Identification of *N*-Acylethanolamine Phosphoglycerides and Acylphosphatidylglycerol as the Phospholipids Which Disappear as *Dictyostelium discoideum* Cells Aggregate†

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ABSTRACT: The cellular slime mold *Dictyostelium discoideum* contains a phospholipid fraction which comprises 10% of the phospholipids in the early developing amoebae and disappears during the aggregation stage of development. As a first step in studying its metabolism, the composition of the fraction has been determined. It was easily isolated by preparative silicic acid thin-layer chromatography because its R_f was considerably higher than most commonly encountered phospholipids. Its R_f was the same as synthetic phosphatidyl-*N*-acylethanolamine and synthetic acylphosphatidylglycerol (also called semilysobisphosphatidic acid). Strong absorption peaks characteristic of amide bonds in the infrared spectrum of the isolated *D. discoideum* phospholipid showed that *N*-acylethanolamine phosphoglycerides were present. The presence of acylphosphatidylglycerol was revealed when mild alkaline hydrolysis of the lipid fraction produced glycerophosphorylglycerol as the only water-soluble, phosphate-containing product. The composition of the fraction was determined by chemical analysis and thin-layer chromatography of the intact phospholipids and their partially or completely hydrolyzed products. The composition of the fraction was 30% diacylglycerophosphoryl-*N*-acylethanolamine, 50% alkenylacylglycerophosphoryl-*N*-acylethanolamine, and 20% acylphosphatidylglycerol. The stereoconfiguration of the glycerophosphorylglycerol moiety of the acylphosphatidylglycerol was found to be *sn*-3-glycerophosphoryl-*sn*-1′-glycerol.

During the life cycle of the cellular slime mold *Dictyostelium discoideum*, changes in membrane structure and function occur. In the vegetative amoebae which use bacteria as a food source, the plasma membrane is actively engaged in phagocytosis, and large numbers of whorl-filled phagolysosomes are present in the cells (Gezelius, 1961; Hohl, 1965). When the bacteria have been consumed, the amoebae commence a developmental program by forming organized aggregates. During this aggregation process, the cells attain the ability to adhere to neighboring cells, and new cell-surface antigens appear which are involved in the mutual adhesion of cells (Beug et al., 1973a,b). Changes in the plasma membrane ultrastructure (Gregg & Yu, 1975) and protein content (Siu et al., 1976; Hoffman & McMahon, 1977; Gilkes et al., 1979) have also been detected. As the cells aggregate, the phagolysosomes disappear (Gezelius, 1961).

Accompanying those alterations in nonlipid membrane components and subcellular organelle content during cell aggregation are qualitative and quantitative changes in the phospholipid composition (Ellingson, 1974). An unknown phospholipid which comprised 10% of the amoebae phospholipids could not be detected in the early cell aggregates. Since phospholipids are predominantly associated with membranes, it is possible that the metabolism responsible for the disappearance of the phospholipid might be useful to study the regulation of membrane turnover. Before a detailed study of the lipid's metabolism could be made, its structure had to be determined. This report presents the spectroscopic, chromatographic, and chemical data which show that the isolated lipid fraction contains a mixture of two different types of phospholipids.

Materials and Methods

Growth of Dictyostelium discoideum. Amoebae of *D. discoideum* NC-4 were harvested after they had grown in association with a prototrophic strain of *Aerobacter aerogenes* on SM-agar plates until the amoebae were at the end of the exponential growth phase (Ellingson, 1974).

Extraction and Purification of the Phospholipid Fraction. Lipids were extracted with chloroform–methanol mixtures as previously described (Ellingson, 1974). The lipids were separated on silicic acid columns with the following sequence of solvents: 8 column volumes of $CHCl_3$, 8 column volumes of 10% CH_3OH in $CHCl_3$, 8 column volumes of 15% CH_3OH in $CHCl_3$, and 8 column volumes of CH_3OH. Most of the unknown phospholipid eluted with solvents containing 10% CH_3OH, and the remainder eluted with the first portion of the 15% CH_3OH solvent, but other phospholipids also eluted with those fractions. Isolation of the pure phospholipid fraction was accomplished by preparative TLC[1] with 0.5-mm-thick silica gel G (Merck) plates developed in $CHCl_3$–CH_3OH–NH_4OH (40:10:1 v/v). The silicic acid containing the desired phospholipid (R_f 0.70; see Figure 3, lane A) was scraped from the plate, and the lipid was eluted by mixing the silicic acid with $CHCl_3$–CH_3OH (1:4 v/v) and then centrifuging the mixture. After the supernatant solution was decanted from the sedimented silicic acid, the elution procedure was repeated three times. The combined supernatant solutions were evaporated to dryness in a rotary evaporator, and the lipid was dissolved in a few milliliters of $CHCl_3$. The purity of the isolated phospholipid fraction was analyzed by TLC with silica gel G (Merck) plates developed in solvent I, $CHCl_3$–CH_3OH–NH_4OH (40:10:1 v/v), and silica gel H (Merck) magnesium silicate plates (Rouser et al., 1968) developed in solvent II, $CHCl_3$–CH_3OH–NH_4OH (65:40:5 v/v) and solvent III,

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[1] Abbreviations used: GPG, glycerophosphorylglycerol; APG, acylphosphatidylglycerol; PNAE, phosphatidyl-*N*-acylethanolamine; TLC, thin-layer chromatography; BHK, cultured baby hamster kidney cells.

