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Alterations in the Phospholipid Composition of *Rhodopseudomonas sphaeroides* and Other Bacteria Induced by Tris

TIMOTHY J. DONOHUE, BRIAN D. CAIN, AND SAMUEL KAPLAN*

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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Alterations in the phospholipid head group composition of most strains of *Rhodopseudomonas sphaeroides*, as well as *Rhodopseudomonas capsulata* and *Paracoccus denitrificans*, occurred when cells were grown in medium supplemented with Tris. Growth of *R. sphaeroides* M29-5 in Tris-supplemented medium resulted in the accumulation of *N*-acylphosphatidylserine (NAPS) to as much as 40% of the total whole-cell phospholipid, whereas NAPS represented approximately 28 an 33% of the total phospholipid when *R. capsulata* and *P. denitrificans* respectively, were grown in medium containing 20 mM Tris. The accumulation of NAPS occurred primarily at the expense of phosphatidylethanolamine in both whole cells and isolated membranes of *R. sphaeroides* and had no detectable effect on cell growth under either chemoheterotrophic or photoheterotrophic conditions. Yeast extract (0.1%) and Casamino Acids (1.0%) were found to be antagonistic to the Tris-induced (20 mM) alteration in the phospholipid composition of *R. sphaeroides*. The wild-type strains *R. sphaeroides* 2.4.1 and RS2 showed no alteration in their phospholipid composition when they were grown in medium supplemented with Tris. In all strains of *Rhodospirillaceae* tested, as well as in *P. denitrificans*, NAPS represented between 1.0 and 2.0% of the total phospholipid when cells were grown in the absence of Tris. [^{32}P]orthophosphoric acid entered NAPS rapidly in strains of *R. sphaeroides* that do (strain M29-5) and do not (strain 2.4.1) accumulate this phospholipid in response to Tris. Our data indicate that the phospholipid head group composition of many *Rhodospirillaceae* strains, as well as *P. denitrificans*, is easily manipulated; thus, these bacteria may provide good model systems for studying the effects of these modifications on membrane structure and function in a relatively unperturbed physiological system.

As one of the major components of biological membranes, phospholipids can be expected to have a significant role in membrane structure and function (51), but information is lacking on the relationship between phospholipid synthesis and the assembly of functional biological membranes. One means of studying the role in membrane structure and function of fatty acyl chains (12) or individual phospholipid species (21, 44, 45) in *Escherichia coli* has been the isolation of mutants defective in specific steps in these pathways. The ability to control experimentally either the fatty acid or phospholipid head group composition in these mutants provides a powerful approach for understanding the role of each of these parameters in proper membrane structure or function or both.

The facultative photoheterotroph *Rhodopseudomonas sphaeroides* provides an attractive system to study membrane assembly and differentiation (27). When growing chemoheterotrophically, *R. sphaeroides* contains a typical gram-negative envelope layer (4). Photoheterotrophic growth conditions induce the differentiation of the cytoplasmic membrane, resulting in the synthesis of the intracytoplasmic membrane (ICM) system, which houses the photosynthetic apparatus of the cell. Studies on the regulation of ICM assembly employing synchronously dividing cell populations have shown that insertion of proteins (17) and photopigments (56) into the ICM occurs continuously throughout the cell cycle and that the cell cycle-specific accumulation of phospholipids within the ICM (17, 18, 35) is the result of the net transfer of phospholipids previously synthesized outside the ICM into this membrane system concurrent with cell division (6).

Phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC)

are the major phospholipid species generally found in all strains of *R. sphaeroides* (6, 9, 28, 36, 48). A new phospholipid, recently identified as *N*-acylphosphatidylserine (NAPS) (14), was discovered in *R. sphaeroides* M29-5 during studies on the cell cycle-specific insertion of phospholipids into the ICM (6). Separate studies on the phospholipid composition of *R. sphaeroides* NCIB 8253 have shown either high levels of phosphatidic acid (32), a very low amount of PC (1), or the existence of an unknown phospholipid (42) with chromatographic properties similar to those of NAPS isolated from strain M29-5. In this paper we show that the growth of most strains of *R. sphaeroides*, as well as the growth of other bacteria, such as *Rhodopseudomonas capsulata* and *Paracoccus denitrificans*, in Tris-supplemented medium results in an alteration in the phospholipid head group composition of the cells and the accumulation of NAPS to as much as 40% of the total phospholipid. These investigations, together with those reported in the accompanying paper (7), show the applicability of using the alteration in phospholipid head group composition resulting from NAPS accumulation as a probe for studying the regulation of membrane structure, function, and assembly in *R. sphaeroides*.

(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

Organisms, media, and growth conditions. Table 1 lists the bacterial strains used in this study. As indicated below, the members of the *Rhodospirillaceae* used and *P. denitrificans* were grown either in a succinic acid minimal medium (35) containing 20 mM phosphate (pH 7.0), a low-phosphate (2 mM phosphate, 20 mM Tris, pH 7.0) version of the same medium (18), or the modification of the malate yeast extract medium of Lascelles (31) described by Al-Bayatti and Takemoto (1). When appropriate, Tris was added to the medium from a sterile stock solution previously adjusted to pH 7.0 with hydrochloric acid. For studies employing *R. sphaeroides* strain M29-5 the medium was supplemented with L-methionine and L-leucine at final concentrations of 50 μg/ml. Cells were adapted to growth in the appropriate medium for at least six culture doublings before use.

Photoheterotrophic cultures of members of the *Rhodospirillaceae* were grown either in completely filled culture vessels or by sparging a liquid culture with a mixture of 95% nitrogen and 5% carbon dioxide, with continuous saturating illumination (56 lx) provided by a bank of Lumline lamps. Chemoheterotrophic cells of *R. sphaeroides* were grown by sparging a liquid culture with a mixture of 25% oxygen, 74% nitrogen, and 1% carbon dioxide, and *P. denitrificans* was grown aerobically with vigorous shaking on a rotary shaker. Cells were grown at 32°C, and growth was monitored turbidimetrically by using a Klett-Summerson colorimeter equipped with a no. 66 filter. One photometer unit was equivalent to approximately 10^7 cells per ml under these conditions.

E. coli W3110 was grown in low-phosphate (0.3 mM) 56-LP (11) medium supplemented with M9 salts (39) and 0.4% (wt/vol) glucose. Cultures of *E. coli* were grown aerobically at 37°C by incubation on a gyrotory shaker or by sparging a culture with the same oxygen-nitrogen-carbon dioxide mixture described above. In these cases culture turbidity was monitored by using a colorimeter equipped with a no. 44 filter. Under these conditions 1 photometer unit corresponded to approximately 5×10^6 cells per ml.

