectional (18), although there is a lag between synthesis of a new phospholipid and its translocation to the outer membrane (20). In our laboratory we have amassed considerable evidence demonstrating

the cell cycle-specific discontinuous incorporation of phospholipids into the ICM (15, 16, 22, 26). We (3) have also shown that this phenomenon results from a net transfer of phospholipids from a discrete site of synthesis into the ICM concurrent with cell division. These data imply the segregation or metabolic isolation of ICM phospholipids from the phospholipids of the other cellular membranes. Indeed, Russell and Harwood (40) reported a slightly elevated PG level in chromatophores, and Marinetti and Cattieu (27) found PE levels reduced in chromatophores compared with whole cells. However, these differences, which we were unable to observe (11), seem insufficient to conclude unequivocally that the ICM contains a selected population of phospholipids. The dramatic difference in the levels of newly synthesized NAPS in the ICM compared with the levels in whole cells strongly supports the segregation of ICM phospholipids. NAPS clearly was not transferred to the ICM in consort with the other phospholipid species under non-accumulating conditions. This may be due to either (i) the physical inaccessibility of newly synthesized NAPS to be transferred to the ICM or (ii) the fact that the time period of the existence of label in newly synthesized NAPS is very brief compared with the time required to transfer phospholipids to the ICM. The causes for this observation remain to be elucidated, but specificity of the transfer mechanism for PG and PE seems unlikely since NAPS was transferred in proportion to its cellular levels when it accumulated in the presence of Tris (3). Elucidation of the mechanism of exclusion of newly synthesized NAPS from the ICM awaits the development of a short-term pulse-chase labeling technique.

Another implication arises from the assumption that phospholipid metabolism is limited to the cell membrane (which is currently under investigation). We have predicted a faster rate of turnover of accumulated NAPS in chemotrophically grown cells than in phototrophically grown cells, due to the absence of ICM in aerated cells. Although the faster NAPS turnover observed in the chemotrophic culture may not be considered compelling, it is consistent with our hypothesis. Further experimentation will be necessary before the merits of this approach can be assessed.

The slow rate of turnover of accumulated NAPS in excess of normal cellular levels frustrated all attempts to observe a direct precursor-product relationship between NAPS and any of the other phospholipid species, should such a relationship exist. However, our data do indicate that NAPS accumulated in the presence of [^{3}H]glycerol can apparently donate radioactivity specifically to the PS branch of phospholipid metabolism (i.e., not PG). NAPS is an apparent metabolic intermediate normally found in all strains of *R. sphaeroides* tested (11). NAPS is not produced by a known enzymatic activity, as described for the phospholipid biosynthetic pathway of *E. coli* (8, 37). CDP-diglyceride synthetase-defective strains of *E. coli* accumulated phosphatidic acid at the expense of PG (18). Phosphatidylserine synthetase-deficient *E. coli* possessed a reduced PE content (37). PS accumulated in a phosphatidylserine decarboxylase mutant at the expense of PE (19). NAPS accumulation and the corresponding decrease in PE supported the hypothesis that a previously undescribed activity shunts the phospholipid biosynthetic pathway at some point on the PS branch of phospholipid metabolism in *R. sphaeroides*.

Resolution of the uncertainties regarding both the biosynthesis of NAPS and the metabolic fate of NAPS awaits demonstration of enzyme activities in vitro. Regardless, the utility of NAPS as an intrinsic membrane probe for movement of phospholipids is abundantly clear.

ACKNOWLEDGMENTS

We thank Neela Kulkarni for excellent assistance in the induction studies and William D. Shepherd for assistance with penicillin-binding studies.

This work was supported by Public Health Service grant GM 15590 from the National Institutes of Health to S.K. and by Postdoctoral Research Fellowship 1-F32-GMO7633-01 from the National Institutes of Health to T.J.D.

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