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$CHCl_3$–acetone–CH_3OH–CH_3COOH–H_2O (33:36:5:10:4 v/v). After TLC, the phospholipid was analyzed for phosphate with a phosphate-detecting spray reagent (Dittmer & Lester, 1964), for primary amino groups with a ninhydrin reagent (Dittmer & Wells, 1969), and for vinyl ethers with Schiff's fuchsin reagent (Dittmer & Wells, 1969). The presence of other organic compounds was checked by using a formaldehyde–sulfuric acid charring reagent (Rouser et al., 1970).

Infrared Spectroscopy. Each phospholipid was deposited as a thin film between NaCl disks, and the infrared spectrum was recorded with a Beckman Model IR-18A infrared spectrophotometer.

Synthesis of Phosphatidyl-N-acylethanolamine, Phosphatidyl-N-acylserine, and Acylphosphatidylglycerol. The phospholipids were synthesized by reacting a fatty acyl chloride with an authentic phospholipid as described by Matsumoto & Miwa (1973). PNAE was synthesized by reacting authentic phosphatidylethanolamine with twice as many micromoles of stearoyl chloride in 5 mL of chloroform containing 1 mL of pyridine for 2 h at room temperature. Phosphatidyl-*N*-acylserine was synthesized by using authentic phosphatidylserine and stearoyl chloride. APG was synthesized by the same procedure by using authentic phosphatidylglycerol and stearoyl chloride, but the reaction time was increased to 6 h. After the reaction had proceeded for the specified amount of time, the reaction mixture was extracted twice with 2 volumes of saturated $NaHCO_3$. Then the chloroform layer was evaporated in a rotary evaporator, and the lipids were dissolved in a few milliliters of $CHCl_3$. The lipids were fractionated on a silicic acid column with the same solvents used in the isolation of the slime mold phospholipid. After evaporation of the solvents under vacuum, the synthesized phospholipids were dissolved in a few milliliters of $CHCl_3$. Sometimes the column fractions were not pure, and in such cases the phospholipid was purified by the same preparative TLC procedure used to isolate the *D. discoideum* phospholipid.

Mild Alkaline and Acid Hydrolysis of the Isolated Phospholipid. Mild alkaline hydrolysis was carried out by dissolving 1 or 2 μmol of lipid phosphate in 1 mL of $CHCl_3$–CH_3OH (1:4 v/v) and then adding 0.1 mL of 1.2 N NaOH in CH_3OH–H_2O (1:1 v/v). The lipid was hydrolyzed for 10 min at 37 °C, and after neutralization with CH_3COOH the deacylated water-soluble products were separated from the nonsaponifiable organic-soluble products as described by Dittmer & Wells (1969). Vinyl ethers were hydrolyzed by treating 1–3 μmol of lipid phosphate dissolved in 1.5 mL of $CHCl_3$ with 1.5 mL of 0.1 N HCl in 99% CH_3OH for 1 h at room temperature. The products were partitioned between aqueous and organic phases according to the procedure of Renkonen (1962). The organic-soluble compounds were analyzed on silica gel G plates in solvent I. The water-soluble components were separated on 0.25-mm cellulose thin-layer plates by using solvent IV, 2-propanol–H_2O–NH_4OH (7:2:1 v/v), solvent V, butanol–CH_3COOH–H_2O (5:3:1 v/v), and solvent VI, butanol–propionic acid–H_2O (45:21:30 v/v). The water-soluble, phosphate-containing compounds were detected on the cellulose TLC plates with a phosphate-detecting spray reagent (Hanes & Isherwood, 1949).

Stereochemical Analysis of Glycerophosphorylglycerol. The slime mold phospholipid was deacylated by the method of Dittmer & Wells (1969), and the water-soluble fraction was concentrated to a small volume. The GPG in the aqueous phase was degraded to glycerol and glycerol phosphate by subjecting it to strong alkaline hydrolysis in 0.1 N NaOH at 100 °C for 36 h (Figure 2, procedure B). The amount of *sn*-glycerol 3-phosphate was assayed spectrophotometrically by using the stereospecific enzyme *sn*-glycerol-3-phosphate dehydrogenase [*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase (EC 1.1.1.8)] as described by Michal & Lang (1974). The glycerol content was determined by the method of Wieland (1974) by using a combination of glycerol kinase [ATP:glycerol 3-phosphotransferase (EC 2.7.1.30)] to convert the glycerol to *sn*-glycerol 3-phosphate and then measuring the amount of *sn*-glycerol 3-phosphate with *sn*-glycerol-3-phosphate dehydrogenase. The enzyme assays were conducted in a Gilford recording spectrophotometer.

