Positional distribution of fatty acids in the major glycerophospholipids of *Paramecium tetraurelia*

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Abstract Fatty acid positional distributions and the fatty acid compositions of diacyl and alkyl acyl analogs of the three major glycerophospholipids of *Paramecium tetraurelia* were examined. Phosphatidylcholine and the phospho- and phosphonyl ethanolamine glycerolipids were separated into diacyl and alkyl acyl species by thin-layer chromatography after phospholipase C digestion, and acetylation. Phosphatidylcholine and the ethanolamine phosphonolipid were predominantly in the alkyl acyl form and phosphatidylethanolamine was predominantly in the diacyl form. The three major glycerophospholipids were also subjected to hydrolysis by phospholipase A_2. Complete and efficient hydrolyses of all three phospholipids were accomplished with the enzyme from porcine pancreas. Sodium tetraborate prevented acyl migration when added to reaction mixtures. Fatty acids released by phospholipase A_2 action, and the lyso-derivatives were separated by thin-layer chromatography. Fatty acid methyl esters were prepared and analyzed by gas-liquid chromatography. Saturated acids were predominantly at C-1 of diacyl phospholipids. Polyunsaturated fatty acids were mainly at C-2, particularly in the alkyl acyl species. An exception was γ-linolenate which was a major acid found esterified to C-1 in the three diacyl phospholipids. Identification of this acid at that position supports the idea that in some ciliates there may be an acyltransferase, specific for γ-linolenate, that esterifies this acid to the glycerophospholipids.—**Kaneshiro, E. S.** Positional distribution of fatty acids in the major glycerophospholipids of *Paramecium tetraurelia*. *J. Lipid Res.* 1980. **21:** 559–570.

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The fatty acid compositions at the C-1 and C-2 positions of the major glycerophospholipids from the ciliate, *Tetrahymena*, were recently reported by Peiringer and Conner (1). The three lipid classes they analyzed were *a*) 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and 1-alkyl, 2-acyl-*sn*-glycero-3-phosphocholine (PC), *b*) 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl) phosphate (PsE) and 1-alkyl, 2-acyl-*sn*-glycero-3-(2-aminoethyl) phosphate (PsE) and *c*) 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl) phosphonate (PnE) and 1-alkyl, 2-acyl-*sn*-glycero-3-(2-aminoethyl) phosphonate (PnE). They found that the C-1 position in these lipids was favored by saturated and iso-acids and the C-2 position was favored by unsaturated, particularly polyunsaturated fatty acids (PUFA). They concluded that acyltransferases in that protozoan exhibited some degree of substrate specificities which resulted in those distributions. Those results were in agreement with fatty acid positional distributions observed in a variety of organisms (2, 3). A striking exception, however, was the presence of γ-linolenate ($18{:}3^{\Delta 6,9,12}$) which was the major acid at C-1 in all three phospholipids. They suggested that either an acyltransferase specific for esterification of γ-linolenate at either C-1 or C-2 of glycerolipids was present in *Tetrahymena*, or that desaturation of esterified fatty acids occurred (1).

In the present study, the fatty acid positional distributions of these phospholipids from another ciliate, *Paramecium*, were examined. The lipid metabolism of *Paramecium* differs from that of *Tetrahymena* in that *Paramecium* requires a sterol (stigmasterol is the most active) and a fatty acid, oleic acid ($18{:}1^{\Delta 9}$), for growth (4). *Tetrahymena* does not have a lipid requirement. Lipids from *Paramecium* have high concentrations of arachidonic acid ($20{:}4^{\Delta 5,8,11,14}$) and other 20 carbon acids (5), while the fatty acids from *Tetrahymena* usually do not include species with chain lengths that exceed 18 carbon atoms (6). As with *Tetrahymena* (7, 8), the major glycerophospholipids of *Paramecium* are PC, PsE and PnE (9, 10) and these contain diacyl as well as 1-alkyl, 2-acyl analogs.[1] Alkyl acyl phospholipids of both *Tetrahymena* (11) and *Paramecium*[1] contain 1-O-

Abbreviations: BHT, butylated hydroxy toluene; FAME, fatty acid methyl esters, GLC, gas–liquid chromatography; MOPS, morpholinopropane sulfonic acid; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1-alkyl,2-acyl-*sn*-glycero-3-phosphocholine; PsE, 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl)phosphate and 1-alkyl,-2-acyl-*sn*-glycero-3-(2-aminoethyl)phosphate; PnE, 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl)phosphonate and 1-alkyl, 2-acyl-*sn*-glycero-3-(2-aminoethyl)phosphonate; PUFA, polyunsaturated fatty acids; S/L, ratios of weight % of fatty acids with <20 C to weight % of fatty acids with 20 C chain lengths; S/U, ratios of weight % of saturated fatty acids to weight % of unsaturated fatty acids; TEAE, triethylaminoethyl; TLC, thin-layer chromatography.