Radioactive labeling, extraction, and quantitation of phospholipids. Steady-state labeling of phospholipids with [^{32}P]orthophosphoric acid (final concentration, 10 μCi/ml) was accomplished by inoculating fresh medi-

TABLE 1. Bacterial strains used

Strain	Phenotype	Source
Rhodopseudomonas sphaeroides 2.4.1	Wild type	W. R. Sistrom
R. sphaeroides R26.1	Blue-green carotenoid$^-$ derivative of 2.4.1	W. R. Sistrom
R. sphaeroides RS2	Wild type	S. Harayama
R. sphaeroides 2.4.7	Wild type	J. Wall (Indiana collection)
R. sphaeroides M29-5	Met$^-$ Leu$^-$ derivative of 2.4.7	W. R. Sistrom
R. sphaeroides NCIB 8253	Wild type	R. A. Niederman
R. sphaeroides WS8	Wild type	W. R. Sistrom
R. sphaeroides L	Wild type	J. Lascelles
R. sphaeroides Y	Wild type	H. Gest
R. capsulata B10	Wild type	B. Yen
R. palustris ATCC 17002	Wild type	N. Pfennig
Rhodospirillum rubrum Ha	Wild type	N. Pfennig
Rhodopseudomonas sp. strain SCJ	Wild type	H. Gest
Rhodopseudomonas sp. strain 81-1	Wild type	H. Gest
Paracoccus denitrificans ATCC 13543	Wild type	B. Wilkinson
Escherichia coli W3110	Wild type	Laboratory strain

um with cells which had been grown previously in the same medium for at least six culture doublings. Growth of the cells was allowed to continue in the presence of label for at least another six culture doublings before sampling (final cell density, approximately 10^9 cells per ml). The exact conditions used for studies of the kinetics of phospholipid labeling with [^{32}P]orthophosphoric acid are described below.

The phospholipids present in whole cells or purified membrane samples were extracted into chloroform (2, 5), and the resulting chloroform fraction was washed once with an equal volume of 1% NaCl (26). The proper partitioning of radioactively labeled phospholipid samples was facilitated by the addition of an excess of unlabeled carrier cells (approximately 200 μg of phospholipid per ml of chloroform) to samples before extraction (45). The solvent systems used for two-dimensional thin-layer chromatographic separation (43), as well as the migration of *R. sphaeroides* phospholipids in this system, have been described previously (14). Phospholipids were detected by staining with iodine vapor, and radioactively labeled phospholipids were scraped directly from the thin-layer plates into scintillation vials, where the amount of radioactivity in each phospholipid species was measured by using a toluene-based scintillant (17). Nonradioactively labeled phospholipids were quantitated by lipid phosphorus assays (3).

Preparation and analysis of membrane fractions. The isolation of ICM-derived membrane vesicles (chromatophores) from photoheterotrophically grown cells was performed essentially as described by Fraley et al. (17). Cell membrane and outer membrane fractions from chemoheterotrophic cells were prepared by the method of Ding and Kaplan (13). Labeling of cell membranes and outer membranes with [^{14}C]acetate was accomplished by growing cells chemoheterotrophically for at least six culture doublings in succinic acid minimal medium supplemented with 40 μg of sodium acetate per ml and 0.5 μCi of sodium [1-^{14}C]acetate per ml.

The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein profile of purified membranes (4, 8) was analyzed on either 10% or 10 to 14% gradient SDS slab gels (120 by 160 by 0.8 mm) prepared by the method of Laemmli and Favre (30). Protein determinations were performed on trichloroacetic acid-precipitated samples by the modified biuret assay described by Munkres and Richards (40), using bovine serum albumin as a standard.

Whole-cell absorption spectra were obtained with a Cary model 14 recording spectrophotometer. Mid-exponential phase photoheterotrophic cultures of *R. sphaeroides* M29-5 were harvested by centrifugation. The cell pellet was washed once in 0.1 M sodium phosphate (pH 7.0)–5 mM EDTA buffer and suspended in approximately one-fourth the original culture volume of the buffer described above, and the cells were disrupted by sonication (17). Cell debris was removed by centrifugation at 10,000 × *g* for 20 min, and the supernatant fraction was used as the material for spectral analysis.

Materials. [^{32}P]orthophosphoric acid (carrier-free) and sodium [1-^{14}C]acetate (57.7 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Silica Gel G was obtained from Supelco Inc., Bellefonte, Pa. Trizma base (Sigma Chemical Co., St. Louis, Mo.) or ultrapure Tris (Boehringer Mannheim Corp., New York, N.Y.) were tested as sources of Tris, and no detectable differences were observed in the effects of these preparations on phospholipid composition. Yeast extract and vitamin-free Casamino Acids were obtained from Difco Laboratories, Detroit, Mich., and the responses of cells to these medium supplements were not affected by the different lot numbers tested or by sterilization via membrane filtration or autoclaving. All other chemicals used were of reagent grade. Autoradiograms were analyzed by using type XAR-5 X-ray film (Eastman Kodak, Rochester, N.Y.).

RESULTS

Effect of Tris on whole-cell phospholipid composition. We previously described the existence of a new phospholipid in *R. sphaeroides* M29-5 grown in a low-phosphate version of the succinic acid minimal medium described above [referred to as P(x) by Cain et al. (6)]. In the course of identifying this new phospholipid as NAPS (14), we found that the accumulation of this phospholipid in *R. sphaeroides* M29-5 was dependent on the presence of Tris in the low-phosphate version of the growth medium. The effect of Tris on the phospholipid composition of *R. sphaeroides* M29-5 is shown in Fig. 1. Growth of *R. sphaeroides* M29-5 in medium supplemented with increasing concentrations of Tris increased the fraction of the total phospholipid accounted for by NAPS. Figure 1 also shows that the accumulation of NAPS occurred primarily at the expense of PE, with only minor reductions in the fractions of the total phospholipid accounted for by PG and PC. The growth rate of strain M29-5 was not affected by growth in medium supplemented with as much as 40 mM Tris; however, the presence of Tris at a concentration greater than 40 mM resulted in an increase in the generation time of the cells (data not shown). The effect of Tris on the phospholipid composition of the cells reached a plateau at a Tris concentration of 40 mM; indeed, NAPS can account for as much as 40 to 45% of the total phospholipid of *R. sphaeroides* M29-5 at these Tris concentrations. Although the compositional data shown in Fig. 1 were obtained after steady-state labeling with [^{32}P]orthophosphoric acid, essentially the same results were obtained when the phospholipid composition was determined by lipid phosphorus assays on samples from unlabeled cells (data not shown). Control experiments have also revealed that the accumulation of NAPS is not dependent on the phosphate concentration of the medium, so that the initial discovery of this phospholipid in the low-phosphate medium (6) was dependent solely on the presence of Tris in the growth medium (Table 2).

