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Purification and Characterization of an *N*-Acylphosphatidylserine from *Rhodopseudomonas sphaeroides*†

Timothy J. Donohue,‡ Brian D. Cain, and Samuel Kaplan*

ABSTRACT: A new phospholipid that can account for up to 40% of the total cellular phospholipid of *Rhodopseudomonas sphaeroides* has been identified. Purification of the phospholipid was accomplished by column chromatography on silicic acid and diethylaminoethylcellulose followed by preparative thin-layer chromatography. A combination of spectroscopic and chemical techniques were used to identify the unknown phospholipid as an *N*-acylphosphatidylserine. Infrared spectroscopy revealed the presence of both ester and amide bonds in the phospholipid. Interpretation of the proton nuclear magnetic resonance spectrum of the new phospholipid indicated the presence of three acyl chains per phospholipid and in all other respects was compatible with the proposed structure of the molecule. Chemical studies confirmed the presence of a glycerylphosphorylserine moiety in the molecule and yielded three fatty acyl chains per hydrolyzed phospholipid. The fatty acid composition of the phospholipid was approximately 85% vaccenic acid, 9% stearic acid, 5% palmitic acid, and 1% palmitoleic acid, which is essentially identical with the fatty acid composition of whole cell phospholipid preparations from *R. sphaeroides*. Chemical synthesis of an *N*-acylphosphatidylserine from beef brain phosphatidylserine and palmitic anhydride gave a product with characteristics similar to those of the naturally occurring material isolated from *R. sphaeroides*.

Phospholipids are a major component of biological membranes, and the fluid mosaic model (Singer & Nicholson, 1972) proposes that a phospholipid bilayer constitutes the basic unit of biological membrane structure. Consequently many investigations of the control of membrane assembly have focused on the control of phospholipid synthesis (Bell & Coleman, 1980; Cronan, 1978; Raetz, 1978) and the relationship between phospholipid synthesis and membrane structure and function.

The facultative photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* provides an attractive system in which to study membrane biogenesis and differentiation [for a recent review, see Kaplan (1978)]. When growing chemoheterotrophically, *R. sphaeroides* contains a typical Gram-negative outer membrane (Baumgardner et al., 1980) and a cytoplasmic membrane. Photoheterotrophic growth conditions induce the differentiation of the cytoplasmic membrane, resulting in the synthesis of the intracytoplasmic membrane system, which houses the photosynthetic apparatus of the cell. Studies on the regulation of intracytoplasmic membrane assembly employing synchronously dividing populations of *R. sphaeroides* have shown that while insertion of protein (Fraley et al., 1978) and photopigments (Wraight et al., 1978) into the intracytoplasmic membrane occurs continuously throughout the cell cycle, accumulation of phospholipids within the intracytoplasmic membrane occurs discontinuously with respect to the cell cycle (Fraley et al., 1979a,b; Lueking et al., 1978). This discontinuity in phospholipid incorporation results from the bulk transfer of phospholipids from outside the intracytoplasmic membrane into the intracytoplasmic membrane concurrent with cell division (Cain et al., 1981).

In the course of studies aimed at determining the regulation of phospholipid biosynthesis and insertion into the intracytoplasmic membrane, an unidentified phospholipid was discovered in *R. sphaeroides* strain M29-5 (Cain et al., 1981). Phosphatidylglycerol (PG),[1] phosphatidylethanolamine (PE), and phosphatidylcholine (PC) are the major phospholipids generally found in strains of *R. sphaeroides* (Cain et al., 1981; Cohen et al., 1979; Kenyon, 1978; Marinetti & Cattieu, 1981). However, there is a report of relatively large amounts of phosphatidic acid in one study performed with *R. sphaeroides* strain N.C.I.B. 8253 (Lascelles & Szilagyi, 1965). The existence of an unknown phospholipid in this same strain of *R. sphaeroides* has also been reported (Onishi & Niederman, 1978). This communication describes the characterization of the unidentified phospholipid from *R. sphaeroides* strain M29-5 by a variety of spectroscopic and chemical means. The results of these studies show that the unidentified phospholipid is an *N*-acylphosphatidylserine (NAPS).

Materials and Methods

Materials. Cellex D (low capacity) and Bio-Gel P2 (minus 400 mesh) were purchased from Bio-Rad Laboratories. Carrier-free [^{32}P]orthophosphoric acid and sodium [^{3}H]acetate (2 Ci/mmol) were obtained from New England Nuclear. Silica gel G (Supelco) was used for thin-layer chromatography, and silica gel H (Brinkman Instruments) was used for column chromatography. Triolein and purified bovine brain PS (P-8518) were obtained from Sigma Chemical Co. Cronex 2DC (Du Pont) film was used for autoradiography. Elemental analysis (Microanalytical Laboratory) and all molecular spectroscopy (National Science Foundation Regional Molecular Instrumentation Facility) were performed at the facilities of the School of Chemical Sciences of the University of Illinois at Urbana-Champaign. Unless noted, all chemicals used were of reagent grade.

Organism, Media, and Growth Conditions. R. sphaeroides M29-5, a leucine and methionine auxotroph of the wild-type strain 2.4.7, was kindly provided by W. R. Sistrom, University

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[1] Abbreviations: GPS, glycerylphosphorylserine; IR, infrared; NAPS, *N*-acylphosphatidylserine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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of Oregon. Except where noted in the text, cells were grown photoheterotrophically in completely filled culture vessels by using the low-phosphate (2 mM phosphate and 20 mM Tris, pH 7.0) modification (Fraley et al., 1979a) of the succinic acid minimal medium previously described (Lueking et al., 1978). Control experiments showed that the growth of *R. sphaeroides* M29-5 was not measurably affected by the low-phosphate medium (generation time of 150–180 min at 32 °C).

For radioactive labeling of phospholipids with [^{32}P]orthophosphoric acid, cells were previously adapted to growth in low-phosphate medium for at least eight generations. Inocula were then diluted into low-phosphate medium supplemented with [^{32}P]orthophosphoric acid at a final concentration of 10 μCi/mL. Growth of the cells was allowed to continue for approximately six culture doublings (final cell density approximately 1×10^9 cells/mL) before sampling.

Bulk Extraction and Isolation of Phospholipids. Phospholipids present in radioactively labeled culture samples were extracted into chloroform (Bligh & Dyer, 1959; Ames, 1968). The resulting chloroform fractions were washed once with an equal volume of 1% (w/v) NaCl (Kanfer & Kennedy, 1963). The proper partitioning of phospholipids was facilitated by the addition of an excess of unlabeled carrier cells (equivalent to approximately 200 μg of phospholipid/mL of chloroform) prior to extraction (Raetz, 1978). Control experiments showed that the presence of NAPS in extracts of *R. sphaeroides* M29-5 was not an artifact of the extraction procedures employed (data not shown).