[1] Kaneshiro, E. S., K. B. Meyer, and D. E. Rhoads. The glyceryl ethers of *Paramecium* phospholipids. Unpublished data.

hexadecyl glycerol (chimyl alcohol). 1-O-Octadec-*cis*-11-enyl glycerol (paramecyl alcohol) has also been identified and quantified in *Paramecium* lipids.[1,2] Analyses of whole cell phospholipids indicated that alkenyl acyl phospholipids (plasmalogens) are not present in *Paramecium* (9).

The present report provides additional information on the structure of *Paramecium* lipids and on the metabolic processes operative in ciliates that are commonly employed as experimental organisms.

MATERIALS AND METHODS

Organisms

Paramecium tetraurelia, strains 51s (parental) and $d_4$95 (pawn, membrane mutant) were grown in the dark at 25°C in Fernbach flasks containing 500 ml of a crude, enriched medium (4). Lipids were extracted from cells at an early stationary phase of growth which was 5 days for strain 51s and 6 days for strain $d_4$95. Cells were concentrated and washed with 250 ml of distilled water by continuous flow centrifugation at 200–250 *g* (12). In some studies, *Paramecium* lipids were labeled with ^{32}P by growing the ciliates in the presence of 100 μCi/liter [^{32}P]H_3PO_4 (Amersham Corporation, Arlington Heights, IL).

General procedures

All solvents were redistilled before use. Butylated hydroxytoluene (BHT, Shell Chemical Co., New York, NY) was added to all solvents at a final concentration of 8 μg/ml (13) and samples were kept under a N_2 atmosphere whenever possible to prevent oxidation of lipids. The BHT was not added to solvents during the preparation of fatty acid methyl esters (FAME) to exclude the BHT peak in GLC analyses of FAME. Large volumes of solvents were removed on a Büchi rotary evaporator (Brinkmann Instruments, Westbury, NY) and small volumes were removed under a stream of N_2 (N-Evap, Organomation Associates, Worcester, MA). Both methods of concentrating lipid samples were done at temperatures <30°C.

Silicic acid for thin-layer chromatography (TLC) (Silica Gel G or H, Merck, Darmstadt, Germany) or column chromatography (Unisil, 100–120 mesh, Clarkson Chemical Co., Williamsport, PA) was prewashed in boiling methanol. The "fines" and impurities were removed by filtration through a 40–60 μm pore size sintered glass filter and additional rinses of methanol were used until the filtrate ran clear. Thin-layer plates were also pre-developed in acetone before use.

Extraction and isolation of phospholipids

Cells were extracted by a modification (9) of the method of Bligh and Dyer (14). Solids were removed by filtration through a 10–15 μm pore size sintered glass filter. The extract was then made biphasic by addition of chloroform and 0.58% NaCl in volumes according to Folch, Lees, and Sloane Stanley (15). The upper phase was reextracted with fresh lower phase and the lower phases were recombined and dried.

Lipids were fractionated by ion-exchange column chromatography on triethylaminoethyl (TEAE)-cellulose by the procedure of Turner and Rouser (16). The fraction containing PC was further purified by preparative TLC on 0.5 mm silica gel G or H. The plates were developed in chloroform–methanol–water 65:35:5 (v/v/v) (13). The band containing PC was visualized by I_2 vapors and scraped with a razor blade. The lipids were eluted from silica gel with chloroform–methanol 1:2 (v/v). The fraction off TEAE-cellulose columns containing PsE and its phosphono analog, PnE, was further fractionated by preparative TLC. The plates were developed in chloroform–glacial acetic acid–water 65:35:5 (v/v/v). The bands were visualized by I_2 vapors and PsE and PnE were recovered as described above.

These fractions were further purified by adsorption column chromatography on 4-cm Unisil columns packed in Pasteur pipettes. The lipids were dissolved in chloroform and applied to the column, which was eluted with 4 ml of chloroform and then 4 ml of methanol. The methanol eluate containing the purified lipid was collected and dried.