Table 2 also shows the effect of Tris on the phospholipid composition of various strains of

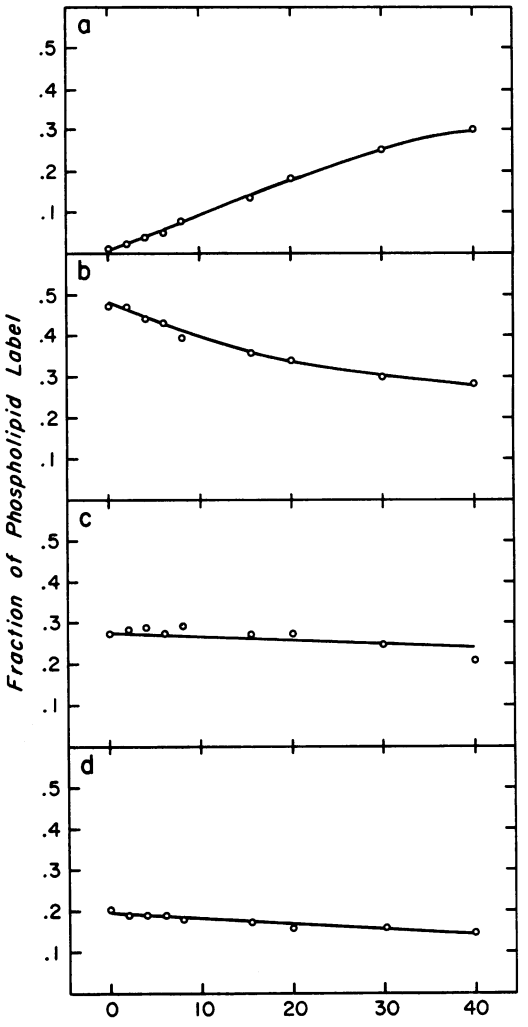

FIG. 1. Effect of Tris on the phospholipid composition of *R. sphaeroides* M29-5. Cells were grown photoheterotrophically and steady state labeled with [^{32}P]orthophosphoric acid (10 μCi/ml) in succinic acid minimal medium supplemented with different concentrations of Tris. At the late exponential phase of growth, 0.6-ml samples were removed, the phospholipids were extracted, and the fraction of the total phospholipid label in each of the phospholipid species was determined after resolution of the phospholipids as described in the text. The generation time of the cells was between 2.5 and 3.0 h in all cases. The data are expressed as the fractions of the total phospholipid label in the individual phospholipid species. The total radioactivity applied per plate was approximately 3 × 10^4 cpm. (a) NAPS. (b) PE. (c) PG. (d) PC.

R. sphaeroides, as well as other members of the *Rhodospirillaceae* and *P. denitrificans*. Although there were some differences in the relative amounts of NAPS present in some of the strains studied, it is evident that with the exception of wild-type strains *R. sphaeroides* 2.4.1 and RS2, all of the *R. sphaeroides* strains tested, as well as *R. capsulata* and *P. denitrificans*, accumulated NAPS when Tris was added to the succinic acid minimal medium. The low levels of NAPS reported for these bacteria grown in medium lacking Tris and the values found for *R. palustris* and *Rhodospirillum rubrum* in both media represent the amounts of NAPS normally present in these cells (approximately 1% of the total phospholipid) plus some small amount of phosphatidylserine (PS), which has chromatographic properties very similar to those of NAPS in the thin-layer chromatography system employed (14, 43; unpublished data). As was the case for the data presented in Fig. 1, the generation time of the bacteria tested was not significantly altered by growth in the presence of 20 mM Tris (data not shown). The data in Table 2 also indicate that accumulation of NAPS by all of the *Rhodospirillaceae* strains tested resulted primarily in a decrease in the relative amount of PE, whereas the accumulation of NAPS by *P. dentrificans* occurred primarily at the expense of PG and to a lesser extent at the expense of PC. The difference in the observed responses of the phospholipid head group composition to Tris between the members of the *Rhodospirillaceae* and *P. denitrificans* may reflect the needs of *P. denitrificans* to maintain what are the normally low levels of PE found in these cells to meet its requirements for PC biosynthesis via methylation of PE (55).

Table 3 shows that growth of *R. sphaeroides* M29-5 under either photoheterotrophic or chemoheterotrophic conditions in the presence of Tris resulted in similar alterations in the whole-cell phospholipid composition. In addition, the phospholipid composition of membranes isolated from either photoheterotrophic or chemoheterotrophic cells was virtually identical to the phospholipid composition of the cells from which the membranes were derived. Thus, when NAPS was accumulated, it was distributed together with the other phospholipid species to all of the membrane systems of the cells.

A comparison of membranes isolated from *R. sphaeroides* M29-5 grown in medium lacking Tris with membranes from cells grown in Tris-supplemented medium revealed no detectable differences in either the ratio of membrane protein to phospholipid, the SDS-PAGE profiles of chromatophores isolated from photoheterotrophic cells, or the SDS-PAGE profiles of cell membranes and outer membranes purified from chemoheterotrophic cells (data not shown). Autoradiographs of SDS-PAGE profiles of membrane samples prepared from [^{14}C]acetate-labeled cells showed that chemoheterotrophic

TABLE 2. Whole-cell phospholipid compositions of various bacterial strains[a]