Large-scale extraction of unlabeled whole cell phospholipids was accomplished by a similar procedure (Folch et al., 1957). The extracted phospholipids were separated from photopigments by repeated precipitation of the phospholipids in ice-cold acetone (Kates, 1975), followed by chromatography on a column (4 × 20 cm) of silicic acid H (Sweeley, 1969). The phospholipid-containing methanol eluate of the silicic acid column was taken to dryness under reduced pressure by rotary evaporation, suspended in a minimal volume of chloroform, and stored in Teflon-lined screw-capped tubes at −20 °C. Thin-layer chromatography (see below) of a portion of this bulk phospholipid sample revealed that the only iodine-reactive material detectable in this fraction comigrated with known *R. sphaeroides* phospholipids.

The large-scale purification of an individual phospholipid species from *R. sphaeroides* was accomplished by using the following general protocol. Bulk phospholipids in chloroform (either with or without the acetone precipitation step) were applied to a column (2 × 20 cm) of Cellex D that had been previously equilibrated with chloroform (Rouser et al., 1963, 1969). Fractions enriched in individual phospholipids were eluted from the column by employing the solvent washes described under Results. The appropriate fraction was taken to dryness by rotary evaporation, and the phospholipids were purified by using preparative thin-layer plates and by employing the solvent system described below. In an attempt to minimize damage to unsaturated fatty acyl chains, thin-layer plates were only minimally stained with iodine. Phospholipids were extracted from the phospholipid-containing regions of thin-layer plates by either of the two extraction techniques cited above. Residual silica gel in the purified phospholipid samples was removed by chromatography on the silicic acid column as described above.

Identification and Quantitation of Phospholipid Species. Individual phospholipid species were resolved by using a two-dimensional thin-layer chromatography system (Poorthius et al., 1976). Silica gel G plates, impregnated with 0.4 M boric acid, used for analytical purposes were 0.25 mm thick while preparative thin-layer plates were 0.5 mm. Chloroform–methanol–water–ammonium hydroxide (70:30:3:2) constituted the first-dimension solvent system while chloroform–methanol–water (65:35:5) was used for development in the second dimension. Plates were dried under a nitrogen atmosphere between the first and second dimension. Detection of phospholipids was accomplished by iodine staining or, in some cases when ^{32}P-labeled samples were used, by autoradiography. The amount of radioactivity in each of the phospholipid species was determined by scraping the phospholipid-containing gel from the thin-layer plates directly into scintillation vials by using a toluene-based scintillation fluid (Fraley et al., 1978). Nonradioactively labeled phospholipids were quantitated by lipid phosphorus assays (Bartlett, 1959).

Spectroscopic Techniques. Infrared (IR) spectra were obtained from KBr pellets containing a mixture of approximately 1–2 mg of phospholipid/100 mg of KBr by using a Beckman IR12 double-beam infrared spectrophotometer. All spectra were recorded at room temperature against a reference KBr pellet.

Proton nuclear magnetic resonance (NMR) spectra were acquired by using a Nicolet NTC 360 spectrometer operating at 360 MHz in the Fourier transform mode with the probe temperature maintained at 16 °C. Spectra were obtained from phospholipid dissolved in deuteriochloroform to a final concentration of approximately 2% (w/v) and required between 100 and 200 scans. Chemical shift values are expressed in parts per million (ppm) relative to tetramethylsilane at 0 ppm (Δ scale).

Preparation of Phospholipid Phosphate Esters. Phospholipids were deacylated for the production of their glycerylphosphoryl esters (hereafter referred to as phospholipid head groups) by using a mild alkaline hydrolysis procedure (Dittmer & Wells, 1969). The phospholipid head groups produced were concentrated by lyophilization and separated from the potassium acetate introduced during the hydrolysis and extraction steps by chromatography on a column (2 × 70 cm) of Bio-Gel P2 that had been previously equilibrated with distilled water. A trace amount of sodium [^{3}H]acetate (approximately 2×10^5 cpm) was added to the sample before chromatography in order to facilitate monitoring of the separation of phospholipid head groups (detected by either ^{32}P determination or lipid phosphorus assays) from the acetate salt. Partitioning of ^{32}P-labeled material into the aqueous phase after mild alkaline hydrolysis was routinely greater than 95%; recovery of ^{32}P from the aqueous phase as *N*-acylglycerylphosphorylserine after desalting was approximately 40%. The desalted phospholipids were chromatographed in two dimensions on Whatman 1 paper sheets (Kates, 1975). Development in the first dimension was accomplished by using saturated phenol–water (100:38 w/v) while the second-dimension solvent system was phenol-saturated water–ethanol–acetic acid (50:5:6). Quantitation of ^{32}P was accomplished by cutting out pieces of the chromatogram corresponding to the regions of an exposed sheet of X-ray film and counting the samples in a Triton–toluene-based scintillant (Fraley et al., 1978). Where indicated in the text, acid treatment of phospholipid head groups was performed in sealed acid-washed ampules.

Phospholipid Transmethylation. Mild alkaline methanolysis (Carter & Gaver, 1967) was performed by dissolving dry lipid samples in 0.5 mL of chloroform–methanol (2:1) in a Teflon-lined screw-capped test tube. After the addition of 0.5 mL of freshly prepared 1 N NaOH in methanol, the sample was incubated at room temperature for 30 min with occasional

shaking. The sample was neutralized with 1 N HCl, and the fatty acyl methyl esters produced were extracted into chloroform after the addition of 5 mL of chloroform–methanol (2:1) and 1.5 mL of H_2O. The lower chloroform phase was removed and taken to dryness under a stream of nitrogen, and the fatty acyl methyl esters were purified by chromatography on a column of silicic acid before analysis for methyl ester content or composition (see below).

In some cases phospholipid fatty acyl chains were transmethylated by using a methanolic hydrochloric acid system recommended for use with *N*-acyl-containing lipids (Kates, 1975). Dry lipid samples were dissolved in 5 mL of methanolic hydrochloric acid (2 N HCl), which was prepared by mixing 10 mL of constant-boiling HCl with 50 mL of methanol. After the addition of 0.8 mL of benzene, the sample was heated for 5 h at 70 °C in a Teflon-lined screw-capped test tube. The fatty acyl methyl esters produced were then extracted 4 times with 5 mL of hexanes and purified by column chromatography before further use (see below).

Fatty acyl methyl esters were purified (Kates, 1975) by elution from a silicic acid H column (1 × 10 cm) with hexanes–ethyl ether (99:1) and concentrated by rotary evaporation before analysis for methyl ester content or composition as described below. Studies using triolein or [^{3}H]acetate-labeled *R. sphaeroides* phospholipids with either of the above transmethylation protocols have shown that transmethylation is quantitative and that there is no detectable degradation of fatty acyl chains under these conditions (data not shown).

