

## In Vivo Metabolic Intermediates of Phospholipid Biosynthesis in *Rhodopseudomonas sphaeroides*

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The in vivo metabolic pathways of phospholipid biosynthesis in *Rhodopseudomonas sphaeroides* have been investigated. Rapid pulse-chase-labeling studies indicated that phosphatidylethanolamine and phosphatidylglycerol were synthesized as in other eubacteria. The labeling pattern observed for *N*-acylphosphatidylserine (NAPS) was inconsistent with the synthesis of this phospholipid occurring by direct acylation of phosphatidylserine (PS). Rather, NAPS appeared to be kinetically derived from an earlier intermediate such as phosphatidic acid or more likely CDP-diglyceride. Tris-induced NAPS accumulation specifically reduced the synthesis of PS. Treatment of cells with a bacteriostatic concentration of hydroxylamine (10 mM) greatly reduced total cellular phospholipid synthesis, resulted in accumulation of PS, and stimulated the phosphatidylglycerol branch of phospholipid metabolism relative to the PS branch of the pathway. When the cells were treated with a lower hydroxylamine dosage (50  $\mu$ M), total phospholipid synthesis lagged as PS accumulated, however, phospholipid synthesis resumed coincident with a reversal of PS accumulation. Hydroxylamine alone was not sufficient to promote NAPS accumulation but this compound allowed continued NAPS accumulation when cells were grown in medium containing Tris. The significance of these observations is discussed in terms of NAPS biosynthesis being representative of a previously undescribed branch of the phospholipid biosynthetic sequence.

Kennedy and co-workers (36) deduced the pathway for biosynthesis of bacterial phospholipids by developing in vitro assays for each of the enzymes required for the synthesis of the major phospholipid species of *Escherichia coli*, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Briefly, phosphatidic acid (PA) is reacted with cytidine triphosphate to form CDP-diglyceride. The cytidine monophosphate moiety of the liponucleotide is exchanged for either *sn*-glycerol-3-phosphate or L-serine by the activities of separate enzymes to generate phosphatidylglycerophosphate (PGP) or phosphatidylserine (PS), respectively. A specific phosphatase converts PGP to PG, whereas decarboxylation of PS generates PE. The enzymes of the phospholipid biosynthetic pathway are virtually ubiquitous throughout the eubacteria studied to date (17, 42). Characterization of *E. coli* mutants defective in each activity (12, 22, 36) and cloning of the genes which encode these enzymes (27, 33) have firmly established the basic details of the pathway in vivo. Synthesis of other lipid-containing compounds such as cardiolipin (23), membrane-bound oligosaccharides (38, 40), and glycolipids (32) result from further metabolism of phospholipids. These advances in understanding phospholipid synthesis in *E. coli*

thus provide a framework facilitating the study of physiologically and metabolically more complex systems.

The facultative photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* is an attractive model system for the investigation of membrane biogenesis and differentiation (16, 24, 25). In addition to the typical gram-negative cytoplasmic and outer membranes (4, 41), an extensive intracytoplasmic membrane (ICM) develops in response to growth under conditions of low oxygen tension, with the quantity of ICM inversely related to the light intensity incident upon the culture (1, 9, 11). Studies which used synchronously dividing cell populations to study ICM assembly have shown that although protein (19) and photopigments (28, 44) are incorporated continuously into the ICM throughout the cell cycle, phospholipid accumulation in the ICM occurs discontinuously with respect to the cell cycle (20, 21, 29). This discontinuity results from a bulk transfer of phospholipid to the ICM concurrent with cell division (6). Recently, owing to the importance of the *Rhodospirillaceae* in the study of bacterial photosynthesis, as well as their utility for the study of membrane differentiation, the phospholipid composition (10, 15, 34, 39) of the photosynthetic membrane and the

transbilayer distribution of phospholipids (2, 13, 31, 45) have generated much interest.

During our studies of ICM assembly, we observed an unidentified phospholipid (6) in addition to the known *R. sphaeroides* phospholipids, PE, PG, and phosphatidylcholine (PC). The presence of an unknown phospholipid was independently reported by Onishi and Niederman (34). We identified this novel phospholipid as *N*-acylphosphatidylserine (NAPS) (14). NAPS appears to be virtually ubiquitous and present at normally low levels throughout the *Rhodospirillaceae* (15), and the inclusion of Tris in the culture medium causes substantial accumulations of NAPS in some strains of *R. sphaeroides*, *Rhodopseudomonas capsulata* and *Paracoccus denitrificans* (15). Although the evidence was consistent with the metabolism of NAPS generating PE, the removal of Tris from the medium failed to result in sufficiently rapid turnover of NAPS to unambiguously establish a precursor-product relationship between NAPS and any other phospholipid (7). Importantly, pulse-labeling experiments suggested that NAPS was an early intermediate in normal lipid metabolism in *R. sphaeroides* (7, 15). In this communication, we have examined in more detail the *in vivo* flow of intermediates through the phospholipid biosynthetic pathways in *R. sphaeroides* by using a more rapid pulse-chase-labeling approach. In addition, the effects of hydroxylamine, a known inhibitor of PS decarboxylase in *E. coli* (37), were examined with respect to phospholipid biosynthesis and, specifically, NAPS metabolism. The results obtained are consistent with the synthesis of PE and PG occurring in *R. sphaeroides* as in other eubacteria. We present evidence that NAPS is neither the product of the direct acylation of PS nor an obligate precursor in the synthesis of PS, and, therefore, we suggest that NAPS is an intermediate on a heretofore undescribed branch of phospholipid metabolism.

#### MATERIALS AND METHODS

**Organism, media, and growth conditions.** *R. sphaeroides* M 29-5 (Met<sup>-</sup> Leu<sup>-</sup>) was grown under photoheterotrophic conditions in Sistrom medium A supplemented with 50 µg/ml each of L-methionine and L-leucine (29). Tris supplementation was used to stimulate NAPS accumulation, as specified for each study. Cultures were illuminated by a bank of lumiline lamps (General Electric Co., Schenectady, N.Y.) at intensities indicated for each experiment. The illumination of 10 W/m<sup>2</sup> was saturating for the growth rate (ca. 2.5 h), whereas 3 W/m<sup>2</sup> limited growth to a generation time of ca. 11 h. All cultures were sparged continuously with a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub>. The temperature was maintained at 32°C unless otherwise noted.