Strain	Medium supplement	% Of total phospholipid[b]			
		NAPS	PG	PC	PE
Rhodopseudomonas sphaeroides 2.4.7	None	0.9 (0.3)	37.0 (2.4)	18.6 (3.5)	45.2 (2.5)
	20 mM Tris	41.3 (3.2)	25.5 (2.0)	14.0 (1.5)	19.6 (1.5)
R. sphaeroides M29-5	None	1.4 (0.3)	33.7 (0.9)	23.5 (1.6)	42.0 (1.8)
	20 mM Tris	33.9 (5.0)	27.3 (2.5)	16.3 (2.4)	22.5 (0.3)
	20 mM Tris[c]	36.0 (1.8)	23.3 (1.2)	13.9 (1.4)	22.4 (1.5)
R. sphaeroides 2.4.1	None	1.3 (0.5)	29.2 (1.0)	19.4 (2.6)	50.0 (3.2)
	20 mM Tris	0.9 (0.3)	31.0 (1.0)	22.6 (4.2)	45.5 (3.5)
R. sphaeroides R26.1	None	0.9 (0.2)	30.2 (1.4)	22.2 (1.9)	44.9 (2.4)
	20 mM Tris	0.9 (0.4)	32.0 (1.0)	25.6 (2.4)	41.6 (2.3)
R. sphaeroides RS2	None	1.0 (0.3)	29.1 (1.2)	25.3 (3.3)	44.5 (2.6)
	20 mM Tris	1.2 (0.6)	33.3 (1.5)	22.2 (4.3)	43.6 (3.6)
R. sphaeroides NCIB 8253	None	1.2 (0.3)	32.3 (2.2)	17.1 (1.4)	49.5 (1.7)
	20 mM Tris	44.9 (10.4)	20.4 (3.2)	11.9 (3.3)	22.9 (4.6)
R. sphaeroides L	None	2.2 (1.3)	32.6 (2.1)	21.0 (1.9)	44.1 (1.3)
	20 mM Tris	47.0 (1.2)	20.5 (0.6)	12.8 (1.4)	19.7 (2.5)
R. sphaeroides WS8	None	2.1 (0.3)	37.0 (0.6)	16.3 (0.8)	44.4 (1.1)
	20 mM Tris	33.2 (2.1)	28.2 (0.6)	13.3 (1.7)	26.3 (1.3)
R. sphaeroides Y	None	1.2 (0.1)	34.4 (1.6)	15.4 (2.2)	49.0 (0.5)
	20 mM Tris	33.8 (1.4)	25.8 (3.2)	14.5 (1.8)	27.8 (0.8)
R. capsulata B10	None	1.5 (0.5)	36.9 (0.3)	18.6 (0.2)	43.3 (0.9)
	20 mM Tris	23.7 (0.8)	28.2 (0.3)	16.3 (0.4)	29.8 (3.7)
R. palustris ATCC 17002	None	2.9 (0.6)	19.6 (1.8)	25.4 (2.5)	51.9 (4.3)
	20 mM Tris	3.4 (0.8)	17.8 (1.1)	22.3 (3.1)	55.1 (2.7)
Rhodospirillum rubrum Ha	None	1.9 (0.8)	35.2 (1.6)	7.8 (1.0)	54.2 (4.3)
	20 mM Tris	3.0 (1.2)	35.8 (3.0)	9.0 (2.0)	52.3 (4.5)
Rhodopseudomonas sp. strain SCJ	None	1.4 (0.3)	34.6 (0.1)	15.6 (1.5)	48.5 (3.5)
	20 mM Tris	22.8 (0.2)	22.6 (0.2)	20.2 (0.3)	36.0 (1.9)
Rhodospeudomonas sp. strain 81-1	None	0.8 (0.2)	31.7 (2.1)	19.9 (1.2)	47.6 (0.8)
	20 mM Tris	28.8 (0.2)	26.3 (1.7)	12.2 (1.8)	32.6 (0.2)
Paracoccus denitrificans ATCC 13543	None	1.2 (0.6)	49.3 (6.2)	27.8 (2.7)	7.9 (1.6)
	20 mM Tris	33.1 (2.6)	37.6 (5.7)	19.5 (1.3)	6.0 (1.9)

[a] The *Rhodospirillaceae* strains were grown photoheterotrophically, and *P. dentrificans* was grown aerobically for at least six culture doublings in succinic acid minimal medium (20 mM phosphate) containing [^{32}P]orthophosphoric acid (10 μCi/ml) and additional supplements where indicated. The phospholipids contained in 0.6-ml culture samples were extracted and resolved by two-dimensional thin-layer chromatography, as described in the text. The total radioactivity applied per plate was approximately 10^4 cpm.

[b] Expressed as the percentage of the total phospholipid label present in each of the four major phospholipid species. These four phospholipid species routinely represent more than 95% of the total phospholipid label. The remainder of the phospholipid label is contained in cardiolipin (approximately 2 to 3%), PS, and the mono- and dimethyl derivatives of PE (14); each of the latter three phospholipid species routinely accounts for less than 1% of the total phospholipid. We have not included data for these minor phospholipid species since the levels of these phospholipids have not been found to be affected significantly by the growth conditions employed. The data shown are the averages of at least four determinations, and the numbers in parentheses are the standard deviations.

[c] Cells were grown and extracts were analyzed as described in footnote *a*, except that the low-phosphate (2 mM) version of succinic acid minimal medium was employed.

growth of *R. sphaeroides* M29-5 in the presence of 20 mM Tris did not result in significant accumulation of or changes in the level of acylation of the major 47-kilodalton outer membrane lipoprotein (4) of these cells (data not shown). The whole-cell absorption spectra (10) of photoheterotrophic cells were also not affected by growth of strain M29-5 in the presence of as much as 40 mM Tris (data not shown).

The effect on phospholipid metabolism produced by Tris could not be duplicated by growth of *R. sphaeroides* M29-5 in the presence of ethanol (22, 23) or phenethylalcohol (41), compounds which are known to perturb phospholip-

TABLE 3. Phospholipid composition of whole cells and membranes isolated from *R. sphaeroides* M29-5[a]

Growth conditions	Supplement	Cellular fraction	% Of total phospholipid[b]			
			NAPS	PG	PC	PE
Photoheterotrophic	None	Whole cells	1.8 (0.2)	35.4 (1.6)	18.0 (1.2)	45.1 (1.5)
		Chromatophores	1.5 (0.1)	35.7 (1.4)	21.0 (1.2)	42.3 (1.8)
	20 mM Tris	Whole cells	37.9 (0.9)	28.3 (1.8)	13.9 (0.5)	20.0 (1.8)
		Chromatophores	35.6 (0.8)	29.3 (1.1)	15.9 (0.3)	19.3 (0.3)
Chemoheterotrophic	None	Whole cells	0.8 (0.2)	33.8 (2.3)	25.4 (3.4)	35.7 (1.7)
		Cell membrane	1.2 (0.5)	36.6 (1.3)	24.1 (2.6)	37.9 (2.3)
		Outer membrane	0.7 (0.3)	34.5 (0.9)	26.7 (2.8)	38.4 (3.2)
	20 mM Tris	Whole cells	40.5 (1.8)	24.4 (0.7)	15.6 (2.2)	19.5 (3.9)
		Cell membrane	38.0 (0.7)	27.0 (1.2)	17.5 (2.4)	17.5 (3.4)
		Outer membrane	39.4 (1.6)	26.1 (2.0)	14.8 (1.5)	19.9 (1.0)