The fatty acyl ester content of samples either before or after transmethylation was determined by measuring the absorbance of acyl ester ferric hydroxamate derivatives (Dittmer & Wells, 1969). Triolein was used as a standard for these studies.

The fatty acid composition of lipid samples was determined by gas chromatography of the fatty acyl methyl esters produced by transmethylation. Gas chromatography was performed on a Hewlett-Packard 5830A gas chromatograph. Fatty acyl methyl esters were separated on a 6-ft glass column (inner diameter 2 mm) packed with either 10% SP-2340 on 100/120 Chromosorb W AW or 3% SP-2100 DOH on 100/120 Supelcoport (Supelco Chemical Co.). Fatty acyl methyl esters were provisionally identified by coelution with known methyl esters. Direct confirmation of the identity of these compounds was provided by subjecting the material present in individual gas chromatography peaks to mass spectroscopy (data not shown). Low-resolution mass spectra of fatty acyl methyl esters were obtained on a VG 7070 mass spectrometer by J. Carter Cook, Jr., of the mass spectroscopy laboratory of the University of Illinois at Urbana-Champaign. In this instance fatty acyl methyl esters were separated on a 6-ft glass column (2-mm inner diameter) packed with 3% OV-17 by using a Varian 3700 gas chromatograph.

All solvents used in the preparation of samples for gas chromatography were of spectral grade, and all glassware was acid washed. The methanol used in transmethylation experiments was dried over type 3A molecular sieves before use. In some cases a known amount of methyl myristate was added to lipid samples before transmethylation in order to quantitate recovery of fatty acyl methyl esters analyzed by gas chromatography. Recovery of the methyl myristate was greater than 85% in all cases.

Synthesis of N-Acylphosphatidylserine. An *N*-acylphosphatidylserine was synthesized from freshly prepared palmitic anhydride and purified bovine brain PS. IR and proton NMR analyses of the purified brain PS (by using the techniques cited above) were consistent in all regards with the previously reported spectra of this phospholipid (Rouser et al., 1963; Salem et al., 1980). Palmitic anhydride was prepared from palmitic acid and dicyclohexylcarbodiimide (Selinger & Lapidot, 1966). The purity of the palmitic anhydride was confirmed by infrared spectroscopy. Purified bovine brain PS (21.0 μmol) was acylated with palmitic anhydride (420 μmol) in chloroform–pyridine (4:1 v/v; Epps et al., 1980) by using *N,N*-dimethyl-4-aminopyridine (20 μmol) as a catalyst (Gupta et al., 1977). The reaction was carried out at room temperature for 24 h, and the phospholipids were purified by elution from a column (4 × 20 cm) of silicic acid with methanol. The NAPS contained in the phospholipid-containing eluate of the silicic acid column was purified by passage over a column of Cellex D (as described under Results), and the material eluted with chloroform–acetic acid (3:1) was treated with trinitrobenzenesulfonic acid (Rothman & Kennedy, 1977) to convert any unreacted PS present to its trinitrophenyl derivative. Since NAPS is only marginally resolved from PS in the two-dimensional TLC system described above, trinitrophenylation of PS was used to convert PS to a form easily separable from the reaction product by TLC. The reaction product, after extraction from the TLC support and chromatography on a column (4 × 20 cm) of silicic acid to remove remaining traces of silica gel, was obtained in approximately 5% yield.

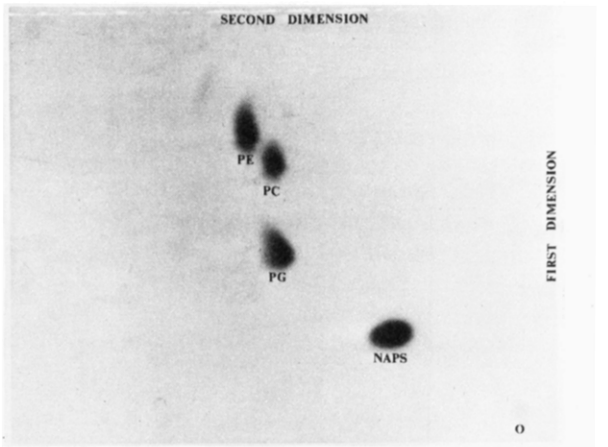

FIGURE 1: Autoradiogram of a two-dimensional thin-layer chromatogram of a chloroform extract of ^{32}P-labeled *R. sphaeroides* M29-5 grown in low-phosphate medium. Extraction of the radioactively labeled phospholipids and separation of the individual phospholipid species were performed as described under Materials and Methods. The symbols refer to the chromatographic origin (O) and the known phospholipids of *R. sphaeroides* M29-5. Total radioactivity applied to the chromatogram was approximately 3 × 10^5 cpm.

Results

Detection of N-Acylphosphatidylserine. Figure 1 shows an autoradiogram of a thin-layer chromatogram of a chloroform extract of ^{32}P-labeled *R. sphaeroides* M29-5 grown in low-phosphate medium. Four major ^{32}P-containing species can be seen, and as indicated in the figure they are PG, PC, PE, and the previously unidentified phospholipid (NAPS). In total, these four phospholipids represent greater than 95% of the total phospholipid as determined either by lipid phosphorus assays by using unlabeled extracts or as the fraction of ^{32}P contained in a chloroform extract after labeling cells for approximately six generations. The remaining 5% of the total phospholipid is comprised of cardiolipin and what are believed to be the mono- and dimethyl derivatives of phosphatidylethanolamine. NAPS can account for as much as 40% of the total phospholipid in log-phase *R. sphaeroides* M29-5 grown in low-phosphate medium. The other major phospholipids PG, PC, and PE represent approximately 25, 15, and 20% of the total

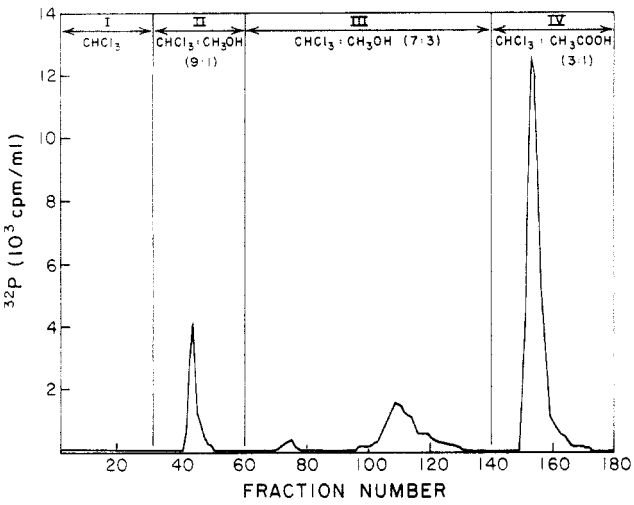

FIGURE 2: Elution profile of a chloroform extract of ^{32}P-labeled *R. sphaeroides* M29-5 on a column (2 × 20 cm) of Cellex D. Radioactively labeled phospholipids were extracted from cells grown in low-phosphate medium as described under Materials and Methods. The phospholipid sample (containing approximately 1.2×10^6 cpm) was applied to the column in chloroform in a total volume of 1 mL, and approximately 7-mL fractions were collected. The composition of the eluting solvents was changed as indicated. The relative amounts of each phospholipid species present in the pooled fractions from each solvent eluate were determined by thin-layer chromatography.

phospholipid, respectively, under these conditions.