Phospholipase C digestion

Purified fractions of PC, PsE and PnE were subjected to phospholipase C digestion by a modification of the method of Uthe and Magee (17). Five to 15 mg of each lipid were dried in 40-ml screw-capped culture tubes. To each sample were added 5 ml of 0.01 M MOPS (morpholinopropane sulfonic acid) buffer (pH 7.3), 1 ml of freshly-prepared 1% sodium deoxycholate, and 2 ml of distilled water. The mixture was subjected to ultrasonic irradiation by submerging the tubes, to the level of their contents, in an ultrasonic cleaner bath (Mettler Electronics, Corporation, Anaheim, CA) at room temperature for 10 min. After sonication, the lipids were evenly dispersed and no particles were visible. The mixture was then preincubated for 10 min at 37°C.

Phospholipase C (EC 3.1.4.3) samples from *Bacillus*

[2] Kaneshiro, E. S., K. B. Meyer, and D. E. Rhoads. Isolation and characterization of 1-O-octadec-*cis*-11-enyl glycerol from *Paramecium* phospholipids. Paper submitted for publication.

cereus and from *Clostridium welchii* (Sigma Chemical Co., St. Louis, MO) were tested for their abilities to hydrolyze these lipids. Twenty units (0.25 mg protein) of the enzyme from *B. cereus* or 10 units (2 mg protein) of the enzyme from *C. welchii* were preincubated at 37°C for 30 min in 1 ml of 0.01 M $CaCl_2$ and adjusted to a final volume of 2 ml with distilled water. This enzyme solution was then added to the lipid sample and the reaction was allowed to progress at 37°C. The mixture was agitated at 15 min intervals on a vortex mixer.

For studies on the kinetics of digestion, lipids prelabeled with ^{32}P (>11.5 μCi/μmol lipid P) were employed. Aliquots (0.75 ml) were removed prior to the addition of enzyme and during the reaction process. These aliquots were fractionated into lipids and water-soluble products (15), and the radioactivities were determined in a liquid scintillation spectrometer (Packard Tri-Carb, Packard Instruments, Downers Grove, IL).

Samples used for the eventual separation into diacyl and alkyl acyl analogs were allowed to incubate for 1.5 hr to ensure complete hydrolysis. The entire reaction mixture was then transferred into a separatory funnel and extracted with 30 ml of chloroform–methanol 2:1 (v/v). The lower phase was removed and the upper phase was reextracted with 20 ml of fresh lower phase. The pooled lower phases were dried and transferred into 1-dram shell vials with Teflon-lined screw caps for acetylation.

Separation of diacyl and alkyl acyl analogs

The diglycerides derived from phospholipase C (*B. cereus*) digestion of phospholipids were acetylated with pyridine and acetic anhydride (18). Diglyceride acetates were separated into diacyl and alkyl acyl species employing petroleum ether (bp 30–60°C)–diethyl ether 8:12 (v/v) (19). For fatty acid analyses, bands were visualized by I_2 vapors and scraped directly into 5-ml conical tubes. Five or 10 μg of heneicosanate (21:0) was added to each sample as an internal standard to quantify the amount of alkali-labile FAME in each species (19). To quantify alkyl acyl and diacyl species by densitometry (Kontes, Vineland, NJ), diglyceride acetates were separated on either K5W precoated analytical plates (Pierce Chemical Co., Rockford, IL) and stained with phosphomolybdate (20), or on Safety-Kotes with charring agent (Applied Science Labs., State College, PA).

Phospholipase A_2 Digestion

Purified PC, PsE and PnE were subjected to phospholipase A_2 digestion by methods similar to those employed for phospholipase C digestions. To each 5-mg lipid sample were added *a*) 5 ml of 0.1 M MOPS buffer adjusted to pH 7.0 or 5 ml of 0.1 M tricine buffer adjusted to pH 8.0 or 8.9; *b*) 1 ml of freshly-prepared 1% sodium deoxycholate or 1 ml of 0.5% Triton X-100; and *c*) 2 ml of distilled water or 1 ml of 0.1 M $Na_2B_4O_7$ and 1 ml of distilled water. The sonified mixtures were preincubated and incubated at 32–37°C when using the porcine enzyme and at 21–23°C when using the snake enzymes. Four different enzyme preparations were tested for their abilities to hydrolyze PC, PsE, and PnE. Phospholipase A_2 (EC 3.1.1.4) from *Naja naja* and *Crotalus adamanteus*, and porcine pancreas were obtained from Sigma Chemical Co. Highly purified phospholipase A_2 from *C. atrox* was obtained from Dr. John Law (University of Chicago, Chicago, IL).