Growth was routinely monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter. A value of 270 µg (dry weight) per ml corresponds to a culture turbidity of 100 photometer units. Cells were observed directly by phase contrast microscopy and enumerated by using a Petroff-Hausser bacteria counter.

**Rapid pulse-chase labeling of lipids.** Cultures were grown for at least eight generations in medium supplemented with 10 µg of glycerol per ml at a light intensity of 3 W/m<sup>2</sup>. Exponentially growing cells were aseptically harvested (12,000 × *g* for 5 min) and washed once with fresh, presparged medium. The cells were inoculated into 200 ml of fresh medium lacking glycerol (8 × 10<sup>8</sup> cells per ml, final concentration) and allowed to grow for 30 min before the addition of [2-<sup>3</sup>H]glycerol (10 Ci/mmol) to a final concentration of 5 µCi/ml. After 30 s, labeling was terminated by injection of an unlabeled chase solution of 25% (vol/vol) glycerol to a final concentration of 1 mg/ml. Vigorous sparging and stirring facilitated rapid mixing of the label and chase solutions.

Culture samples were withdrawn, and their volumes (ca. 9 ml) were measured and then mixed with 25 ml of methanol. Samples (5.5 ml) were then added to tubes containing 2 ml of chloroform for lipid extraction.

**Hydroxylamine treatment.** Exponentially growing cultures (light intensity, 10 W/m<sup>2</sup>) were pre-labeled with [<sup>32</sup>P]orthophosphoric acid (100 µCi/ml, final concentration) for one generation. Hydroxylamine was added to the culture from a 1 M sterile stock solution; final concentrations of hydroxylamine are noted in the details of each experiment. Culture samples were withdrawn, and 1.4-ml samples were added to tubes containing 6 ml of chloroform-methanol (1:2) for lipid extraction.

**Extraction and quantitation of lipids.** Lipids were extracted by the method of Blich and Dyer (3, 5), and the resulting chloroform phases were washed once with 4 ml of 1% NaCl. During rapid pulse-chase studies, authentic NAPS, PA, and PS (10 µg each) were included in the extraction mixture to facilitate visualization of the resolved phospholipid species after chromatography. Application of the two-dimensional thin-layer chromatography (TLC) system of Poorthuis et al. (35) to *R. sphaeroides* phospholipids has been described elsewhere (14). Figure 1 shows an extension of this approach to the resolution of the major phospholipids of *R. sphaeroides* from known intermediates in phospholipid metabolism. Lipids were detected by iodine-vapor staining, and the spots were scraped directly into scintillation vials. <sup>3</sup>H-containing samples were quantitated by using a Triton X-100 toluene scintillation cocktail; <sup>32</sup>P samples were counted in a toluene-based scintillant. Recoveries of radioactive phospholipids from the thin-layer plates (as estimated by comparison of the sum of the radioactivity in individual phospholipids to the total chloroform soluble radioactivity) was routinely 90% or greater.

PGP, which does not move sufficiently away from the chromatographic origin in this chromatography system to allow unambiguous quantitation, was identified by enzymatic conversion to PG. Radioactive samples were initially chromatographed on TLC plates, and the origin region was collected. Unlabeled *R. sphaeroides* phospholipids were added, and the

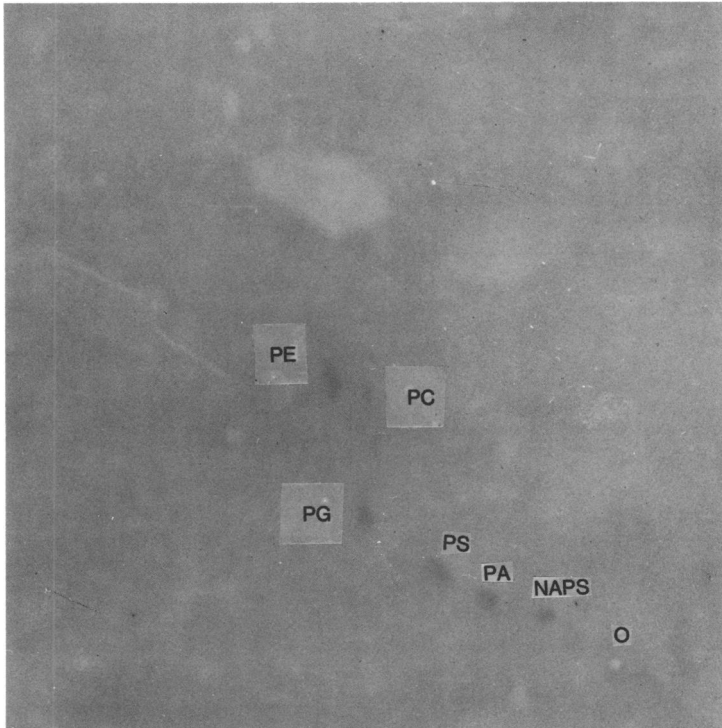


FIG. 1. Resolution of *R. sphaeroides* phospholipids from PA and PS by two-dimensional TLC (35). Phospholipids (200  $\mu\text{g}$  dissolved in 60  $\mu\text{l}$  of chloroform) were spotted on the plate (0.25-mm Silica gel G impregnated with 0.4 M boric acid). Chloroform-methanol-water-ammonium hydroxide (70:30:3:2) constituted the first dimension, whereas the second dimension was chloroform-methanol-water (65:35:5); the plate was dried under a nitrogen atmosphere between dimensions. Phospholipids were visualized by using iodine vapor staining. To facilitate photography, this plate was sprayed with a soluble starch suspension (5 g/100 ml of water). The resulting violet stain was virtually permanent.

lipids were extracted into chloroform as before. The extract was reduced to dryness under a stream of  $\text{N}_2$  and then resuspended by sonication into an aqueous buffer (38). The lipid suspension was brought to 1 ml with 0.1 M Tris-hydrochloride (pH 8.0). The mixture was incubated at 37°C for 1 h with 16 U of alkaline phosphatase. Control experiments with authentic PGP under identical conditions generated >99% conversion of PGP to PG (data not shown). The products were separated chromatographically, and radioactivity was measured as above.