As can be seen in Figure 1 NAPS does not migrate far from the origin in either of the two solvent systems used. Other phospholipids that are known (Poorthius et al., 1976) or might be expected (Kates, 1975) to migrate to the same general region in this two-dimensional system are phosphatidic acid, phosphatidylglycerol phosphate, PS, and lyso derivatives of PC. Proof that NAPS is not any of these phospholipids will be presented in later sections, but briefly the lack of reactivity of NAPS with either amino-specific reagents such as ninhydrin or trinitrobenzenesulfonic acid or choline-specific stains was preliminary evidence that NAPS was neither PS nor a lyso derivative of PC.

Purification and Elemental Analysis of NAPS. In order to facilitate the structural identification of the unknown phospholipid, a purification scheme yielding milligram quantities of pure phospholipid was developed. Figure 2 shows an elution profile for a chloroform extract of ^{32}P-labeled *R. sphaeroides* M29-5 on a column of Cellex D (Rouser et al., 1963, 1969). The sample was applied to the column in chloroform, and as expected no detectable ^{32}P-labeled material was removed upon washing the column with 200 mL of chloroform. Upon analysis of the pooled and concentrated chloroform–methanol (9:1) eluate (200 mL) by TLC, it was found to be approximately 95% PC and 5% PE. The chloroform–methanol (7:3) eluate (approximately 600 mL) contained approximately 80% PE with small amounts of NAPS (5%), PG (5%), PC (3%), CL (2%), and the presumed mono- and dimethyl derivatives of PE (5%). Subsequent elution of the column with 300 mL of chloroform–acetic acid (3:1) resulted in the removal of another ^{32}P-containing fraction which when pooled, concentrated, and analyzed by TLC contained approximately 90% NAPS and lesser amounts of PG (5%), PE (3%), and PC (2%). The elution of NAPS from Cellex D with chloroform–acetic acid (3:1) was unexpected since this is a solvent mixture previously reported to remove only weakly acidic materials such as free fatty acids and bile acids (Rouser et al., 1963, 1969). Thus NAPS is the first phospholipid reported to be removed from DEAE-cellulose in high yields

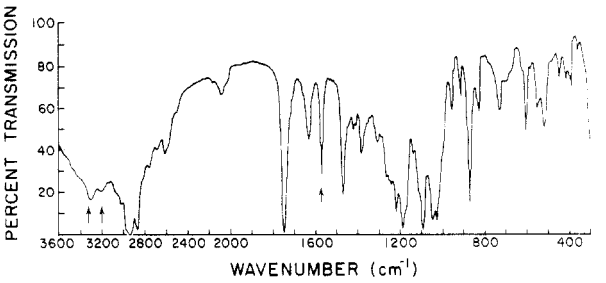

FIGURE 3: Infrared spectrum of pure NAPS. The sample (approximately 1.7 mg) was examined as a KBr pellet. Arrows at 1575, 3200, and 3300 cm^{-1} indicate absorption signals attributable to an amide bond.

with chloroform–acetic acid (3:1). Approximately 90% of the ^{32}P applied was recovered in fractions II–IV (Figure 2) plus the highly enriched PG-containing fraction obtained by subsequent elution of the Cellex D column with 300 mL of chloroform–acetic acid (3:1) containing 10 mM ammonium acetate.

Chromatography of phospholipid samples for preparative purposes on the same column yielded similar results although the relative purity of each of the pooled fractions described above was slightly diminished (data not shown). With preparative samples the entire eluate from one solvent wash was collected in a single vessel. The NAPS present in the chloroform–acetic acid (3:1) eluate was purified via preparative TLC. Following extraction of NAPS from the TLC support, traces of silica gel were removed by chromatography of the material on a column of silicic acid H (see Materials and Methods). At this stage NAPS was the only iodine-reactive material detectable on a two-dimensional TLC plate. The reactivity of the purified phospholipid with group-specific stains and its migration on TLC plates were unchanged from those of the material initially present in crude phospholipid samples (data not shown). With this protocol 10–15 mg of pure NAPS was routinely isolated from approximately 8 g (wet weight) of *R. sphaeroides* M29-5 grown photoheterotrophically in low-phosphate medium. This is approximately 50% of that expected assuming 10% of the dry weight of the cell was phospholipid and NAPS constituted at a maximum 40% of the total phospholipid of the cell. However, NAPS can vary between 20 and 40% of the total phospholipid under these growth conditions.

Elemental analysis of pure NAPS revealed that by weight NAPS was composed of 58.1% carbon, 9.3% hydrogen, 3.5% phosphorus, and 1.4% nitrogen. Pure *R. sphaeroides* PG was found to contain 4.2% phosphorus by weight, so that if the assumption is made that NAPS contains one phosphorus per molecule, then the finding that nitrogen accounted for 1.4% of the weight of NAPS revealed that NAPS contains approximately one (0.9) nitrogen atom per phospholipid molecule. A minimum molecular weight for NAPS of approximately 1000 was obtained from the elemental analysis data and the assumption that there was one nitrogen atom per phospholipid molecule.

Infrared Spectroscopy. Figure 3 shows the infrared spectrum of a sample of pure NAPS. As noted in previous studies the ability to derive complete and definitive structural information from IR spectra of phospholipids is limited by the existence of many IR-absorbing functional groups in the molecule (Rouser et al., 1963; Salem et al., 1980). Although the IR spectrum of NAPS was similar to those of other glycerophosphatides, the strong absorption bands at approximately 1575, 3200, and 3300 cm^{-1} (see arrows in Figure 3) were indicative of the presence of an amide bond in the molecule.