Chemical Analyses of the Phospholipid Fraction and Its Hydrolysis Products. The compounds were analyzed for acyl esters (Snyder & Stephens, 1959), phosphate (Bartlett, 1959), and vicinal glycol groups (Ansell & Spanner, 1963). Total hydrolysis of the phospholipid was accomplished with 4 M HCl at 110 °C for 4 h, and after the hydrolysate was neutralized with 4 M NaOH ethanolamine was determined as described by Axelrod et al. (1953). Ethanolamine was identified on TLC cellulose plates by using butanol–CH_3COOH–H_2O (5:3:1 v/v). Vinyl ether content was determined by an iodine spectrophotometric method (Gottfried & Rapport, 1963). The amount of vinyl ethers was also measured by analytical TLC. The phospholipid was subjected to mild acid hydrolysis (Renkonen, 1962) to hydrolyze the vinyl ether bonds, and then 100 μmol of the hydrolyzed lipid phosphate was chromatographed on silica gel G TLC plates in solvent I. The phospholipids were detected by the phosphate-detecting spray (Dittmer & Lester, 1964), and the areas containing the phospholipids were scraped from the plate and analyzed for phosphate as previously described (Ellingson, 1974). The amounts of unhydrolyzed phospholipid, which had contained no vinyl ethers, and the hydrolyzed phospholipid, which had contained vinyl ethers, were determined. The recovery of phosphate from the plates was greater than 95% of the amount applied.

Results

Characteristics of the D. discoideum Phospholipid Fraction. The *D. discoideum* phospholipids were eluted from a silicic acid column with increasing amounts of CH_3OH in $CHCl_3$, and a fraction which eluted mainly with 10% CH_3OH in $CHCl_3$ was detected when the column fraction was analyzed by silicic acid TLC in solvents I, II, and III. Its high R_f corresponded to the phospholipid previously found to disappear during the aggregation stage of development (Ellingson, 1974). This phospholipid fraction was isolated by preparative TLC, and when it was subjected to TLC in solvents I, II, and III only one spot was detected by the phosphate-detecting spray or the charring spray reagent. The presence of plasmalogens was indicated by a positive reaction with Schiff's fuchsin spray reagent, and the absence of primary amino groups was revealed by the failure to react with ninhydrin. The phospholipid had the same R_f as synthetic PNAE and APG in TLC solvents I, II, and III, and its R_f was markedly different from those of all other common phospholipids, including the other acidic phospholipids, cardiolipin, phosphatidylglycerol, and phosphatidic acid.

Chemical Characterization of the Synthetic Phospholipids. Reaction of phosphatidylethanolamine with an acyl chloride produced a ninhydrin-negative phospholipid which migrated farther than phosphatidylethanolamine in silicic acid TLC solvents I, II, and III. The synthetic lipid had an R_f of 0.70 (Figure 3, lane A) in TLC solvent I. When it was subjected to mild base hydrolysis and the products were partitioned between aqueous and organic phases, over 98% of the lipid

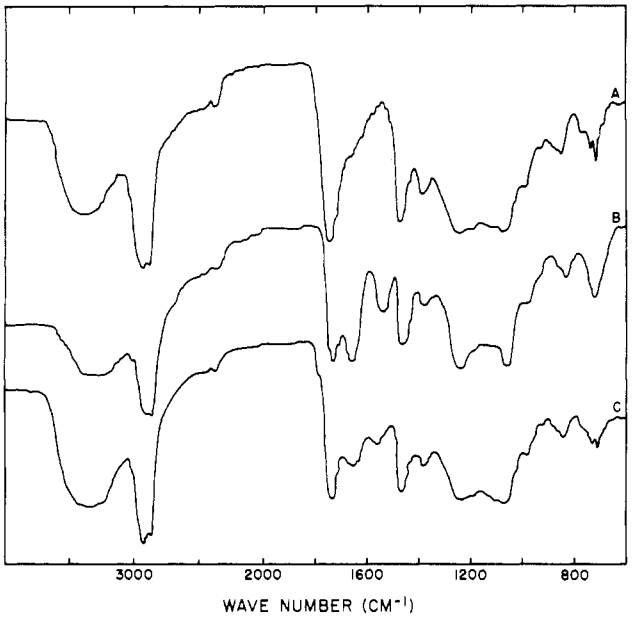

FIGURE 1: Infrared spectra of synthetic acylphosphatidylglycerol (A), synthetic phosphatidyl-*N*-acylethanolamine (B), and the isolated *D. discoideum* phospholipid fraction composed of 20% acylphosphatidylglycerol and 80% phosphatidyl-*N*-acylethanolamine and its plasmalogen form (C).

phosphorus was recovered in the organic layer. The only phospholipid present did not react with ninhydrin and had a very low R_f of 0.06 (Figure 3, lane C) when analyzed by TLC. Its molar ratio of vicinal diol to ethanolamine to phosphate was 1:1:1, which is the theoretical ratio expected for *sn*-glycero-3-phosphoryl-*N*-acylethanolamine.

When phosphatidylglycerol reacted with an acyl chloride, a phospholipid was produced which migrated farther than phosphatidylglycerol on silicic acid TLC. The major product had an R_f of 0.7 in solvent I (Figure 3, lane A) and a molar ratio of acyl ester to vicinal diol to phosphate of 3:0:1, which is characteristic of acylphosphatidylglycerol.