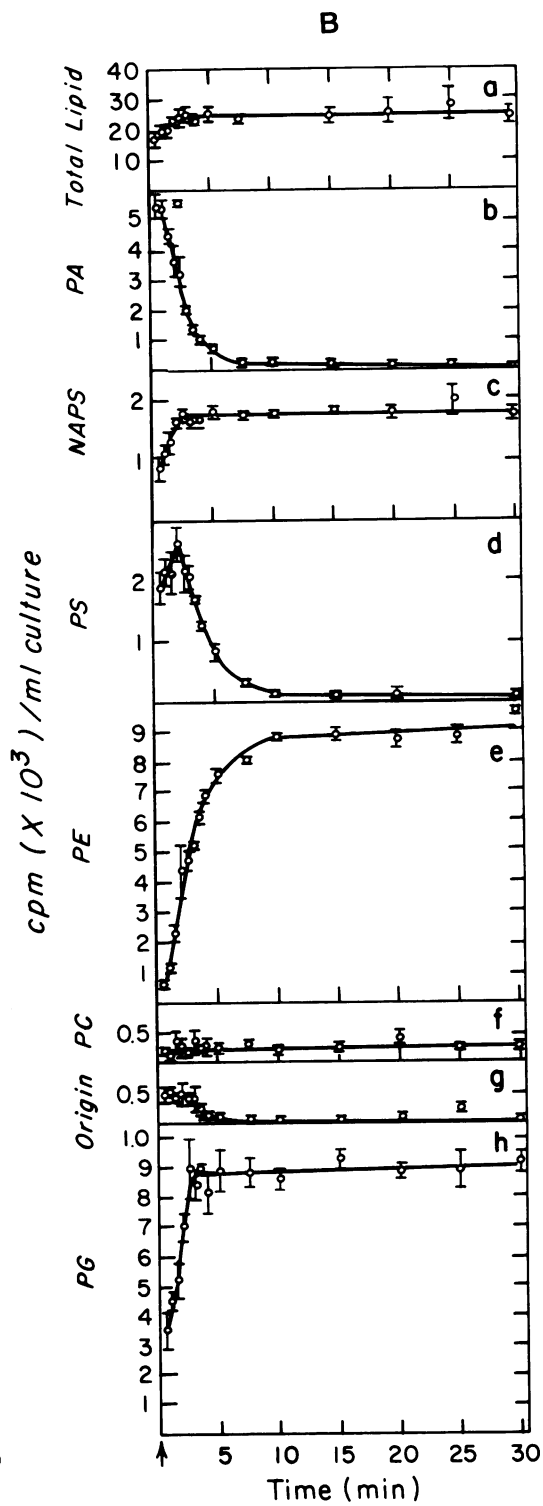
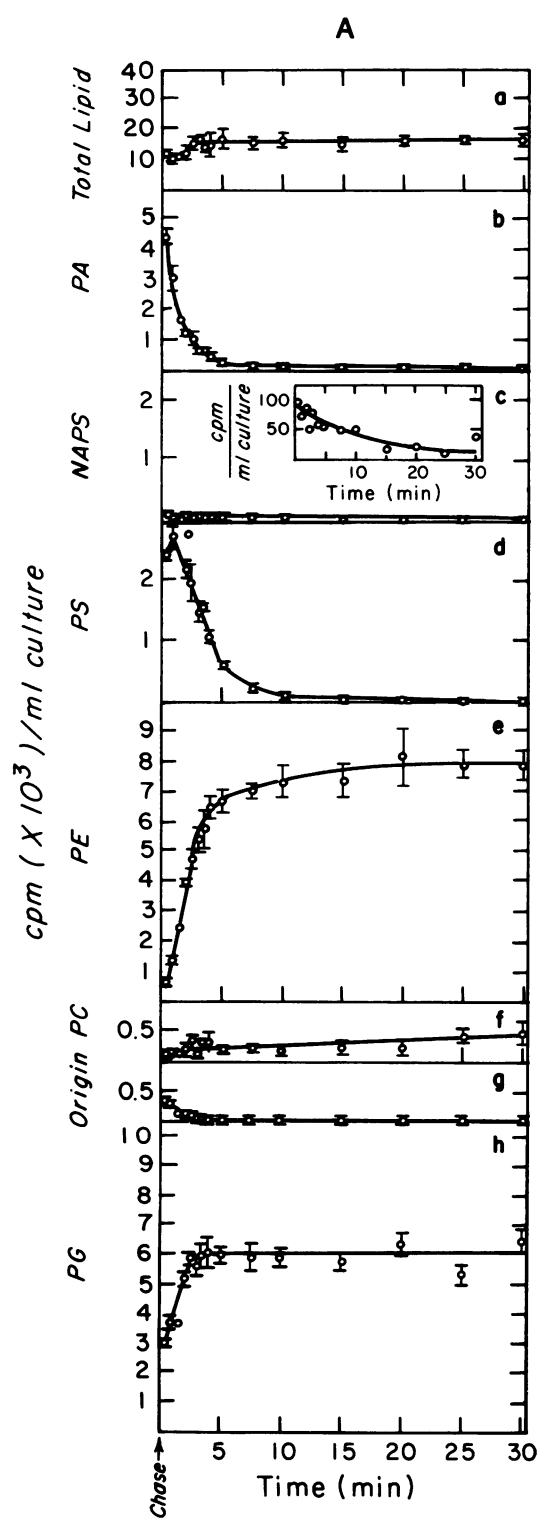
**Materials.** All solvents were of reagent grade and used without further purification. Silica gel G was purchased from Supelco Inc., Bellefonte, Pa. [ $^3\text{H}$ ]glycerol (10 Ci/mmol) and [ $^{32}\text{P}$ ]orthophosphoric acid (carrier free) was obtained from New England Nuclear Corp., Boston, Mass. Alkaline phosphatase (bovine intestine, type VII-S), PS (bovine brain), and PA (from egg yolk lecithin) were the products of Sigma Chemical Co., St. Louis, Mo. The PGP standard was the kind gift of Christian Raetz, University of Wisconsin, Madison.