[a] Cells were grown as indicated and as described in the text; all other conditions were as described in Table 2, footnote *a*. The total radioactivity applied per plate was at least 10^4 cpm.
[b] See Table 2, footnote *b*.

id metabolism in *E. coli* (data not shown). Furthermore, growth of *R. sphaeroides* M29-5 in the presence of Procaine, an anesthetic known to cause accumulation of precursor forms of membrane and periplasmic proteins in *E. coli* (33), did not result in NAPS accumulation.

Kinetics of phospholipid labeling. Previously published studies have shown that [^{32}P]orthophosphoric acid enters NAPS [referred to as P(x) by Cain et al. (6)] rapidly in *R. sphaeroides* M29-5 and that it requires approximately 15 min to attain steady-state labeling of this phospholipid in the low-phosphate version of succinic acid minimal medium containing 20 mM Tris. Figure 2 shows that [^{32}P]orthophosphoric acid entered NAPS very rapidly in both strain M29-5 and strain 2.4.1 but that as a function of time the fraction of phospholipid label contained in NAPS decreased more rapidly in strain 2.4.1 than in strain M29-5. This difference became even more pronounced after longer labeling periods, as steady-state labeling of phospholipids was achieved (6, 7; data not shown). Labeling kinetics of this nature were consistent with the observation that the steady-state level of NAPS in strain M29-5 was approximately 30-fold higher than the level in strain 2.4.1 when the cells were grown in medium containing 20 mM Tris (Table 2). As expected, the kinetics of phospholipid labeling in strain M29-5 in medium lacking Tris (i.e., when NAPS was not accumulated) were virtually identical to those shown in Fig. 2 for strain 2.4.1 (the strain which did not accumulate NAPS in the presence of Tris [7]). The kinetics of phospholipid labeling shown in Fig. 2 are consistent with the hypothesis that NAPS is an early intermediate in phospholipid biosynthesis in both strain 2.4.1 and strain M29-5, and they may suggest that turnover of newly synthesized NAPS is more rapid in cells which are not accumulating this phospholipid. Although this is a possible explanation for the kinetic data obtained, definitive conclusions on this point await the results of a pulse-chase experiment on the same time scale as the experiment shown in Fig. 2. However, because of our difficulty in effectively chasing [^{32}P]orthophosphoric acid over such a brief period of time in *R. sphaeroides* (6), we are currently attempting to perform these experiments with a precursor such as [^{3}H]glycerol.

It should be noted that, with the possible exception of the values shown for PG in Fig. 2, the fractions of the total label present in the individual phospholipid species do not represent the steady-state amounts of these phospholipids in these cells because of the extremely short time of exposure of the cells to [^{32}P]orthophosphoric acid. The relatively slow rate at which label enters PC over the time course of this experiment is consistent with the results of other studies showing the relatively slow kinetics of PC labeling in *R. sphaeroides* (6, 7, 32), which undoubtedly result in part from the existence of a large pool of PE through which the label must pass.

Because PS is known to migrate similarly to NAPS in the thin-layer chromatographic system employed (14, 43) and since PS might also be expected to be an early intermediate in phospholipid biosynthesis in these cells, phospholipid samples for this experiment were treated with trinitrobenzenesulfonic acid (47) to derivatize any ^{32}P-labeled PS to a form easily separable from NAPS (14). The fraction of the total ^{32}P accounted for by PS was no more than 4% of the total phospholipid label in any of the samples (N. Kulkarni and S. Kaplan, unpublished data), and thus the data for the fraction of the total phospholipid label in PS are not included in Fig. 2.

Effect of yeast extract and Casamino Acids on the phospholipid composition of *R. sphaeroides*. In a recent study (1), Al-Bayatti and Takemoto

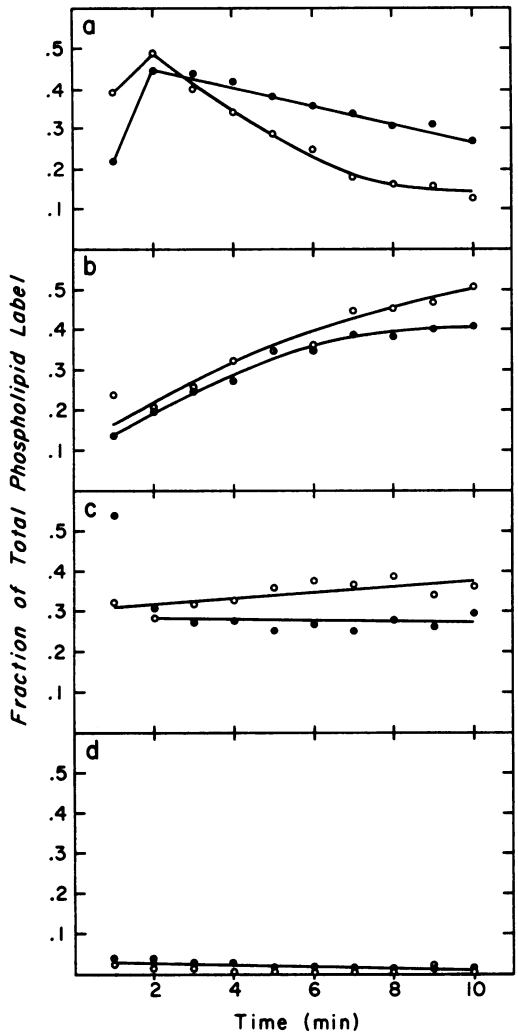

FIG. 2. Kinetics of phospholipid labeling in *R. sphaeroides* M29-5 (●) and 2.4.1 (○). Cells were grown photoheterotrophically for at least six culture doublings in the low-phosphate version of the succinic acid minimal medium (containing 20 mM Tris) described in the text. At zero time exponentially growing cells were transferred to fresh low-phosphate medium supplemented with [^{32}P]orthophosphoric acid (100 μCi/ml). At different times 1.4-ml samples were removed, the phospholipids were extracted, and the fraction of the total phospholipid label present in each of the individual phospholipid species was determined after resolution of the phospholipids as described in the text. The data are expressed as the fractions of the total phospholipid label present in the individual phospholipid species. The discrepancy between the value shown for the fraction of the total phospholipid label present in PG in strain M29-5 after 1 min of labeling and the remainder of the values shown for this phospholipid in (c) is probably the result of an error in the analysis of this sample. We have included the raw data for this sample in order to be complete despite the fact that this discrepancy was not observed in three independent repetitions of this experiment (data not shown). The total radioactivity applied per plate was between 1 × 10^3 and 1.3 × 10^4 cpm. (a) NAPS. (b) PE. (c) PG. (d) PC.