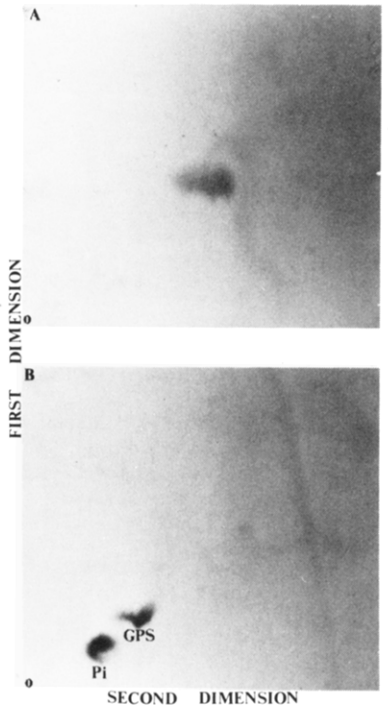

FIGURE 4: Autoradiogram of a two-dimensional paper chromatogram of a ^{32}P-labeled preparation of NAPS head group. Mild alkaline hydrolysis of pure NAPS, desalting of the resulting glycerylphosphoryl esters, and chromatography on Whatman 1 paper were as described under Materials and Methods. The chromatographic origin (o) and the migration of authentic samples of glycerylphosphorylserine (GPS) and inorganic phosphate (P_i) are indicated. Panel A shows the migration of an untreated sample of ^{32}P-labeled NAPS head group, and panel B shows the migration of the products produced after treatment of a sample of ^{32}P-labeled NAPS head group with 8 N acetic acid for 16 h at 110 °C. Total radioactivity applied to the chromatograms in each case was approximately (1–2) × 10^5 cpm. Recovery of ^{32}P in acetic acid treated samples was routinely greater than 85%.

Absorption at these wavenumbers was not found in the spectra of other pure glycerophosphatides, but strong signals in these regions are characteristic of the amide bond of sphingolipids (Rouser et al., 1963). In contrast, strong absorption at 1750 cm^{-1} (a region of absorption assigned to the stretching of the ester carbonyl groups of glycerophosphatides) is not seen in sphingolipids (Rouser et al., 1963; Dittmer & Wells, 1969). Therefore, absorption by NAPS at 1750 cm^{-1} as well as 1575, 3200, and 3300 cm^{-1} indicated the presence of both the ester carbonyl groups found in all glycerophosphatides in addition to an amide bond.

Analysis of NAPS Head Group. Figure 4 shows an autoradiogram of a two-dimensional paper chromatogram of a ^{32}P-labeled sample of the NAPS head group. The ^{32}P-labeled material (panel A) migrated in both the first ($R_f \simeq 0.52$) and second ($R_f \simeq 0.50$) dimensions with R_f values distinct from those of other known nitrogen-containing phospholipid head groups (Kates, 1975).

Figure 4 also shows the migration of the ^{32}P-labeled NAPS head group after treatment with 8 N acetic acid for 16 h at 110 °C (panel B). Approximately 5% of the ^{32}P migrated to the region of the chromatogram where the untreated NAPS head group migrates. The remaining 40 and 55% of the radioactivity applied migrated in both dimensions with R_f values identical with those of glycerylphosphorylserine and inorganic phosphate, respectively (Kates, 1975). This was in contrast to the less than 2% of the ^{32}P present in an untreated NAPS head group sample that migrated with glycerylphosphorylserine and inorganic phosphate (see panel A). When glycerylphosphorylserine (prepared from purified bovine brain PS) was chromatographed together with an acid-treated sample of the ^{32}P-labeled NAPS head group, the ^{32}P-containing material marked GPS in panel B of Figure 4 comigrated with the glycerylphosphorylserine (detected by ninhydrin staining). The observation that a significant fraction of ^{32}P-labeled material became reactive with trinitrobenzenesulfonic acid after acetic acid hydrolysis of a ^{32}P-labeled NAPS head group sample was also consistent with the production of glycerylphosphorylserine (data not shown).

Acetic acid has been used for protein hydrolysis (Ingram, 1963; Landon, 1977) so that at least one effect of the acetic acid in the experiments described may be the cleavage of an amide bond in the NAPS head group. The streaking of the untreated NAPS head group sample (panel A of Figure 4) relative to that observed for the acetic acid hydrolysis products (panel B) was a reproducible phenomenon that could be due to interaction of the *N*-acyl chain of the unhydrolyzed head group sample with the paper during development of the chromatogram.

Independent confirmation of the occurrence of serine and glycerol in NAPS was also obtained after hydrolysis of NAPS in constant boiling (6 N) HCl at 110 °C for 3 or 6 h. Glycerol phosphate and serine were identified as water-soluble products of acid hydrolysis by using the paper chromatography system described above (Kates, 1975). The presence of serine in the hydrolysates was also confirmed by analysis of a portion of the sample on an amino acid analyzer.

Fatty Acid Analysis. Analysis of the acyl ester content of NAPS as well as bulk phospholipid from *R. sphaeroides* M29-5 indicated approximately 2 acyl ester equiv per equiv of phosphorus (Table I). Acid (2 N HCl) or base (0.5 N NaOH) catalyzed transmethylation increased the acyl ester to phosphorus ratio of NAPS to approximately 3 while the ratio for bulk phospholipid remained at 2. The data in Table I also show that there were no detectable differences between the fatty acid composition of NAPS and bulk phospholipid from *R. sphaeroides* M29-5. Thus (1) the recovery of three fatty acyl methyl esters after treatment of NAPS with methanolic hydrochloric acid, (2) the detection of one nitrogen per NAPS molecule together with the lack of reactivity of NAPS with amino group specific reagents, (3) the indication of an amide bond in the IR spectrum of NAPS, and (4) the identification of a glycerylphosphorylserine moiety from a head group preparation of NAPS were all consistent with the unknown phospholipid being an *N*-acylphosphatidylserine. However, the finding that mild alkaline methanolysis of the phospholipid also resulted in the formation of approximately 3 equiv of fatty acyl methyl ester per molecule was unexpected.

When the aqueous phase obtained after partitioning of the methyl esters produced by alkaline methanolysis of the phospholipid was dried, subjected to transmethylation with methanolic hydrochloric acid, and analyzed by gas chromatography, fatty acyl methyl esters were found. The fatty acid composition of the methyl esters found in this fraction was similar to that shown for the phospholipid samples in Table I (data not shown). The recovery of methyl esters in this fraction represented approximately 2% of the amount present in the original sample. Thus it appears that a small fraction of the amide-linked fatty acids in NAPS (approximately 6%) survived mild alkaline methanolysis and partitioned into the aqueous phase as *N*-acylglycerylphosphorylserine. No detectable methyl esters were found in the aqueous phase from a mild alkaline hydrolysate of the same amount (7 mg) of a bulk phospholipid sample from *R. sphaeroides* M29-5 in which