## RESULTS

**Metabolism of phospholipid intermediates.** Our previous kinetic studies suggested that NAPS

was an early intermediate in phospholipid metabolism normally present in small quantities in *R. sphaeroides* (7, 15). A direct test of this hypothesis required the development of a rapid pulse-chase-labeling technique to facilitate the observation of the label moving through NAPS relative to the other phospholipid species. [ $^2\text{H}$ ]glycerol was selected as the label since previous experiments with [ $^{32}\text{P}$ ]orthophosphoric acid have shown that this label would be unsuitable for use over the time scale required in these studies (6, 26). A reduction of light intensity was used to limit the growth of the culture and, therefore, to extend the effective length of the labeling period (data not shown). Control experiments had shown no effects on NAPS accumulation or bulk phospholipid composition with changing light intensity (15). In initial studies, Tris was omitted from the medium so that any turnover of NAPS would not be masked by its accumulation.

Figure 2A shows the results of this experiment. The glycerol chase solution effectively stopped label incorporation into lipids within 2.5



min (Fig. 2Aa). The initially large quantity of label in PA, the first identifiable glycerol-containing intermediate in phospholipid biosynthesis, rapidly dissipated as the label moved into other phospholipids (Fig. 2Ab). As expected for a midpathway intermediate, PS labeling increased as PA declined (presumably via CDP-diglyceride; see below), with a maximum at 1.5 min after injection of the chase (Fig. 2Ad). Afterward, the label in PS dropped with a coincident accumulation of label in PE (Fig. 2Ad and e), clearly demonstrating a precursor-product relationship between these phospholipids. As expected, the short time scale of this and subsequent experiments was inappropriate to allow significant PE conversion to PC (Fig. 2Af) (6, 26).

The small quantity of radioactivity initially present at the origin dropped sharply within 3 min (Fig. 2Ag). This region represents the sum of CDP-diglyceride, PGP, and perhaps other compounds which do not reproducibly migrate from the origin. In the 0.5- and 1.0-min samples, at least 20% of the origin counts were shown to be converted to PG by using alkaline phosphatase, proving that this portion of the origin label was PGP. Interestingly, as the origin label approached zero, PG label reached its maximum (Fig. 2Ah). Apparently, the *in vivo* lifetime of PGP was much shorter than that of PS, the equivalent intermediate on the other branch of the phospholipid biosynthetic pathway. Although there is some residual radioactivity entering lipids during the first 2.5 min after the addition of the chase, PG appeared kinetically to be derived primarily from PA.

Although a steady decrease in the amount of NAPS label was seen, NAPS represented only approximately 1% of the total lipid label at the earliest time point after the addition of the glycerol chase and only 0.3% of the total label by 2.5 min after the chase (Fig. 2Ac). Unfortunately, owing to the lack of sufficient labeling of NAPS, no precursor-product relationship could be discerned between NAPS and the other phospholipids. However, the decrease in NAPS label

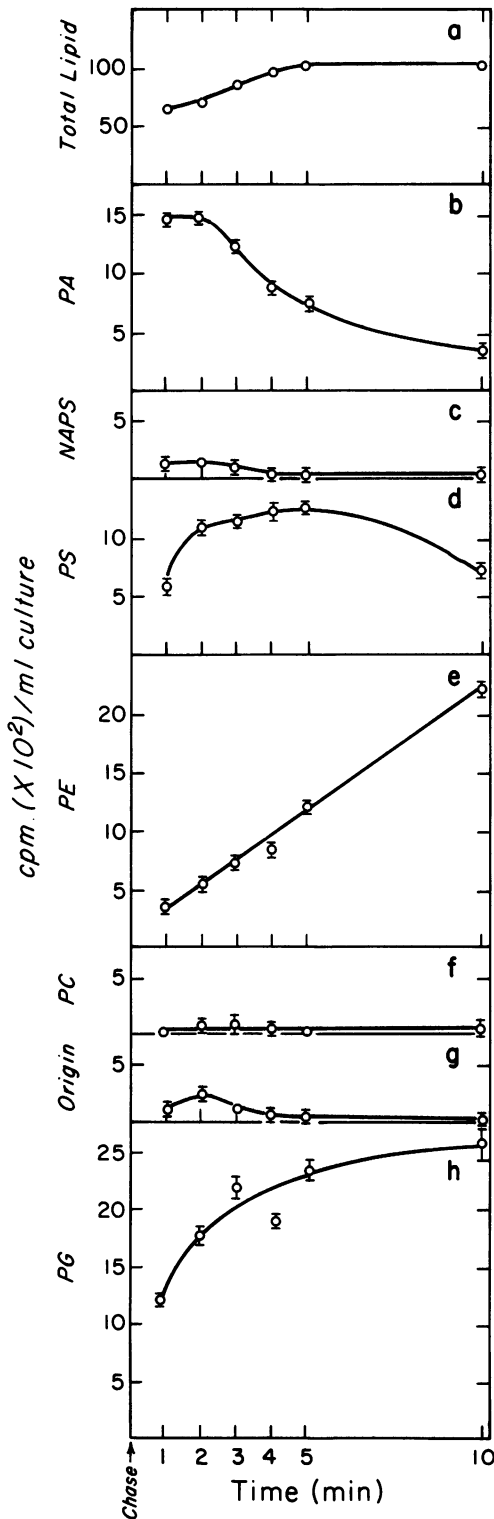
over the course of the experiment supported the hypothesis that NAPS was an early metabolic intermediate, albeit with a relatively short lifetime.

To increase the quantity of label in NAPS by virtue of its accumulation, the pulse-chase experiment was repeated but with 20 mM Tris in the medium. The results of this experiment are shown in Fig. 2B.