For studies on the kinetics and nature of hydrolysis, ^{32}P-labeled lipid products were separated by solvent partition (15). The lipids in the lower phase were separated on thin-layer plates developed in chloroform–methanol–water 65:35:5 (v/v/v) (21) and the radioactivities in the upper phase material, lyso compounds and parent compounds were determined as described above. Recoveries of radioactivities compared to the total cpm at t = 0 was 94.8% (±1.2 S.E., n = 107).

Samples for the eventual analyses of fatty acids were digested by 100 units of the porcine pancreatic enzyme in 5 ml of 0.1 ml tricine buffer (pH 8.0), 1 ml of 1% sodium deoxycholate, and 2 ml of distilled water. For hydrolysis of PsE, 1 ml of 0.1 M $Na_2B_4O_7$ and 1 ml of distilled water were used instead of 2 ml of distilled water. Digestions were allowed to proceed to completion at 32–37°C which was 1 hr for PsE, 2 hr for PC, and 3 hr for PnE.

Fatty acid analysis

Fatty acid methyl esters from phospholipids, diglyceride acetates, free fatty acids, and lyso derivatives were prepared for GLC by a modification (9) of the micro-method of MacGee and Allen (22). Saponifiable FAME were analyzed on a 6 ft glass column packed with 10% EGSS-X on 100–120 mesh Gas Chrom P (Applied Science Labs.) in a Hewlett-Packard 5830A reporting gas chromatograph (Hewlett-Packard, Avondale, PA) operated in the dual column mode. Column temperature was initially maintained at 150°C for 30 min then increased to 220°C at 1°/min. The injection temperature was 270°C and the flame ionization detector temperature was 250°C. Nitrogen carrier gas flow was 15 ml/min. The integrator slope sensitivity was set at 0.1.

Fatty acid compositions (weight %) were analyzed in several parameters which included % total saturated fatty acids and % total PUFA. Values for S/U (ratios

of weight % of saturated fatty acids to the weight % of unsaturated fatty acids) and S/L (ratios of weight % of fatty acids with <20 C to weight % of fatty acids with 20 C chain lengths) were also calculated.

The total weight of FAME in a given sample was estimated by the peak area of heneicosanate relative to the sum of the other FAME peaks. The relative amounts of diacyl and alkyl acyl species within a given phospholipid were estimated by calculating the weight of FAME derived from each fraction. The weights of FAME from diacyl diglyceride acetates were divided by two since they contain two saponifiable fatty acids. The relative percentage of the analogs was calculated by comparing the relative percentage of fatty acids recovered from each analog.

The weights of FAME from C-1 and C-2 of phospholipids were also calculated from peak areas. Relative percentages of diacyl and alkyl acyl analogs were estimated from these values by subtracting the total weight of C-1 FAME from that of C-2. The difference, which represents unsaponifiable alcohols at C-1, was then divided by the total weight of C-2 FAME. It was assumed that all C-2 acids were saponifiable since we have not detected 1-acyl, 2-alkyl or 1,2-dialkyl phospholipids in *Paramecium* lipids.[1]

Effect of I_2 on FAME

Since I_2 reacts with double bonds of unsaturated acids, the effect of I_2 vapors on the proportions of FAME from samples subjected to conditions employed in this study was examined. Total lipids from *Paramecium* and a mixture of known fatty acids were analyzed with and without exposure to I_2. Samples dissolved in chloroform–methanol 2:1 (v/v) (plus BHT) were applied to Silica Gel G plates under a stream of N_2. Spots of replicate samples were scraped and processed as described above. Another set of replicate samples was exposed to I_2 vapors for <1 min, placed under a stream of N_2, and FAME prepared. Quantitation by GLC indicated that the proportions of FAME in both the *Paramecium* lipids and the mixture of authentic fatty acids were not different in untreated and treated samples.

RESULTS

Phospholipase C digestion

Phospholipase C from *C. welchii* and *B. cereus* were both effective in hydrolyzing [^{32}P]PC as judged by the release of water-soluble ^{32}P radioactivity as well as loss of ^{32}P radioactivity from the lipid. The hydrolysis of PC by the *C. welchii* enzyme was complete in 10 min. The enzyme from *B. cereus* was also effective in cleaving PsE and PnE, but the enzyme from *C. welchii* did not catalyze the release of ^{32}P-labeled water-soluble products from these lipids. Hydrolyses of the three phospholipids by the *B. cereus* enzyme were complete in 1 hr. In studies in which higher concentrations (10–35 mg lipid/reaction) were used with the *B. cereus* enzyme, differences in the rates of digestion of PC, PsE, and PnE were observed. Rates of hydrolysis of substrates were in the order: PC > PsE > PnE.