Table I: Fatty Acid Content and Composition of NAPS and Bulk *R. sphaeroides* M29-5 Phospholipid

sample	acyl ester to phosphorus ratio[a]		equiv of fatty acyl methyl ester recovered per mol[b,c]	% of the total fatty acid[b]			
	untreated	acid-catalyzed transmethylated		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$
NAPS	1.7 ± 0.3	2.8 ± 0.2	3.1 ± 0.3	5.6 ± 0.4	1.0 ± 0.2	9.5 ± 0.8	84.2 ± 1.6
alkaline-treated NAPS			2.9 ± 0.2	5.0 ± 0.2	1.4 ± 0.2	9.2 ± 0.5	83.8 ± 1.7
bulk phospholipid[d]	2.2 ± 0.3	2.0 ± 0.3	2.1 ± 0.2	6.1 ± 0.6	0.8 ± 0.1	11.3 ± 0.2	81.9 ± 1.3
alkaline-treated phospholipid			1.8 ± 0.1	4.6 ± 0.3	1.0 ± 0.1	8.9 ± 0.3	85.4 ± 1.1

[a] Determined colorimetrically (see Materials and Methods). Values shown are the average of five determinations. [b] Determined by gas chromatography of the fatty acyl methyl esters produced via transmethylations (see Materials and Methods). Values shown are the average of at least three determinations. [c] Based on quantitation of the recovery of the fatty acyl methyl esters released by transmethylation as detected by gas chromatography (see Materials and Methods). [d] Bulk phospholipid sample containing PG, PC, and PE (prepared as described under Materials and Methods) from *R. sphaeroides* M29-5 grown under conditions in which NAPS accounts for less than 1% of the total phospholipids.

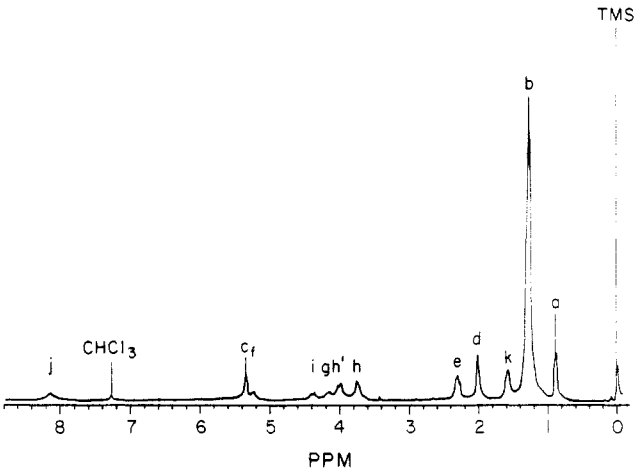

FIGURE 5: Proton NMR spectrum (360 MHz) of pure NAPS in deuteriochloroform. The spectrum was obtained from a 2% phospholipid solution, and chemical shift values are referenced to tetramethylsilane at 0 ppm (Δ scale). The signal at approximately 7.26 ppm is from residual chloroform in the deuteriochloroform. The letters above the individual peaks in the spectrum refer to the class of protons responsible for the production of that signal (see Figure 6).

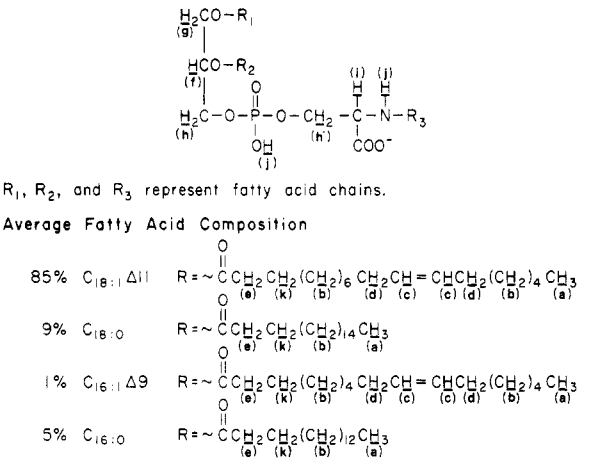

FIGURE 6: Structure of *N*-acylphosphatidylserine. Letters in parentheses refer to the class of protons responsible for the production of individual signals in the NMR spectrum shown in Figure 5. The fatty acid composition of NAPS shown is based on the data given in Table I.

NAPS represented less than 1% of the total phospholipid.

^1H NMR Spectroscopy. The ^1H NMR spectrum (360 MHz) of a purified preparation of NAPS is shown in Figure 5. Assignment of the signals in the spectrum to individual classes of protons present in NAPS was accomplished by use of spectra of model compounds (Pouchert & Campbell, 1974), by theoretical considerations (Dyer, 1965), and by comparison of the spectrum of the phospholipid with previously published proton NMR spectra of phospholipids performed at 60 (Chapman & Morrison, 1966) or 100 MHz (Salem et al., 1980). The letters in parentheses in the following section refer to the signals corresponding to each class of protons in the spectrum of NAPS (see Figure 5 and the representation of NAPS shown in Figure 6). The only signal that was eliminated by the addition of a small amount of D_2O to the sample was the 2 proton signal at 8.16 ppm (j). Table II summarizes both the chemical shift values and the number of protons responsible for the production of the individual signals in the NMR spectrum.

Published NMR spectra of fatty acids (Pouchert & Campbell, 1974; Salem et al., 1980) or phospholipids (Chapman & Morrison, 1966; Salem et al., 1980) recorded at either 60 or 100 MHz invariably show an intense signal at approximately 1.3 ppm from the methylene protons of the fatty acyl chain (b) along with a downfield resonance shoulder in the 1.5–1.8-ppm region. To our knowledge the 360-MHz proton NMR spectrum shown in Figure 5 represents the first instance in which a distinct peak in the 1.6-ppm region (k) has been observed for a phospholipid. Figure 7 shows the results of the spin-decoupling experiments used to assign the peak at 1.59 ppm (k) to the methylene protons on carbons β

Table II: Summary of Proton NMR Data

class of protons[a]	chemical shift[b]	no. of protons	
		obsd	expected[c]
a	0.88	8	9 (6)
b	1.27	61	63 (42)
c	5.34	4	5 (3)
d	2.02	8	10 (7)
e	2.29	5	6 (4)
f	5.23	1	1 (1)
g	4.16	2	2 (2)
h	3.74	4	2 (2)
h'	3.98	4	2 (2)
i	4.36	1	1 (1)
j	8.16	2	2 (2)
k	1.59	6	6 (4)

[a] As shown in the spectrum and the structure of NAPS. [b] Reported in ppm relative to Me_4Si set at 0 ppm. [c] Assuming the structure of NAPS shown in Figure 7. The numbers in parentheses are the numbers of protons expected assuming only two fatty acyl chains per phospholipid molecule.