Infrared Spectrum of the Synthetic Phospholipids and the D. discoideum Phospholipid Fraction. The infrared spectrum of the isolated phospholipid fraction exhibited strong absorption peaks at 1540 and 1650 cm^{-1} (Figure 1, trace C) which are characteristic of amide-containing compounds. It also had a strong peak at 1720 cm^{-1} due to the presence of acyl ester bonds. The spectrum is very similar to the one for the authentic PNAE (Figure 1, trace B) synthesized from phosphatidylethanolamine and stearoyl chloride and the ones of PNAE published by other investigators (Somerharju & Renkonen, 1979; Gray, 1976; Matsumoto & Miwa, 1973; Dawson et al., 1969). The other phospholipids known to contain amide bonds are sphingomyelin and phosphatidyl-*N*-acylserine, but both of these lipids had significantly lower R_f values than PNAE when chromatographed in solvents I, II, and III. The infrared spectrum of synthetic APG does not contain the strong peaks at 1540 and 1650 cm^{-1} but does contain the acyl ester peak at 1720 cm^{-1} (Figure 1, trace A). Since PNAE, APG, and the isolated *D. discoideum* phospholipid fraction have the same chromatographic mobilities on silicic acid (Figure 3, lane A), the infrared spectrum of the isolated phospholipid fraction (Figure 1, trace C) clearly shows that the amide bond of PNAE is present in the *D. discoideum* phospholipid.

Characterization of the D. discoideum Phospholipid. The series of hydrolytic procedures used to characterize the

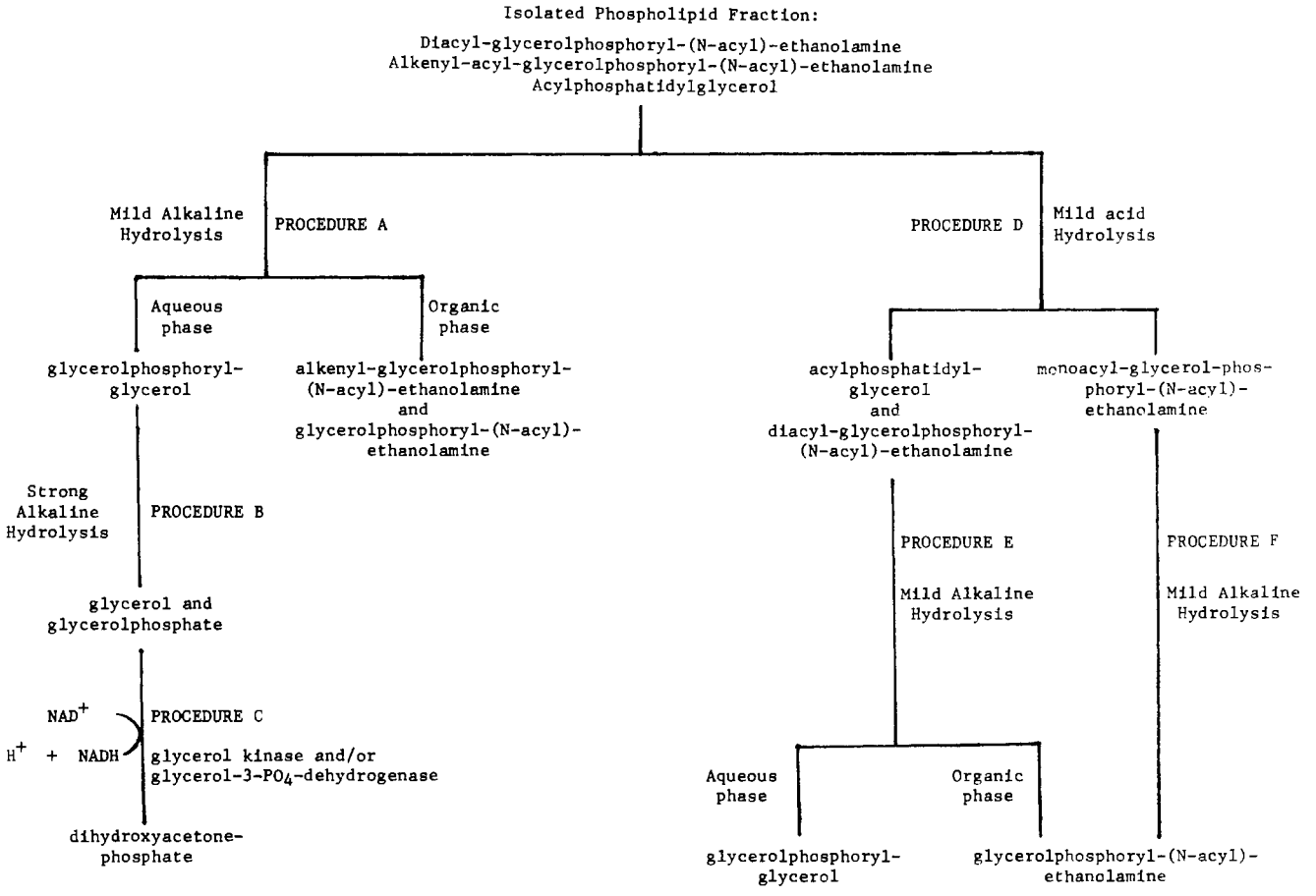

FIGURE 2: Stepwise degradation of the isolated *D. discoideum* phospholipid fraction by acid and base hydrolysis.