reported a very low level of PC in *R. sphaeroides* NCIB 8253 when cells were grown in a modification of the malate yeast extract medium originally described by Lascelles (31). Table 2 shows that *R. sphaeroides* NCIB 8253, as well as all other *Rhodospirillaceae* strains tested, contained levels of PC consistent with previously reported values (6, 9, 14, 28, 32, 36, 48) when cells were steady state labeled in succinic acid minimal medium. Therefore, we investigated whether growth in the malate yeast extract medium resulted in a diminution in the level of PC and found that all of the *R. sphaeroides* strains tested (strains 2.4.1, 2.4.7, M29-5, and NCIB 8253) contained normal amounts of PC (i.e., approximately 15 to 20% of the total phospholipid) when cells were steady state labeled in this medium (Table 4).

In the course of these experiments, we found that Tris was relatively ineffective in stimulating the accumulation of NAPS when *R. sphaeroides* strains 2.4.1, 2.4.7, M29-5, and NCIB 8253 were grown in the malate yeast extract medium (Table 4). Subsequent studies showed that yeast extract alone was capable of preventing the Tris-induced accumulation of NAPS even in the succinic acid minimal medium in which NAPS accumulation was first described (Table 4). Figure 3 shows that increasing concentrations of yeast extract or Casamino Acids had a dramatic effect on the phospholipid composition of strain M29-5, with 0.1% yeast extract or 1.0% Casamino Acids being sufficient to virtually eliminate any accumulation of NAPS produced by 20 mM Tris. As expected from previous experiments (Fig. 1 and Table 2), the relative amount of PE was reciprocally related to the decreased levels of NAPS, whereas the fraction of the total phospholipid accounted for by PG remained relatively static over the range of yeast extract and Casamino Acid concentrations employed. Interestingly, the relative amount of PC increased abruptly from approximately 13 to 25% of the total phospholipid at yeast extract concentrations between 0.05 and 0.1%, whereas this effect was not observed with Casamino Acids. The exact reason for this abrupt increase is not known at this time, but it has been observed during several independent experiments. However, the fact that the relative amount of PG did not increase until after the level of PE had reached a plateau may reflect the necessity or availability of PE for PC biosynthesis, since methylation of PE appears to be the primary route for PC biosynthesis in *R. sphaeroides* (6, 7, 20).

TABLE 4. Effect of yeast extract on the phospholipid composition of *R. sphaeroides*[a]

Strain	Medium[b]	Supplements		% Of total phospholipid[c]			
		Tris (mM)	Yeast extract (%)	NAPS	PG	PC	PE
NCIB 8253	MYE			1.9 (1.0)	33.2 (1.5)	21.4 (0.8)	43.5 (2.0)
	MYE	20		2.5 (0.9)	29.5 (0.9)	23.9 (1.5)	44.1 (2.9)
2.4.1	MYE			1.4 (0.6)	29.5 (0.6)	12.1 (0.5)	56.3 (1.4)
	MYE	20		1.1 (0.4)	30.4 (1.9)	11.8 (0.2)	56.2 (3.3)
2.4.7	MYE			1.3 (0.2)	31.2 (0.9)	25.4 (1.6)	42.0 (1.0)
	MYE	20		2.7 (0.5)	33.7 (1.7)	22.1 (3.4)	42.0 (3.2)
M29-5	MYE			1.4 (0.5)	32.2 (1.6)	23.4 (1.2)	42.7 (1.5)
	MYE	20		1.7 (0.7)	32.0 (1.3)	21.9 (0.9)	44.3 (0.4)
	S			2.4 (0.2)	34.6 (0.4)	21.0 (0.3)	41.6 (0.5)
	S	20		37.9 (1.0)	28.3 (1.4)	13.9 (0.5)	20.0 (2.0)
	S		0.2	1.2 (0.4)	30.7 (0.9)	28.6 (0.6)	39.7 (1.1)
	S	20	0.2	3.1 (0.2)	31.1 (0.2)	25.3 (0.3)	40.9 (0.5)
	S	50	0.2	4.3 (0.6)	30.5 (3.1)	26.3 (2.3)	37.4 (1.3)

[a] Cells were grown photoheterotrophically in medium for at least six culture doublings before being transferred to fresh medium for steady state labeling of phospholipids as described in the text. All other conditions were identical as described in Table 2, footnote *a*. The total radioactivity applied per plate was approximately 10^4 cpm.

[b] MYE, Modification of the malate yeast extract of Lascelles (31), as described in the text (1) (the yeast extract concentrations in this medium was 0.2%); S, succinic acid minimal medium (20 mM phosphate), as described in the text.

[c] See Table 2, footnote *b*.

The cause of the antagonistic effect of yeast extract or Casamino Acids on the Tris-induced alterations in phospholipid head group composition remains to be determined. Evidence that this phenomenon may be mediated by the nonspecific saturation of a transport system(s) required for Tris transport came from the fact that vitamins (10 μg/ml each), such as ascorbate, pyridoxal, riboflavin, and panthothenic acid, or an individual amino acid, such as serine (1 mg/ml), did not alter the Tris-induced changes in phospholipid head group composition. However, when an equimolar mixture of the 20 amino acids commonly found in proteins was added to the medium at a final concentration analogous to that shown in Fig. 3 for Casamino Acids, it produced qualitatively although not quantitatively similar results on phospholipid head group composition (data not shown).

DISCUSSION

The data presented above outline two ways in which the phospholipid composition of *R. sphaeroides* can be manipulated experimentally without deleterious effects on cell growth or viability. Growth of various strains of *R. sphaeroides*, as well as *R. capsulata* and *P. denitrificans*, in minimal medium supplemented with Tris resulted in an increase in the fraction of the total phospholipid accounted for by NAPS, whereas the presence of yeast extract or Casamino Acids in Tris-containing media overcame the effect of Tris in altering the phospholipid composition of *R. sphaeroides*.