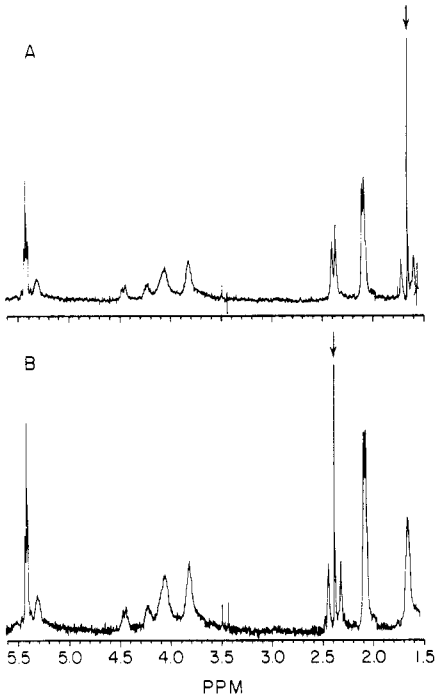

FIGURE 7: Proton NMR spectrum (360 MHz) of pure NAPS after irradiation of the sample at 1.59 (A) and 2.29 ppm (B). All other conditions were as in Figure 5.

to fatty acyl carbonyl groups. Irradiation of the sample at 1.59 ppm (panel A, see the arrow) reduced the 2.29-ppm signal (assigned to the methylene protons on carbons α to the fatty acyl carbonyl groups, e) to a doublet. Figure 7 also shows the results obtained when the sample was irradiated at 2.29 ppm (panel B). In this instance the only effect seen in the spectrum was a sharpening of the 6 proton signal at 1.59 ppm.

It should be noted that all the methylene protons of known phospholipid head groups give rise to a broad band in the region of 3.0–4.5 ppm in NMR spectra of phospholipids acquired at either 60 (Chapman & Morrison, 1966) or 100 MHz (Salem et al., 1980). Although relatively distinct signals for these protons (g, h, h′, i) were produced in the 360-MHz spectrum of NAPS shown in Figure 5, the broadness of the signals in the 3.5–4.5-ppm region of the spectrum made integration of the area under these peaks somewhat difficult, increasing the error involved in the calculation of the number of protons responsible for these signals. The assignment of these proton classes in NAPS to specific NMR signals was supported by theoretical considerations (Dyer, 1965) as well as spectra of model compounds (Pouchert & Campbell, 1974), glycerylphosphorylserine (data not shown), and other phospholipids (Chapman & Morrison, 1966). Attempts to more rigorously assign the NMR signals in the 3.5–4.5-ppm region of the spectrum to particular proton classes by the use of spin-decoupling experiments similar to those described above were not completely successful due to our inability to completely saturate these signals.

Properties of Synthetic N-Acylphosphatidylserine. An *N*-acylphosphatidylserine was prepared from bovine brain PS and palmitic anhydride as described under Materials and Methods. Palmitic acid represented less than 1% of the total fatty acid of bovine brain PS (Salem et al., 1980; data not shown). The purified reaction product had two-dimensional thin-layer chromatographic properties identical with those of the material isolated from *R. sphaeroides* M29-5, and when a 1-mg sample of the synthetic phospholipid was applied to a column (2 × 20 cm) of Cellex D, approximately 72% of the phospholipid was recovered in the chloroform–acetic acid (3:1) eluate. In addition the infrared spectrum of the synthetic NAPS displayed absorption signals attributable to an amide bond, which were not present in the starting materials (data not shown). The susceptibility of the amide-linked fatty acid in NAPS to alkaline methanolysis was directly confirmed by the specific release of methyl palmitate from the synthetic phospholipid under these conditions, and 3 equiv of fatty acyl methyl esters with the expected fatty acid composition (i.e., approximately one-third methyl palmitate) was recovered after acid methanolysis of the synthetic phospholipid.

Discussion

By a combination of spectroscopic and chemical techniques, a newly discovered phospholipid has been identified as an *N*-acylphosphatidylserine. Infrared spectroscopy revealed the presence of both ester and amide bonds in the molecule, and information derived from proton NMR spectroscopy was consistent with the proposed structure and indicated the presence of three acyl chains per phospholipid molecule. Independent confirmation of the structure was obtained by the identification of a glycerylphosphorylserine moiety in the phospholipid and by the recovery of three fatty acyl chains per hydrolyzed phospholipid molecule. On the basis of the fatty acid composition of NAPS presented in Table I, the molecular weight of an average phospholipid molecule was 1046; a minimum molecular weight of approximately 1000 was calculated from elemental analysis of the phospholipid.

The removal of both the amide- and ester-linked fatty acids by mild alkaline methanolysis was unexpected since the conditions employed had previously been shown to be specific for cleavage of ester-linked acyl chains (Carter & Gaver, 1967; Dittmer & Wells, 1969; Kates, 1975). Indeed, recent studies have found the *N*-acyl bond in *N*-acylphosphatidylethanolamines to be resistant to mild alkaline hydrolysis under conditions similar to those employed in this study (Ellingson, 1980; Epps et al., 1980). One possible explanation for the apparent selective susceptibility of the amide-linked fatty acid in NAPS to alkaline methanolysis is that the proximity of the carboxyl group of serine destabilizes the serine *N*-acyl linkage sufficiently to allow nucleophilic attack under alkaline conditions. The lability to alkaline methanolysis of the *N*-acyl linkage in NAPS was confirmed in studies with the synthetic phospholipid. Thus it appears that not all *N*-acyl fatty acid linkages in phospholipids are resistant to mild alkaline methanolysis. Our ability to recover fatty acyl methyl esters when the aqueous phase of a mild alkaline hydrolyzed preparation of NAPS was subjected to transmethylation suggests that some finite amount (approximately 6%) of the *N*-acyl linkages of NAPS remains intact under these alkaline hydrolysis conditions (0.5 N NaOH in anhydrous methanol).

The conditions used for the production of phospholipid head groups by mild alkaline hydrolysis of ^{32}P-labeled NAPS (0.1 N NaOH in aqueous methanol) were slightly different from those used to esterify fatty acyl chains. The *O*-seryl phosphate ester bond is known to be labile under aqueous alkaline conditions (Greenstein & Winitz, 1961), so NAPS might be expected to undergo cleavage at this position during alkaline hydrolysis. Cleavage of NAPS at the seryl phosphate bond during alkaline methanolysis would produce glycerol phosphate. Hydrolysis of NAPS at this site could account for the previous identification of what we believe to be the same phospholipid from *R. sphaeroides* N.C.I.B. 8253 as phosphatidic acid (Lascelles & Szilagyi, 1965). Identification of the phospholipid as phosphatidic acid in the previous study was based solely on the identification of glycerol phosphate

as a water-soluble product following alkaline methanolysis of a ^{32}P-labeled phospholipid mixture from *R. sphaeroides* N.C.I.B. 8253.