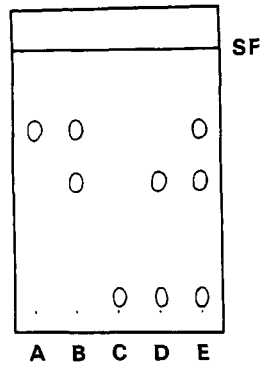

FIGURE 3: Thin-layer silica gel G chromatogram of the isolated *D. discoideum* phospholipid fraction and its organic-soluble degradation products. The chromatogram was developed in solvent I. (A) Isolated phospholipid fraction; also synthetic phosphatidyl-*N*-acylethanolamine or synthetic acylphosphatidylglycerol. (B) Products produced by mild acid hydrolysis of the isolated phospholipid. (C) Products produced by mild alkaline hydrolysis of either the compounds in lane B or the synthetic phosphatidyl-*N*-acylethanolamine. (D) Products produced by mild alkaline hydrolysis of the isolated phospholipid. (E) Products produced by partial alkaline hydrolysis of synthetic phosphatidyl-*N*-acylethanolamine. SF, solvent front.

phospholipid fraction are shown in Figure 2. These procedures were carried out on the phospholipid isolated from several different preparations of cells, and the same results were obtained from each preparation.

When the synthetic PNAE was subjected to partial hydrolysis by mild alkaline conditions, three ninhydrin-negative components are detected by TLC (Figure 3, lane E), unhydrolyzed PNAE (R_f 0.70), monoacylglycerophosphoryl-*N*-acylethanolamine (R_f 0.48), and deacylated glycerophosphoryl-*N*-acylethanolamine (R_f 0.06). After treatment of 1 μmol of the isolated *D. discoideum* phospholipid with mild acid to hydrolyze the vinyl ether bonds (Figure 2, procedure D), over 98% of the phosphate remained organic soluble, and two phospholipids were detected on silica gel G thin-layer plates developed in solvent system I (Figure 3, lane B). When 100 nmol of the mild acid hydrolyzed lipid phosphate was chromatographed, 55 nmol was present in a compound which had an R_f (0.70) the same as that of the synthetic PNAE and the original *D. discoideum* phospholipid, and it was that portion of the phospholipid fraction which had contained only acyl ester bonds and no vinyl ether bonds. It contained the APG and PNAE. The other 45 nmol was detected in the spot with the lower R_f (0.48) derived from the phospholipid molecules which had contained vinyl ether bonds. It was monoacylglycerophosphoryl-*N*-acylethanolamine and was identified from the following facts. When the *D. discoideum* phospholipids remaining after mild acid hydrolysis (R_f's 0.70 and 0.48 shown in Figure 3, lane B) were hydrolyzed by mild alkaline conditions (Figure 2, procedures E and F), they both yielded the same organic-soluble phospholipid which was ninhydrin negative and had the same low R_f (0.06) as the base-hydrolyzed synthetic PNAE (Figure 3, lane C). The base-hydrolyzed slime mold phospholipid with the low R_f (0.06) was identified as glycerophosphoryl-*N*-acylethanolamine after isolation by preparative TLC and analysis for specific chemical components. The results in Table I show that it had a phosphorus to vicinal diol to ethanolamine ratio which was consistent with the theoretical ratio of 1:1:1, expected for glycerophosphoryl-*N*-acylethanolamine. In all the analyses, a sample containing a known amount of lipid phosphate was analyzed for other components. The amount of lipid phosphate used for ethanolamine analysis was 0.7–1.0 μmol, and for vicinal diol determinations it was 0.25–0.5 μmol. The validity

Table I: Analysis of Authentic Phospholipids and the Organic-Soluble Product Formed from the *D. discoideum* Phospholipid by a Combination of Mild Alkaline and Mild Acid Hydrolyses

lipid analyzed	product (mol/mol of phosphate)		
	total phosphate	vicinal diol	ethanolamine
phosphatidylglycerol	1.0	1.1	0
phosphatidylethanolamine	1.0	0	1.0
phosphatidyl-*N*-acylethanolamine	1.0	0	1.0
glycerophosphoryl-*N*-acylethanolamine (acid and base hydrolyzed *D. discoideum* lipid)	1.0	1.1	1.0

of the ethanolamine analysis was checked by determining the phosphorus to ethanolamine ratio in authentic phosphatidylethanolamine and the synthetic PNAE. The accuracy of the vicinal diol method was established by obtaining the theoretical phosphorus to vicinal diol ratio of authentic phosphatidylglycerol (Table I).

All of the evidence suggests that hydrolysis of the vinyl ether and acyl ester bonds in the *D. discoideum N*-acylethanolamine phosphoglycerides produced glycerophosphoryl-*N*-acylethanolamine which had an R_f of 0.06 on silica gel G thin-layer plates developed in solvent I.

When 120 nmol of the isolated phospholipid fraction was analyzed by the iodine spectrophotometric analysis for vinyl ethers, the molar ratio of vinyl ether bonds to phosphorus was 0.5:1.0. If the analysis was made by TLC of 100 nmol of the mild acid hydrolyzed phospholipid, the ratio was 0.45:1.0. Thus, the two independent methods both indicate that 45–50% of the phospholipid molecules are plasmalogens. As already stated, only one hydrolyzed phospholipid is produced by mild acid hydrolysis of the *D. discoideum* phospholipid fraction (Figure 3, channel B), indicating only one type of plasmalogen molecule is present.

Detection of Acylphosphatidylglycerol in the D. discoideum Phospholipid. The presence of APG was detected by analyzing the deacylated, water-soluble, and phosphorus-containing compound produced by mild base hydrolysis of the *D. discoideum* phospholipid fraction (Figure 2, procedure A). The product was analyzed by TLC and by chemical analysis to determine whether it was the expected GPG.