Phospholipase A_2 digestion

Unless otherwise stated, reaction mixtures contained 5 mg of lipid, 5 ml of 0.1 M tricine buffer (pH 8.0), 1 ml of 1% sodium deoxycholate, and 2 ml of distilled water. Digestions employing 50 units of the *N. naja* enzyme (0.1 mg protein) proceeded rapidly (**Fig. 1A**). The products of hydrolysis of ^{32}P-labeled PC and PnE were relatively stable, however, lyso PsE levels decreased as water-soluble counts increased. Acyl migration or phospholipase A_1 or lysophospholipase activities could produce these effects.

Using 20 or 50 units (20 or 50 μg protein) of the *C. adamanteus* enzyme and tricine buffer at pH 8.9, similar rapid digestion rates were obtained for PC (Fig. 1B). Digestions of the ethanolamine lipids, PsE and PnE, however, were slower than those achieved with the *N. naja* enzyme. Using 50 or 20 units of the enzyme, 53% and 41% respectively, of PnE was digested in 5 hr. Complete digestion of PnE was not observed after 11 hr of incubation but was complete after overnight incubations (22–24 hr total incubation time). Loss of lyso PsE with the appearance of the water-soluble product was also observed with this enzyme.

Highly purified samples of phospholipase A_2 from *C. atrox* (50 μg/reaction mixture) efficiently hydrolyzed PC and PnE (Fig. 1C). Again, the characteristics of PsE digestion were similar to those obtained using the other snake enzymes, i.e., lyso PsE was lost and water-soluble ^{32}P radioactivity increased with time. Using 10 or 50 μg of this enzyme, lipids were also digested in 5 ml of MOPS buffer (pH 7.0), 1 ml of 0.5% Triton X-100, 1 ml of 0.1 M $Na_2B_4O_7$, and 1 ml of distilled water. Under these conditions, lyso PsE did not decrease and water-soluble counts did not increase with time (Fig. 1D). Digestion of PC and PnE were slower than those obtained with this enzyme under the "standard" conditions (see Fig. 1C).

Digestion of all three lipids by porcine pancreatic phospholipase A_2 was rapid and complete. At 20 units (30 μg protein) per assay, digestions were similar to those obtained employing the enzyme from *C. atrox*