The increased total lipid label (35%) in Fig. 2Ba over Fig. 2Aa was reflected in a proportionate increase in most phospholipid species, with the exception of PE (see below), and probably reflected a variance in label uptake over the brief pulse period. The chase was, however, equally effective with the incorporation of label ceasing 2 to 2.5 min after the addition of the excess glycerol (Fig. 2Ba). Loss of the PA label went largely as described above (Fig. 2Bb). As expected, the presence of Tris in the medium caused the accumulation of NAPS so that immediately after the addition of the chase, NAPS represented 6.6% of the total lipid label rising to a maximum of 7.9% of the total label within 2.5 min after the addition of the chase under these conditions (Fig. 2Bc). Simultaneously, PS labeling peaked, and as the label moved out of PS into PE, none could be seen entering NAPS (Fig. 2Bd). Indeed, NAPS labeling achieved its maximum level well ahead of the plateau in labeling of PE at 10 min (Fig. 2Be). As previously reported (7, 15), NAPS accumulation occurred largely at the expense of PE (i.e., the total lipid label increased 35% in experiment B but the absolute quantity of label in PE only increased slightly). In contrast, the final level of PG labeling in the second experiment did reflect the increase in total lipid label.

As we demonstrated above, all suspected intermediates between PA and PG were kinetically "short-lived." The kinetics of NAPS labeling in this study closely resembled the PG kinetics and were clearly distinct from those observed for PS and PE. The direct implication of these observations was that the accumulation of label into NAPS from PA was inconsistent with the

FIG. 2. Kinetics of rapid [ $^3\text{H}$ ]glycerol pulse-chase-labeling of phospholipids in illumination-limited slow growing cultures of *R. sphaeroides* in the absence (experiment A) and presence (experiment B) of 20 mM Tris. The cells were labeled with [ $^3\text{H}$ ]glycerol (5  $\mu\text{Ci/ml}$ ) for 30 s, and then an unlabeled chase solution (25% [vol/vol] glycerol, 1 mg/ml final concentration) was added. Culture samples from which phospholipids were extracted were removed, and the individual species were resolved by TLC. Each sample analyzed by TLC was the equivalent of 1.5 ml of cells, and the radioactivity contained in each phospholipid species was determined by counting samples for 10 min. The data shown are expressed in terms of counts per minute of  $^3\text{H}$  in each phospholipid species per milliliter of culture. The error bars indicate one standard deviation from the mean of quadruplicate samples. Space limitations precluded including error bars for the data points shown in the insert of (Ac), but the range for these values is plus or minus 25% in the worst cases. Recovery of  $^3\text{H}$  from the thin-layer plates (as estimated by comparison of the sum of the radioactivity in the individual phospholipid species to the total chloroform-soluble radioactivity) averaged 92%. (a) Total cellular phospholipid radioactivity. (b through h) Radioactivity of the indicated phospholipid species.



presence of any "long-lived" intermediates (i.e., PS) between PA and NAPS, and therefore NAPS was a primary kinetic product of PA.

To test our supposition, another pulse-chase experiment was performed with cells unperturbed by Tris but growing at a lower temperature (20°C). We reasoned that cooling in addition to low incident light would slow cell growth further and should therefore decrease the relative labeling period as well as expand the resolution of the flow of label through phospholipid intermediates during the period immediately after the addition of the chase. If NAPS were not a product of PS metabolism but rather a product of an earlier intermediate (i.e., PA or CDP-glyceride), then the maximum extent of NAPS labeling might be expected to precede or coincide with the peak in PS labeling.

The low incubation temperature decreased the overall rate of phospholipid metabolism (as evidenced by the drop in the total lipid label) and increased the time necessary for the chase to be totally effective (Fig. 3a). The decrease in PA labeling was also slowed as a result of the slower chase kinetics and low temperature (Fig. 3b). As predicted, the maximum in NAPS labeling (3.2% of the total lipid label) occurred at 2 min, well in advance of the PS peak at 5 min (Fig. 3c and d). Overall phospholipid metabolism was slowed to such an extent in this study that PS conversion to PE was not complete over the time scale of this experiment (Fig. 3e). Interestingly, the maximum in labeling of NAPS coincided with a peak in labeling at the origin (Fig. 3g) and a maximum in labeling of PA (Fig. 3b). Unfortunately, the low quantity of label at the origin precluded the identification of the labeled compound(s) in this experiment. As in the previous studies, PG labeling reached its zenith coincident with the PS-labeling peak (Fig. 3h).

**Effect of hydroxylamine.** Since the experiments described above suggested that NAPS either preceded PS in the biosynthetic pathway or was on an entirely different branch of the pathway, we reasoned that blockage of the PS branch might cause NAPS accumulation if NAPS were an obligate precursor to PS. In view of the precursor-product relationship between

FIG. 3. Kinetics of rapid pulse-chase-labeling of phospholipids in a cooled, low-light culture of *R. sphaeroides*. The experimental protocol was exactly as described for Fig. 2A, except that the temperature was reduced to 20°C throughout the labeling and sampling periods. Sample size, handling, and analysis was performed exactly as described in the legend to Fig. 2. (a) Total cellular phospholipid radioactivity. (b through h) Radioactivity of the indicated phospholipid species.