When the products produced by mild alkaline hydrolysis of 1 μmol of slime mold phospholipid were partitioned between the organic phase and the aqueous phase (Figure 2, procedure A), 190–210 nmol or about 20% of the phosphate was rendered water soluble. The remainder was found in the organic layer, and the total recovery of phosphate from both layers was over 98% of the amount used for hydrolysis. If the base hydrolysis was repeated or if mild acid hydrolysis was performed on the remaining organic-soluble compounds, no decrease in organic-soluble phosphorus-containing products was observed. The only water-soluble phosphorus-containing compound produced by the base hydrolysis had the same chromatographic properties as the water-soluble GPG produced by mild alkaline hydrolysis of authentic phosphatidylglycerol when analyzed by cellulose TLC in solvent systems IV, V, and VI.

The water-soluble deacylated product produced by mild alkaline hydrolysis of the *D. discoideum* phospholipid was analyzed for its molar ratio of phosphorus to vicinal diols to glycerol to see if it had the theoretical ratio of 1:2:2 expected for GPG. The results in Table II show that the water-soluble product had a ratio which was well within the expected the-

Table II: Mild Alkaline Hydrolysis Water-Soluble Products of Bacterial Phosphatidylglycerol and *D. discoideum* Acylphosphatidylglycerol

lipid hydrolyzed	product (mol/mol of phosphate)			
	total phosphate	vicinal diol	glycerol[a]	*sn*-glycerol 3-phosphate[b]
phosphatidylglycerol (bacterial)	1.0	2.1	2.0	0.25
acylphosphatidylglycerol (*D. discoideum*)	1.0	2.0	1.8	0.24

[a] Twice the amount of free glycerol formed by strong alkaline hydrolysis of the water-soluble product. See text for details. [b] The amount of *sn*-glycerol 3-phosphate formed by strong alkaline hydrolysis of the water-soluble product. See text for details.

oretical ratio. For vicinal glycol determination, the amount of deacylated phosphate analyzed was 60–70 nmol, and the glycerol standard had been subjected to the base-hydrolysis procedure. The amount of glycerol was determined spectrophotometrically by the method discussed in the next section.

Determination of the Stereoconfiguration of Glycerophosphorylglycerol. The GPG produced by mild alkaline hydrolysis of bacterial phosphatidylglycerol and *D. discoideum* phospholipid was subjected to strong alkaline hydrolysis to yield glycerol and glycerol phosphate (Figure 2, procedure B). The cleavage occurs with equal probability on both sides of the phosphorus. This results in the formation of 1 mol of glycerol and 1 mol of glycerol phosphate per mol of GPG hydrolyzed, with the phosphate distributed equally between the glycerol molecules. Because the reaction proceeds through a cyclic intermediate, 42–44% (Brown et al., 1958; Baer & Kates, 1948) of the glycerol phosphate formed will be α-glycerol phosphate, and the remainder will be β-glycerol phosphate. Thus, each of the glycerol molecules will contain 21–22% of the total phosphorus in the α position. So if each glycerol in the original GPG was present as *sn*-glycerol 3-phosphate, 42–44% of the total phosphorus in the hydrolyzed product will be *sn*-glycerol 3-phosphate. If one of the original glycerols formed *sn*-glycerol 3-phosphate, then only 21–22% of the phosphorus-containing products would be *sn*-glycerol 3-phosphate.

The amount of *sn*-glycerol 3-phosphate was assayed by using the stereospecific enzyme *sn*-glycerol-3-phosphate dehydrogenase, which will react with only *sn*-glycerol 3-phosphate (Figure 2, procedure C). The validity of the procedure was checked by assaying specific amounts of commercially obtained *sn*-glycerol 3-phosphate and DL-α-glycerol phosphate which would produce changes in absorbance between 0.311 and 0.622. Since the glycerol moieties of bacterial phosphatidylglycerol are known to be of opposite configuration (Benson & Mirjano, 1961; Chang & Kennedy, 1967), 21–22% of its phosphate will be *sn*-glycerol 3-phosphate, so its deacylated, water-soluble product was used as a control for assaying the hydrolysis products. The results in Table II show that both bacterial phosphatidylglycerol and *D. discoideum* phospholipid yielded 24–25% *sn*-glycerol 3-phosphate, indicating the two glycerol moieties of the *D. discoideum* APG are of the opposite configuration.

A further check of the strong alkaline hydrolysis procedure was performed by measuring the amount of free glycerol produced, since one molecule of free glycerol should be produced for every GPG molecule hydrolyzed. Glycerol was determined by using a combination of *sn*-glycerol-3-phosphate dehydrogenase and glycerol kinase to convert glycerol to *sn*-glycerol 3-phosphate. The value obtained was doubled, and the molar ratio of glycerol to phosphate was calculated. The results in Table II show that the deacylated, water-soluble, and phosphorus-containing compound derived from both the bacterial phosphatidylglycerol and the *D. discoideum* phospholipid contained the expected two molecules of glycerol. The validity of the method was established by assaying known amounts of glycerol or combinations of *sn*-glycerol 3-phosphate and glycerol.