A survey of the available literature reveals what appears to be inconsistencies in the reported phospholipid composition of various strains of *R. sphaeroides* (Cain et al., 1981; Cohen et al., 1979; Kenyon, 1978; Marinetti & Cattieu, 1981; Russell & Harwood, 1979). We have been able to detect at least trace amounts of NAPS in all strains of *R. sphaeroides* tested (including wild-type strains 2.4.1, 2.4.7, and N.C.I.B. 8253), and we have found that the accumulation of NAPS is induced by Tris in all of the strains tested except for *R. sphaeroides* strain 2.4.1 (unpublished data). These facts plus the suspected inadvertent identification of NAPS as phosphatidic acid (for possible reasons cited above) should help to resolve some of the discrepancies in the reported phospholipid composition of *R. sphaeroides*. A more detailed physiological characterization of the growth conditions that result in an alteration of phospholipid metabolism and the accumulation of NAPS to as much as 40% of the total phospholipid will be the subject of a future communication.

A relatively high proportion of vaccenic acid has been previously reported for both whole cell phospholipid (Kenyon, 1978) and individual phospholipid species (Russell & Harwood, 1979) derived from *R. sphaeroides*. The finding that the fatty acid composition of NAPS is not significantly different from that of whole cell phospholipid suggests that the amide-linked fatty acyl chain is derived from the same fatty acid pool used as the source of ester-linked phospholipid fatty acids under those growth conditions that lead to the accumulation of NAPS. Definitive proof of this fact awaits our ability to selectively deacylate and analyze the ester- and amide-linked fatty acyl chains of this phospholipid. In this regard it would also be of interest to analyze and compare the composition of the amide-linked fatty acids of NAPS purified from cells that are and are not accumulating this phospholipid. It is possible that the fatty acid composition of the amide-linked chains might not be the same in these two cases and that the overall fatty acid composition reported for NAPS herein is partly a reflection of the cell satisfying the increased demand for amide-linked fatty acyl chains imposed by the accumulation of this phospholipid by using fatty acids that it can synthesize in the greatest abundance.

On the basis of the specificity of the *R. sphaeroides* glycerol-3-phosphate acyltransferase, we might expect the synthesis of the *N*-acyl linkage to require an acyl carrier protein donor (Lueking & Goldfine, 1975). Other mechanisms such as the PE-dependent transacylation reaction implied in the synthesis of *N*-acylphosphatidylethanolamine in a *Butyrivibrio* species (Matsumoto & Miwa, 1973) or the acyl phosphate dependent, cyanide-induced acylation of amino acids indentified in *Clostridium kluyveri* (Stadtman et al., 1952; Katz et al., 1953) are also feasible for the synthesis of the *N*-acyl linkage.

N-Acyl phospholipids have been found in other systems. Although a phospholipid that represents approximately 4% of the total phospholipid of sheep red blood cells has been tentatively identified as an *N*-acylphosphatidylserine (Nelson, 1970), our ability to compare the properties of the phospholipid isolated from *R. sphaeroides* with those of the material from sheep red blood cells was limited by the paucity of information supplied on the techniques used to identify the sheep red blood cell phospholipid. *N*-Acylphosphatidylethanolamines constitute a relatively small fraction of the total phospholipid in plant seeds (Dawson et al., 1969), a *Butyrivibrio* species (Matsumoto & Miwa, 1973), *Dictyostelium* (Ellingson, 1980), and infarcted regions of canine myocardium (Epps et al., 1980). At present it is not clear in any system exactly why or how *N*-acyl phospholipids are produced. One hypothesis currently under investigation is that NAPS is an intermediate involved in the donation of amide-linked fatty acids to the outer membrane lipoprotein (Baumgardner et al., 1980) or lipopolysaccharide (Drews et al., 1978) of *R. sphaeroides*.

The conditions that lead to the accumulation of NAPS in membranes of *R. sphaeroides* M29-5 have no detectable effect on the growth of the cells under either chemoheterotrophic or photoheterotrophic conditions. Our ability to experimentally control the accumulation and turnover of NAPS in membranes of *R. sphaeroides* should allow us to use this phospholipid as a tool to study the regulation, synthesis, and degradation of phospholipids in this organism. Extending our ability to control the level of NAPS within membranes of *R. sphaeroides* to our knowledge of the assembly of the various membrane systems of this bacterium makes NAPS a very useful probe for monitoring the synthesis, assembly, and turnover of both phospholipids and membranes in a procaryotic cell capable of membrane differentiation.

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Inhibition and Inactivation of Estrogen Synthetase (Aromatase) by Fluorinated Substrate Analogues†

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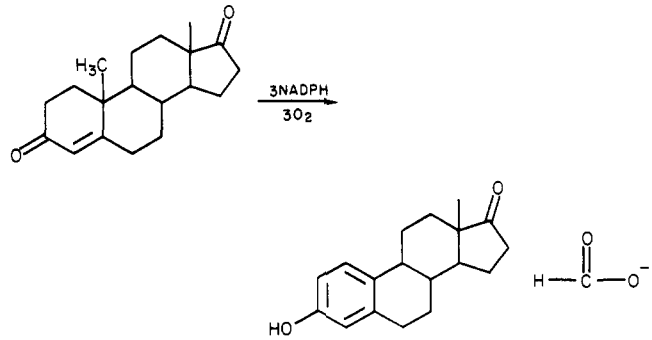
ABSTRACT: 19,19-Difluoroandrost-4-ene-3,17-dione (**1**) and 19-fluorcandrost-4-ene-3,17-dione (**2**) have been synthesized, and the interaction of these compounds with the estrogen synthetase (aromatase) activity of human placental microsomes has been studied. **1** has been found to cause time-dependent, irreversible inactivation of this enzyme (K_i = 1 μM, k_{inact} = 0.023 min^{-1}). A possible mechanism of this process is enzymatic generation of an acyl fluoride through oxidation of **1**. Compound **2** does not cause inactivation, and this substrate analogue has been shown to be converted to estrone in high yield by this enzyme system.

Estrogen synthetase (aromatase) is a key enzyme that is involved in the conversion of androgens (male sex hormones) into estrogens (female sex hormones). Aromatase and androgen 5α-reductase are the enzymes that catalyze reactions of an important branch point in biosynthetic steroid transformations. Since the androgen/estrogen balance is considered to be a factor in many biological processes (Motohashi et al., 1979), work in this laboratory and elsewhere has focused on the development of specific inhibitors of the above enzymes (Blohm et al., 1980; Brodie et al., 1977; Covey et al., 1981; Marcotte & Robinson, 1981; Metcalf et al., 1981; Schade & Schubert, 1979; Schwarzel et al., 1973). In particular, the extraordinary importance of aromatase has led to much work with preparations of microsomes from human term placenta



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