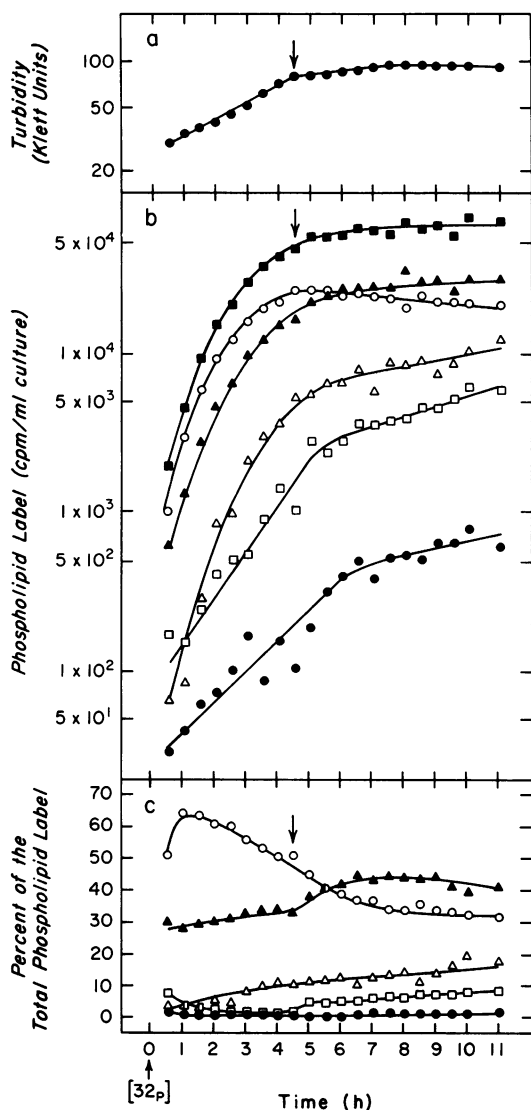


FIG. 4. Effect of the addition of a bacteriostatic concentration of hydroxylamine to an exponentially growing culture of *R. sphaeroides*. [ $^{32}\text{P}$ ]orthophosphoric acid (100  $\mu\text{Ci/ml}$ ) was added to the cells at zero time. After 3 h, 1 M hydroxylamine was added to a final concentration of 10 mM. Culture samples (1.4 ml) were removed for phospholipid extraction, and the individual phospholipid species were resolved by TLC. (a) Culture turbidity. (b) Radioactivity of total phospholipids and individual phospholipid species expressed as counts per minute per milliliter of culture. (c) Percent of the total phospholipid label in the individual phospholipid species. Symbols:  $\blacksquare$ — $\blacksquare$ , total phospholipids;  $\circ$ — $\circ$ , PE;  $\blacktriangle$ — $\blacktriangle$ , PG;  $\triangle$ — $\triangle$ , PC;  $\square$ — $\square$ , PS;  $\bullet$ — $\bullet$ , NAPS.

PS and PE, hydroxylamine (an inhibitor of *E. coli* PS decarboxylase known to cause PS accumulation in vivo [37]) was investigated for this purpose. To avoid ambiguities generated by the relatively slow equilibration of [ $^{32}\text{P}$ ]orthophosphoric acid with the intracellular phosphate pool of *R. sphaeroides*, cultures were pre-labeled for a generation before the addition of the hydroxylamine.

Since 10 mM hydroxylamine had previously been reported to cause accumulation of PS in vivo in *E. coli* (37), our initial studies used hydroxylamine at a final concentration of 10 mM. Growth of the culture was severely retarded immediately upon treatment with 10 mM hydroxylamine, and eventually growth ceased 3 h later, as determined by both turbidity measurements and direct cell counts (Fig. 4a). No abnormal cell morphology was revealed by microscopic examination ( $\times 100$ ) of *R. sphaeroides*. Incorporation of  $^{32}\text{P}$  into phospholipids paralleled growth (Fig. 4b). The PE portion of the label decreased throughout the experiment, reflecting both the accumulation of PC from PE, and PS accumulation after hydroxylamine treatment (Fig. 4c). Interestingly, 10 mM hydroxylamine, although not causing any detectable accumulation of label into NAPS, resulted in additional accumulation of label into PG (Fig. 4c) apparently by perturbing the flow of label through the PS branch of phospholipid metabolism.

In an attempt to study the effect of hydroxylamine in cells capable of increased incorporation of  $^{32}\text{P}$  into phospholipids after hydroxylamine treatment, we elected to reduce the hydroxylamine concentration. A suitable hydroxylamine dose was empirically determined to be 50  $\mu\text{M}$  (data not shown). The experimental protocol was in all other respects exactly as described above.

The growth rate of the culture decreased in response to 50  $\mu\text{M}$  hydroxylamine but did not cease as it had in cells treated with 10 mM hydroxylamine (Fig. 5a). In contrast, the incorporation of  $^{32}\text{P}$  into phospholipids virtually ceased between 1 and 5 h after hydroxylamine treatment (Fig. 5b). Phospholipid synthesis then resumed at a rate faster than culture growth. Label in PE, PC, and PG generally reflected this whole cell pattern, whereas NAPS synthesis also lagged but did not recover over the course of the experiment (Fig. 5b). PS responded immediately to the addition of hydroxylamine by accumulating relative to total phospholipid but then declined, returning to pretreatment levels 5 h later (Fig. 5c). This reversal of PS accumulation coincided precisely with the resumption of phospholipid synthesis, but at present we are unable to explain either the transient nature of PS accumulation or the timing of its reversal.

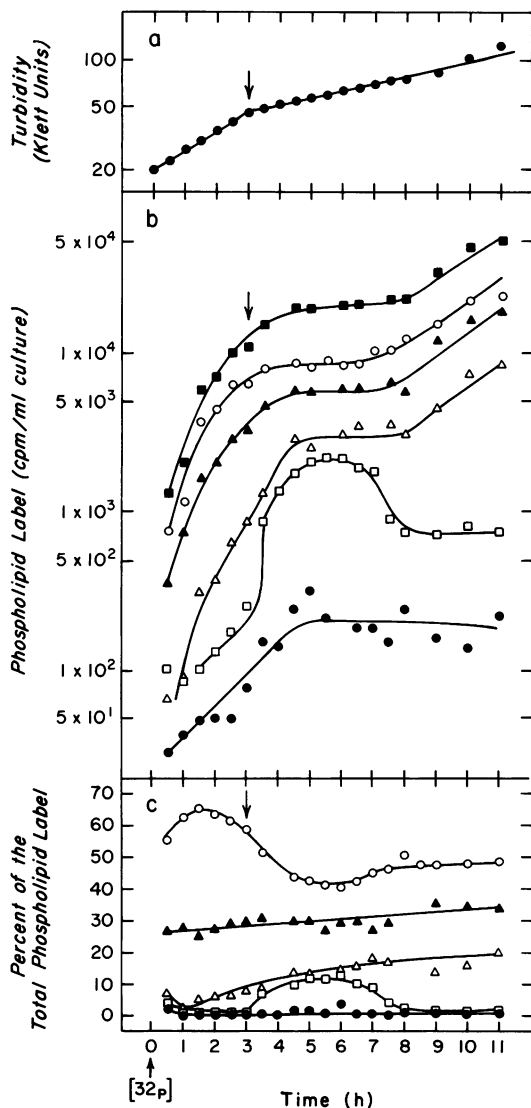


FIG. 5. Effect of the addition of a growth-reducing concentration of hydroxylamine to an exponentially growing culture of *R. sphaeroides*. The experimental protocol was as described for Fig. 4, except that the hydroxylamine concentration was 50  $\mu\text{M}$ . (a) Culture turbidity. (b) Radioactivity of total phospholipids and individual phospholipid species expressed as counts per minute per milliliter of culture. (c) Percent of the total phospholipid label in the individual phospholipid species. Symbols:  $\blacksquare$ — $\blacksquare$ , total phospholipids;  $\circ$ — $\circ$ , PE;  $\blacktriangle$ — $\blacktriangle$ , PG;  $\triangle$ — $\triangle$ , PC;  $\square$ — $\square$ , PS;  $\bullet$ — $\bullet$ , NAPS.