The information in this paper and elsewhere (7, 14) helps resolve some of the apparent inconsistencies in the phospholipid composition of *R. sphaeroides* reported in the literature (6, 9, 28, 32, 36, 48). Lascelles and Szilagyi (32) measured the relative rate of phospholipid synthesis in *R. sphaeroides* NCIB 8253 after transferring chemoheterotrophic cells from yeast extract-containing medium lacking Tris to photosynthetic conditions in the same medium supplemented with 50 mM Tris and [^{32}P]orthophosphoric acid and reported the presence of high amounts of phosphatidic acid. The data in Table 4 show that NAPS represents approximately 5% of the total phospholipid when *R. sphaeroides* M29-5 is grown in medium supplemented with 0.2% yeast extract and 50 mM Tris. Previous studies alluded to some of the properties of NAPS which could have allowed it to be identified inadvertently as phosphatidic acid (14), and the data in the accompanying paper show that the fraction of the total phospholipid accounted for by NAPS does not reach a new steady-state value following two generations after the addition of 20 mM Tris to an exponential phase culture of *R. sphaeroides* M29-5 (7). Thus, the amounts of radioactivity present in individual phospholipids in the earlier study (32) were probably not a reflection of the steady-state levels of these compounds. We were unable to confirm the recently reported absence of PC in *R. sphaeroides* NCIB 8253 (1), but other studies have shown that label enters PC very slowly in *R. sphaeroides* (6, 7, 32), and unfortunately Al-

Bayatti and Takemoto specified no conditions for the length of labeling in their study (1). In addition, the difficulty of reproducibly separating PG, PC, and PE in many one-dimensional thin-layer systems, such as those used by Al-Bayatti and Takemoto (1), is the reason why we routinely employ a two-dimensional system to separate our phospholipid species (14, 43). Finally, the data in Table 3 were not consistent with there being any enrichment for specific phospholipids in either steady-state-labeled membranes or cells grown under different physiological conditions (36, 48).

Onishi and Niederman (42) have recently reported the existence of an unknown phospholipid (designated PX) in *R. sphaeroides* strain NCIB 8253, which has chromatographic and chemical properties similar to those of the unknown phospholipid originally discovered in strain M29-5 (6), which we subsequently identified as NAPS (14). Onishi and Niederman concluded that accumulation of this phospholipid was related to "radiant energy flux" since slowly growing photoheterotrophic cells contained less of this phospholipid than cells grown in the presence of high light. However, these authors also found that slow-growing, nutrient-limited aerobic cells (whose metabolism is not controlled by light [27]) contained more of the unknown phospholipid than rapidly growing chemoheterotrophic cells, and thus they suggested that the levels of this phospholipid were dependent on the growth medium in some unspecified manner (42).

We were unable to detect any light-dependent control of the accumulation of NAPS in *R. sphaeroides* M29-5 when cells were grown photoheterotrophically in media with or without Tris (data not shown), and the alterations in phospholipid head group composition produced by Tris which we described above did not result in changes in the growth rate of the cells or in any of the membrane-associated parameters which we tested. We have shown in this study that the levels of NAPS present in photoheterotrophic cells of *R. sphaeroides* M29-5 are dependent not only on the level of Tris in the growth medium but also on the relative levels of other medium supplements, such as yeast extract and Casamino Acids. In addition, in the accompanying manuscript (7) we show that new steady-state levels of phospholipids are not achieved for at least two culture doublings after a change in growth medium. Obviously, the complex interplay of all of these variables on the labeling of phospholipids with ^{32}P must be stringently controlled before statements can be made concerning steady-state phospholipid composition. However, because we have been unable to determine either the exact composition of the growth medium employed or the conditions of phospholipid labeling used in the study of Onishi and Niederman (42), we are unable at this time to provide an exact explanation for the differ-

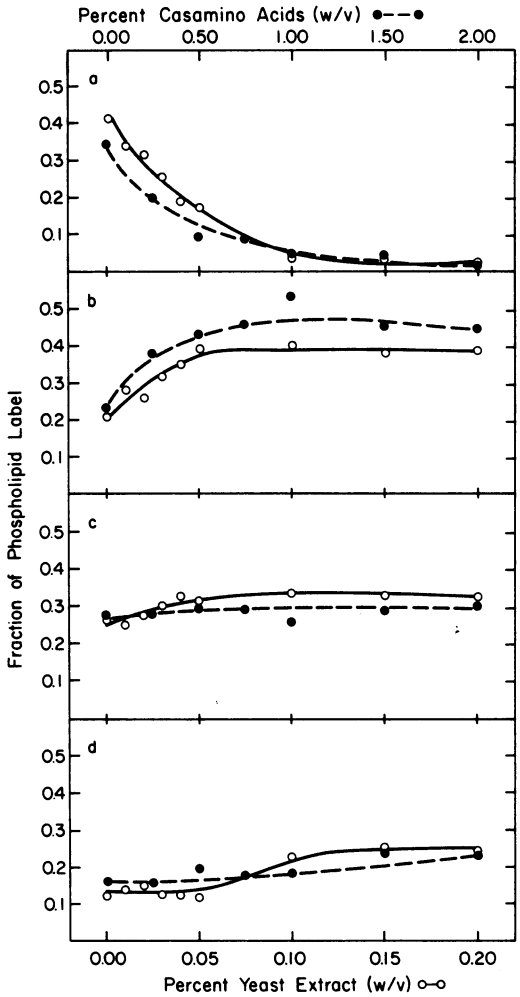

FIG. 3. Effect of yeast extract (○) and Casamino Acids (●) on the phospholipid composition of *R. sphaeroides* M29-5. Cells were grown photoheterotrophically for at least six culture doublings in succinic acid minimal medium supplemented with 20 mM Tris and different concentrations of yeast extract before being transferred to fresh medium for steady state labeling of phospholipids with [^{32}P]orthophosphoric acid (10 μCi/ml). At the late exponential phase of growth, 0.6-ml samples were removed, the phospholipids were extracted, and the individual phospholipid species were resolved as described in the text. The generation time of the cells was between 2.5 and 3.0 h in all cases. The data are expressed as the fractions of the total phospholipid label present in the individual phospholipid species. The total radioactivity applied per plate was between 3×10^3 and 8×10^3 cpm. (a) NAPS. (b) PE. (c) PG. (d) PC. Note the difference in scale on the x-axis for yeast extract and Casamino Acids.

ences in the results reported by these authors and ourselves.