Table III: Analysis of the *D. discoideum* Phospholipid Fraction and Authentic Phosphatidylglycerol

lipid analyzed	product (mol/mol of phosphate)			
	total phosphate	ester	vinyl ether	vicinal diol
phosphatidylglycerol	1.0	2.1	0	1.1
D. discoideum phospholipid fraction	1.0	1.9	0.5	0

Determination of the Composition of the D. discoideum Phospholipid Fraction. All the results presented indicate that the isolated phospholipid fraction is a combination of three types of molecules. Mild alkaline hydrolysis converts about 20% of the phospholipid into water-soluble GPG, and no more GPG is produced if mild acid hydrolysis is subsequently carried out. The GPG is derived from APG, so 20% of the fraction is APG. The spectrophotometric I_2 method and TLC method both showed that about 50% of the fraction is the plasmalogen molecule, alkenylacylglycerophosphoryl-*N*-acylethanolamine. The remaining 30% is diacylglycerophosphoryl-*N*-acylethanolamine. A phospholipid fraction with the proposed composition would contain 17 acyl ester bonds and 5 alkenyl ether bonds for every 10 molecules of phosphate, and its theoretical molar ratio of the combined ester plus vinyl ether bonds to phosphate would be 2.2:1.0. The molar ratio found for the isolated lipid fraction was 2.4:1.0 (Table III), which lends support to the probability that the composition deduced from the chemical degradation results is correct.

Discussion

Two rare phospholipids containing a GPG moiety are bis(acylglycerol) phosphate (also referred to as lysobisphosphatidic acid) and APG (also referred to as semilysobisphosphatidic acid). In rat liver (Wherrett & Huterer, 1972), rabbit alveolar macrophages (Mason et al., 1972), and BHK cells (Brotherus & Renkonen, 1977), the secondary lysosomes are enriched with bis(acylglycerol) phosphate. It is also associated with the secondary lysosomes filled with multilamellar structures in patients with type A Niemann Pick disease (Rouser et al., 1968; Kamoshita et al., 1969) and in livers of drug-treated rats (Tjiong et al., 1978; Tjiong & Debuch, 1978). APG has been identified in *Salmonella typhimurium* (Olsen & Ballou, 1971), degenerating BHK cells (Somerharju et al., 1977), and in liver lysosomes of chloroquine-treated rats (Tjiong & Debuch, 1978).

The *N*-acylethanolamine phosphoglycerides have been found in a *Butyrivibrio* species (Hazelwood & Dawson, 1975) and in bovine erythrocyte stroma (Matsumoto & Miwa, 1973). *N*-Acylethanolamine phospholipids have been detected in some cells with a significant amount of catabolic activity. The PNAE is present in seeds during early stages of germination (Dawson et al., 1969), a time during which many degradative enzymes are activated. It comprised 26% of the phospholipids in granular cells of mammalian epidermis (Gray, 1976), which are a degenerating type of cell and have increased catabolic activities. *N*-Acylethanolamine phospholipids comprise 4–6% of the phospholipids in the infarcted areas of ligated canine

myocardium, which contain degenerating cells induced by the myocardial ischemia (Epps et al., 1979). The diacyl and alkenylacyl forms of *N*-acylethanolamine phosphoglycerides have been found in degenerating BHK cells induced to carry out high levels of autolysis (Somerharju & Renkonen, 1979).

Degenerating BHK cells contain whorl-filled autophagic vacuoles and a phospholipid fraction containing 20% APG and 80% *N*-acylethanolamine phosphoglycerides, which is the same proportion of the two lipids in *D. discoideum* amoebae. This APG–PNAE fraction in degenerating BHK cells can comprise 6.5% of the phospholipids (Brotherus et al., 1977). *D. discoideum* amoebae contain numerous whorl-filled phagolysosomes (Gezelius, 1961; Hohl, 1965) which are carrying out a high level of catabolic activity. Since they also contain APG and PNAE, they are another example of cells containing these lipids and large numbers of secondary lysosomes. A subcellular fraction enriched in acid phosphatase and the PNAE–APG fraction has been isolated from *D. discoideum* amoebae (J. S. Ellingson, unpublished experiments), and future investigations should reveal whether there is a relationship between lysosomes and PNAE and APG in *D. discoideum*.

In the phospholipids with GPG "backbone" isolated from BHK cells (Somerharju et al., 1977), rat liver, rabbit lung, and pig lung (Joutti et al., 1976), the stereoconfiguration of the two glycerol moieties is unique. Both glycerol moieties had the same configuration and were present as *sn*-glycerol 1-phosphate molecules. These phospholipids are the only known glycerol lipids which do not contain an *sn*-glycerol 3-phosphate moiety. In contrast, the APG in *D. discoideum* had a GPG "backbone" with glycerol moieties of opposite configuration. The differences in the stereoconfiguration of APG between mammalian cells and *D. discoideum* suggest that their metabolism is different. The APG in *D. discoideum* cells may arise from the acylation of bacterial phosphatidylglycerol during the digestion process, since its stereoconfiguration is the same as bacterial phosphatidylglycerol and *E. coli* APG (Olsen & Ballou, 1971). APG is not an undigested bacterial phospholipid because it is not present in the *A. aerogenes* used as a food supply for the amoebae (Ellingson, 1974).