Clearly blockage of the PS branch of phospholipid metabolism with either high or low concentrations of hydroxylamine was insufficient to result in NAPS accumulation. Although this precluded the use of hydroxylamine to study a possible precursor-product relationship between

NAPS and PS, we performed one additional study in which 50  $\mu\text{M}$  hydroxylamine was added to cells growing in medium supplemented with 5 mM Tris. In this way, any relationship, either synergistic or antagonistic between the effects of Tris on phospholipid metabolism and the blockage of PE synthesis by hydroxylamine could be observed.

Cell growth proceeded essentially as before (Fig. 6a). However, in contrast to the earlier studies, which had revealed a lag in  $^{32}\text{P}$  incorporation into phospholipids followed by relatively rapid synthesis of phospholipid, inclusion of Tris in the medium allowed bulk phospholipid synthesis to more closely parallel cell growth after the addition of hydroxylamine (Fig. 6b). The lags in PE, PG, and PC labeling produced by hydroxylamine were apparent but not as pronounced as those seen in the absence of Tris (Fig. 6b). Again PS accumulated to virtually the same level (12% of the total phospholipid label) as before only to decline 5 to 6 h after the addition of hydroxylamine (Fig. 6c). With Tris in the medium,  $^{32}\text{P}$  incorporation into NAPS continued after the addition of hydroxylamine so that NAPS accounted for a greater proportion of the total phospholipid label after cells were treated with hydroxylamine (12% than it did before the addition of this compound (8%). This continued NAPS labeling after the addition of hydroxylamine may account in part for the absence of a significant lag in total phospholipid labeling in this culture (see above) and suggests that the incorporation of  $^{32}\text{P}$  into NAPS under these conditions is independent of  $^{32}\text{P}$  incorporation into the other phospholipids.

## DISCUSSION

This work represents an *in vivo* characterization of the intermediary metabolism of phospholipids in *R. sphaeroides*. *R. sphaeroides* is well suited to the pulse-chase-labeling approach employed since the metabolic activity of the cell can be regulated physiologically by changing the light intensity incident upon the culture (9, 16). The cells undergo normal exponential growth, albeit at a relatively long generation time, under reduced illumination. This enabled us to observe the unperturbed flow of label through the phospholipid biosynthetic intermediates.

Although not all the steps are readily discernible *in vivo*, the data presented here indicate that phospholipid biosynthesis in *R. sphaeroides* is generally consistent with the scheme of bacterial phospholipid synthesis originally deduced by Kennedy and co-workers (36). PA was the earliest identifiable glycerol-containing phospholipid and is presumably the product of the *sn*-glycerol-3-phosphate acyltransferase activity found in *R. sphaeroides* (8, 30). The level of CDP-diglycer-



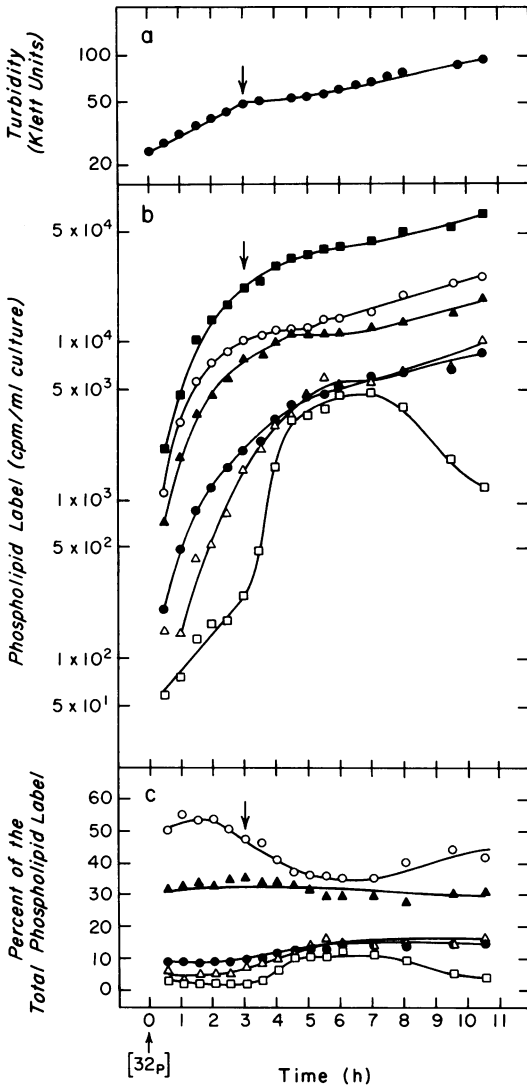


FIG. 6. Effect of the addition of a growth-reducing concentration of hydroxylamine to an exponentially growing culture of *R. sphaeroides* in the presence of Tris. The experimental protocol was as described for Fig. 4, except that the hydroxylamine concentration was 50  $\mu$ M, and 5 mM Tris was included in the medium. (a) Culture turbidity. (b) Radioactivity of total phospholipids and individual phospholipid species expressed as counts per minute per milliliter of culture. (c) Percent of the total phospholipid label in the individual phospholipid species. Symbols: ■—■, total phospholipids; ○—○, PE; ▲—▲, PG; △—△, PC; □—□, PS; ●—●, NAPS.

ide labeling was so low as to be undetectable even under the rapid pulse-chase conditions employed. Minute quantities of PGP were also present, but these experiments do not conclusively demonstrate that PGP is an obligate pre-

cursor to PG. Apparently, the *in vivo* lifetimes of both CDP-diglyceride and PGP are very short; hence, kinetically PG and PS both appear to arise directly from PA.