The inability of Tris to alter the phospholipid composition of *R. sphaeroides* 2.4.1 (Table 2) explains why NAPS accumulation was not reported in some earlier studies (9, 36). An important caveat resulting from this observation is that statements which equate *R. sphaeroides* 2.4.1 with strain NCIB 8253 (19, 52) are clearly not valid in terms of the differential effects of Tris on phospholipid metabolism. We have also recently observed differences between these two strains with regard to their phage sensitivities (D. Baumgardner and S. Kaplan, unpublished data), plasmid complements (C. J. Fornari and S. Kaplan, unpublished data). Subtle differences between individual isolates of *R. sphaeroides* are not unexpected as we begin to characterize these bacteria in more detail biochemically.

To our knowledge, this report represents the first instance in which Tris has been shown to alter the phospholipid head group composition of growing cells. There is very little knowledge of the enzymology of phospholipid biosynthesis in the *Rhodospirillaceae*. Assuming that the phospholipid biosynthetic enzymes are membrane associated, as they are in other gram-negative bacteria (45), we argue that Tris exerts its effect on phospholipid metabolism at the membrane level. Tris has been shown to alter other membrane-associated activities, such as outer membrane permeability (24, 25), susceptibility of outer membrane proteins to chemical modification (50), calcium uptake (29, 54), sodium-potassium ATPase activity (34), recycling of insulin receptors (37), cross-linking of thylakoid peptides (53), and the ability of bacteria to take up DNA (15a), and a recent study indicates that purified chromatophores are permeable to Tris (38). The fact that we were unable to find any detectable differences in the ratios of protein to phospholipid, the SDS-PAGE profiles, or the spectral properties of the various membrane fractions analyzed implies that the effects of Tris observed in this study were restricted to phospholipid metabolism.

A recent study has shown that Tris, as well as other alkylamines, is capable of inactivating the pyridoxal phosphate-dependent serine dehydratase of *E. coli* (15) by forming a Schiff base intermediate analogous to that identified in hydroxylamine-mediated inactivation of the PS decarboxylase of *E. coli* (49). The mechanism by which Tris alters phospholipid metabolism is unknown at this time, but our ability to detect NAPS in *R. sphaeroides* 2.4.1 (Fig. 2), along with the data presented elsewhere on the kinetics of NAPS accumulation after the addition of Tris to a growing culture of cells, suggests that NAPS accumulation is the result of a Tris-induced alteration in the flow of intermediates through a preexisting pathway for phospholipid biosynthesis in *R. sphaeroides* (7). Studies are currently in progress on the PS branch of the phospholipid biosynthetic pathway in *R. sphaeroides* in order to localize the site of Tris action and study the relationship (if any) between the strain-specific response to Tris reported here and a similar strain-specific accumulation of PS in *R. sphaeroides* M29-5 and 2.4.1 when cells are grown in the presence of hydroxylamine (M. Singer and S. Kaplan, unpublished data).

The ability of Tris to alter phospholipid head group composition by causing an accumulation of NAPS in *R. sphaeroides*, *R. capsulata*, and *P. denitrificans* is intriguing in view of the close phylogenetic relationship proposed for these two members of the *Rhodospirillaceae* and *P. denitrificans* (16). This is further highlighted by our ability to detect but not cause the accumulation of NAPS in other more distantly related members of the *Rhodospirillaceae*. A wide diversity of bacteria have yet to be surveyed in a systematic manner, but preliminary results indicate the presence in *E. coli* W3110 of a phospholipid which has chromatographic properties similar to those of NAPS and which can be purified by using the protocol originally developed for the purification of NAPS from *R. sphaeroides* (unpublished data). Although NAPS appears to account for only a relatively minor fraction of the total phospholipid of *E. coli* (less than 0.1% of the total), our ability to detect NAPS in bacteria which do not accumulate this phospholipid to a significant extent may highlight the overall biological occurrence and significance of both NAPS and what might be a previously unidentified branch of PS metabolism in these cells.

The ratio of serine-derived glycerophosphatides to polyglycerophosphatides remains relatively constant under all conditions tested in *R. sphaeroides*, so that the accumulation of an acidic phospholipid such as NAPS to as much as 40% of the total phospholipid might be expected to have drastic effects on the net charge of the membranes, especially in view of the fact that the accumulation of NAPS occurs primarily at the expense of PE, a dipolar ionic phospholipid. The fact that cell growth, as well as the other membrane-associated parameters tested, is not affected by this alteration in phospholipid composition argues that these changes, as well as any alterations in phospholipid head group asymmetry (36) or fluidity of the membrane introduced by the accumulation of a triacylated phospholipid, are compensated for in some as-yet-unknown manner in order to allow for proper membrane function. Thus, it appears that the members of the *Rhodospirillaceae* are much more tolerant of

changes in phospholipid head group composition than other gram-negative bacteria, such as *E. coli*, which often form filaments or show a marked inhibition of growth as the proportion of acidic phospholipids is increased to this extent (21, 44, 46).

The conditions which led to NAPS accumulation produced no detectable changes in the fatty acid composition of this or other phospholipids of *R. sphaeroides* (14), so that any alterations in membrane structure or function should be directly attributable to changes in either polar head group composition or membrane fluidity imposed by the introduction of large amounts of an *N*-acylphospholipid in the membrane. Our ability to control experimentally the phospholipid composition of membranes of *R. sphaeroides* gives us a tool with which to analyze the effects of this altered phospholipid composition on membrane structure and function in an unperturbed physiological system. The data in the accompanying paper (7) detail the kinetics of NAPS accumulation and turnover in *R. sphaeroides* M29-5. In addition, it has been shown that when our ability to manipulate the level of NAPS within membranes of *R. sphaeroides* is superimposed upon our knowledge of the assembly of the various membranes of this bacterium, NAPS becomes a very useful tool for probing the regulation of synthesis, assembly, and turnover of both phospholipids and membranes in a procaryote capable of membrane differentiation (7).

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ADDENDUM IN PROOF

Subsequent to the acceptance of this manuscript, Onishi and Niederman (J. Bacteriol. **152**:1067, 1982) published an erratum that addressed some of the points we raise in the discussion of this manuscript with regards to the composition of the growth medium used by these authors.

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