It has been previously shown that the *D. discoideum* phospholipid fraction containing APG and PNAE comprised 2% of the phospholipids in amoebae which have just emerged from spores, represented 10% of the phospholipids in amoebae grown on bacteria, and had disappeared in 14-h aggregates (Ellingson, 1974). This accumulation and disappearance of PNAE could possibly be used to study the regulation of the enzymes responsible for the synthesis and degradation of a membrane phospholipid. Not much is known about the metabolism of PNAE. A species of *Butyrivibrio* synthesizes PNAE by reaction in which two molecules of phosphatidylethanolamine are converted to a molecule of PNAE and one of lysophosphatidylethanolamine by intermolecular transacylation (Hazelwood & Dawson, 1975). Since *D. discoideum* ethanolamine phosphoglycerides contain vinyl ethers and those in the *A. aerogenes* food source do not (Ellingson, 1974), it seems that the 60% of *N*-acylethanolamine phosphoglycerides which are plasmalogens are most likely synthesized from *D. discoideum* phospholipids. Gray (1976) has indicated a phospholipase C may be involved in the catabolism of PNAE in granular skin cells. Future experiments should determine how PNAE is formed and degraded in *D. discoideum*. This system may be very useful for investigating the biogenesis and turnover of membranes in a eukaryotic cell.

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Primary Structure of Murine Major Histocompatibility Alloantigens: Amino Acid Sequence of the Cyanogen Bromide Fragment Ia (Positions 139–228) from the H-2K^b Molecule†

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ABSTRACT: The complete amino acid sequence of the cyanogen bromide (CNBr) fragment Ia (CN-Ia) from the murine histocompatibility antigen H-2K^b has been obtained by using radiosequence methodology. This glycopeptide is the largest CNBr cleavage product of the H-2K^b molecule and extends from position 139 to position 228. The sequence determined for CN-Ia was Ala-Ala-Leu-Ile-Thr-Lys-His-Lys-Trp-Glu-Gln-Ala-Gly-Glu-Ala-Glu-Arg-Leu-Arg-Ala-Tyr-Leu-Glu-Gly-Thr-Cys-Val-Glu-Trp-Leu-Arg-Arg-Tyr-Leu-Lys-Asn-Gly-(Asn)-Ala-Thr-Leu-Leu-Arg-Thr-*Asp*-Ser-Pro-Lys-Ala-His-Val-Thr-His-His-Ser-Arg-Pro-*Asp*-*Asp*-Lys-Val-Thr-Leu-Arg-Cys-Trp-Ala-Leu-Gly-Phe-Tyr-Pro-Ala-*Asp*-Ile-Thr-Leu-Thr-Trp-Gln-Leu-Asn-Gly-Glu-Glu-Leu-Ile-Gln-*Asp*-Met. The data were obtained by analysis of fragments derived by thrombic, tryptic, chymotryptic, and V8 protease digestion of CN-Ia. A carbohydrate moiety is attached to Asn at position 176. Homology between this 90 amino acid stretch of H-2K^b and HLA-B7 [Orr, H. T., Lopez de Castro, J. A., Lancet, D., & Strominger, J. L. (1979) *Biochemistry 18*, 5711] is 68%, and differences are noted at positions 176, 177, and 178 which in the H-2 molecule are the attachment region for a second carbohydrate moiety. No carbohydrate was detected in this position for HLA-B7 [Orr, H. T. Lopez de Castro, J. A. Lancet, D., & Strominger, J. L. (1979) *Biochemistry 18*, 5711].

The classical histocompatibility antigens are the products of the polymorphic genes at the K, D, and L regions of the murine H-2 major histocompatibility complex (MHC)[1] [see reviews by Klein (1975, 1979), Snell et al. (1976), and Vitetta & Capra (1978)]. These products are integral cell surface glycoproteins containing ~340 amino acids (Schwartz et al., 1973), and they exist in the membrane in association with β_2-microglobulin, a polypeptide of molecular weight 12 000 (Rask et al., 1974; Silver & Hood, 1974; Natori et al., 1975). Although the primary function of these antigenic products remains obscure, recent data suggest that they play a role in the recognition of virally induced and other cell surface antigens, in a process termed "associative recognition" [see reviews by Paul & Benacerraf (1977), Shearer & Schmitt-Verhulst (1977), and Zinkernagel & Doherty (1979)].

Possibly related to their postulated role in cell recognition is the remarkable polymorphism of the K and D gene products, a property first discovered in early serological analysis of laboratory mouse strains [see reviews by Klein (1975) and Snell et al. (1976)] and more recently verified in wild mice (Zaleska-Rutczynska & Klein, 1977). An understanding of the molecular basis for the polymorphism as well as the function–structure relationships will be more approachable when the primary structures of the K, D, and L products have been elucidated.

Because of the difficulty in obtaining significant amounts of H-2 antigens in purified form, the most successful biochemical studies have utilized radiolabeled material obtained from cells cultured in the presence of radioactive amino acids. Primary structural studies of the MHC product coded for by the K gene of the *H-2^b* haplotype have also utilized radiochemical methods. Five major CNBr fragments of K^b were isolated, and they were provisionally aligned as follows: CN-IIIn (23 residues), CN-IIIa (29 residues), CN-Ib (86 residues), CN-Ia (90 residues), and CN-Ic (56 residues) (Ewenstein et al., 1978). The amino acid sequence of residues 1–173 has been reported (Uehara et al., 1980). The present report describes the sequence of CN-Ia (residues 139–228) and with data in the following paper (Martinko et al., 1980)

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[1] Abbreviations used: MHC, major histocompatibility complex; PTH, phenylthiohydantoin; H-2K$^b_{pap}$, H-2K^b glycoprotein derived by papain cleavage of the NP-40 solubilized H-2K^b molecule; β_2-m, β_2-microglobulin.

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