The transient labeling of PS before the accumulation of label in PE, as well as the accumulation of label in PS at the expense of PE in response to hydroxylamine treatment, confirmed the precursor-product relationship between these lipids. In *R. sphaeroides*, PE conversion to PC occurs on a much slower time scale than that under consideration in these studies (6, 26). The above inferences regarding short-lived intermediates in the phospholipid biosynthetic pathway are also supported by our ability to demonstrate the following enzyme activities in cell-free extracts of *R. sphaeroides*: (i) cytidine triphosphate-PA cytidyltransferase, (ii) CDP-diglyceride:sn-glycero-3-phosphate phosphatidyltransferase, (iii) PGP phosphatase, (iv) CDP-diglyceride-L-serine phosphatidyltransferase, (v) PS decarboxylase and, (vi) S-adenosylmethionine-PE *N*-methyltransferase (B. D. Cain, T. J. Donohue, and S. Kaplan, manuscript in preparation).

The presence of NAPS does not alter the conclusion that *R. sphaeroides* phospholipids are synthesized in a manner typical of other eubacteria. The disappearance of the label initially present in NAPS in the absence of Tris supports the hypothesis that NAPS is an early intermediate in phospholipid metabolism (Fig. 2 and 3). This leads to consideration of the central question, what is the location of NAPS in phospholipid metabolism? We could envision the following possibilities: (i) NAPS is the product of an activity which acylates PS, (ii) NAPS is an obligate intermediate between CDP-diglyceride and PS, and (iii) NAPS is generated from an early intermediate (i.e., CDP-diglyceride or PA) but is a precursor in the synthesis of some lipid-containing compound other than the conventional phospholipids identified in *R. sphaeroides*. In the latter case, PS might still be a product of the utilization of the amide-linked fatty acid of NAPS in some as yet unidentified enzyme reaction, but the primary mechanism of PS synthesis would be via the activity of L-serine-CDP-diglyceride phosphatidyltransferase.

NAPS labeled faster than PS (Fig. 3) arguing that NAPS is not the product of direct acylation of PS. Furthermore, the accumulation of NAPS in response to Tris resulted in a reduction of PS synthesis (Fig. 2B), indicating that NAPS accumulation is, in some antagonistic way, related to PS synthesis. PG synthesis was not affected to the same degree, but previous studies have shown that the steady-state amount of PG was only slightly reduced when NAPS was accumulated in excess of normally low cellular levels

(15). These observations would seem to exclude acylation of PS as the primary mechanism of NAPS synthesis *in vivo*.

Two lines of evidence suggest that NAPS is not an obligate precursor in the synthesis of PS. First, our ability to detect the presence of L-serine-CDP-diglyceride phosphatidyltransferase in cell-free extracts suggests that PS can indeed be synthesized directly from CDP-diglyceride without any intermediate step. Second, since the presence of Tris did not affect the extent of PS accumulation in response to hydroxylamine (Fig. 5 and 6), we conclude that NAPS synthesis is independent of the PS branch of phospholipid synthesis. Therefore, the antagonistic relationship between the accumulation of NAPS and the synthesis of the PS-derived phospholipids was not the result of blocking an activity which converts NAPS to PS. Rather, it appears that the expansion of the NAPS pool competes with the PS branch (and to a much lesser extent the PGP branch) of phospholipid metabolism for phosphodiglycerides. Thus, we feel the experiments presented are inconsistent with NAPS being either a product of PS acylation or an obligate intermediate between CDP-diglyceride and PS.

The opening of the NAPS pool to accumulation by Tris probably results from an alteration of some equilibrium within the normal flow of intermediates through the PG, PS, and what we propose to be the NAPS-derived branches of phospholipid metabolism. Although the data presented indicate that NAPS is kinetically derived from PA *in vivo*, we believe it is more likely that NAPS synthesis is the result of an exchange of *N*-acylserine for the CMP moiety of CDP-diglyceride. The synthesis of *N*-acylated amino acids from acyl coenzyme A donors has been reported in cell-free extracts of *Clostridium kluyveri* (43). The data presented here indicate that NAPS is not the primary precursor to PS *in vivo*, but unfortunately they do not address questions regarding conversion of NAPS to PE and PC, possibly via PS, suggested in our earlier studies (7), which presumably results from the utilization of the *N*-acyl-linked fatty acid of NAPS. The use of hydroxylamine to alter phospholipid metabolism by inhibition of PS decarboxylase revealed that hydroxylamine allowed an apparent NAPS accumulation only when cells were grown in the presence of Tris. This observation excludes any possibility of a similar mode of action for Tris and hydroxylamine *in vivo*, in spite of the evidence that both compounds are capable of eliciting similar responses from many Schiff-base-catalyzed enzyme reactions *in vitro* (18). The specificity of NAPS accumulation in response to Tris is also documented by the inability of physiological buffers

such as morpholinopropane sulfonic acid (28), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (B. D. Cain, unpublished data), or a series of Tris or serine analogs (M. Singer, unpublished data) to result in the accumulation of NAPS. The specific ability of Tris to allow the expansion of the NAPS pool and thus result in the accumulation of this phospholipid over its normally low level is highlighted by the observation that NAPS accumulation does not occur in cells in which the flow of intermediates through the PS-derived branch of phospholipid metabolism has been sufficiently disturbed by hydroxylamine to result in an accumulation of label in PG.

Finally, having identified the position of NAPS relative to the other phospholipid species in *R. sphaeroides*, we are in a position to test for activities capable of converting NAPS to PS or PE *in vitro* and to design meaningful *in vivo* experiments to monitor the utilization of specific chemical components of NAPS. Success in these areas as well as in designing a strategy for screening mutants defective in NAPS metabolism is central to our ability to elucidate the mechanism of Tris perturbation of phospholipid metabolism and, more importantly, the precise role of NAPS in cellular metabolism.

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