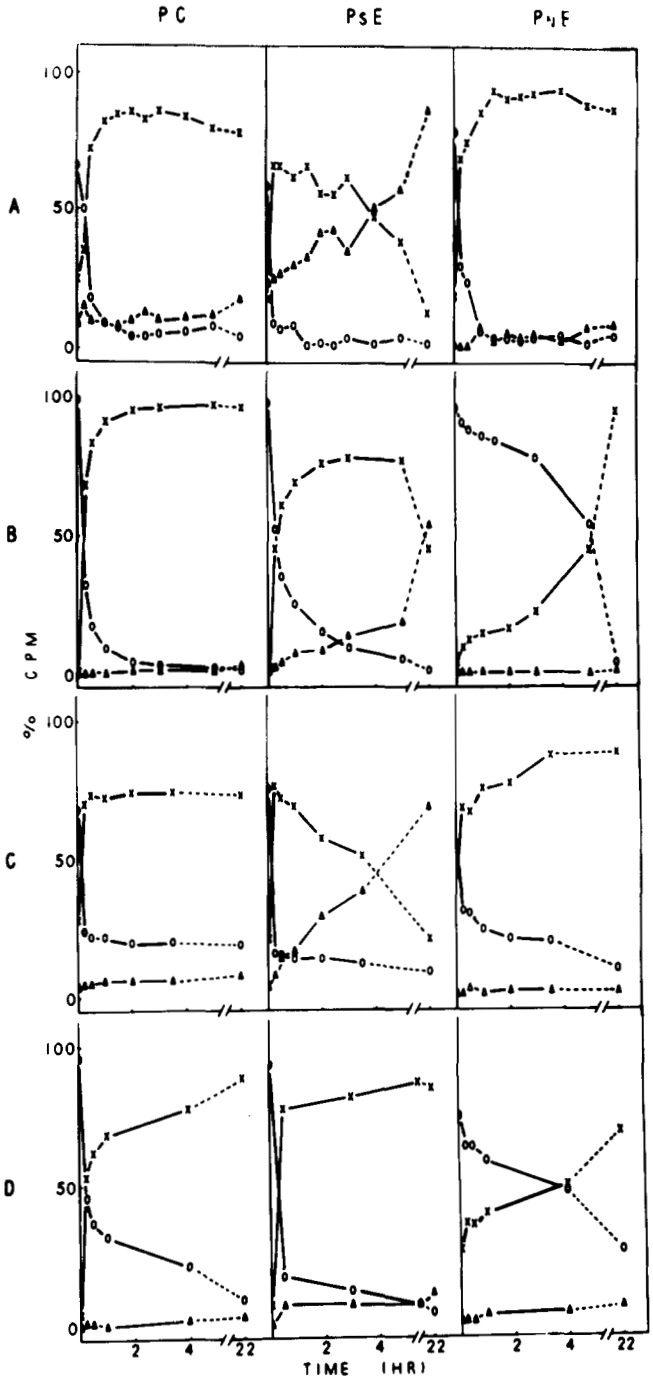

Fig. 1. Hydrolyses of ^{32}P-labeled PC, PsE, and PnE from *Paramecium* by phospholipase A_2. A) Each reaction mixture contained 50 units (0.1 mg protein) of the *N. naja* enzyme, deoxycholate, and tricine buffer adjusted to pH 8.9. Figures are composites of two separate experiments. Acyl migration during hydrolysis of PsE was indicated by the loss of lyso PsE and increase in the water-soluble radioactivity. Acyl migration may have also occurred during the digestion of PC. B) Each reaction contained 20 units (20 mg protein) of the *C. adamanteus* enzyme, deoxycholate, and tricine buffer adjusted to pH 8.9. Acyl migration occurred during digestion of PsE. PnE was completely hydrolyzed only after an extended period of incubation. C) Each reaction mixture contained 50 μg of the *C. atrox* enzyme, deoxycholate, and tricine buffer adjusted to pH 8.9. Acyl migration in PsE occurred using this purified enzyme preparation. D) Each reaction mixture contained 0.01 mg of the *C. atrox* enzyme, Triton X-100, $Na_2B_4O_7$, and MOPS buffer adjusted to pH 7.0. Under these conditions, rates of digestion of PC and PnE were slower than under conditions illustrated in C, however, the presence of borate prevented acyl migration during the hydrolysis of PsE. ○, cpm in the phospholipid; ×, cpm in the lyso derivative; △, cpm in the aqueous phase of the solvent partition (15).

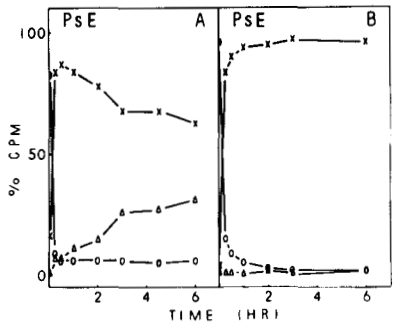

Fig. 2. Hydrolyses of ^{32}P-labeled PsE from *Paramecium* by porcine pancreatic phospholipase A_2 without (A) and with (B) borate. The presence of borate prevented acyl migration. Each reaction mixture contained 100 units (150 μg protein) of the enzyme, deoxycholate, and tricine buffer adjusted to pH 8.0. ○, cpm in the phospholipid; ×, cpm in the lyso derivative; △, cpm in the aqueous phase of the solvent partition (15).

(Fig. 1C). At 100 units (150 μg protein) per assay, digestions improved (**Figs. 2** and **3**). Addition of $Na_2B_4O_7$ to the PsE reaction mix prevented loss of lyso PsE and production of the ^{32}P-labeled water-soluble product. Using 100 units of this enzyme, Triton X-100, MOPS buffer and $Na_2B_4O_7$, as described above for the *C. atrox* enzyme digestions, PsE was digested in 1 hr, PnE in 3 hr, but PC was not digested even after overnight incubations. Under similar conditions, but eliminating the $Na_2B_4O_7$, the rate of digestion of PC was still slow. The porcine pancreatic enzyme was used for studies on the positional distribution of fatty acids as described in Methods and in Figs. 2B and 3.

Estimation of diacyl and alkyl acyl analogs

Densitometry of TLC separations of diacyl and alkyl acyl diglyceride acetates after phospholipase C digestion and acetylation was used to estimate the relative amounts of each analog (**Table 1**). Also, employing fatty acid internal standard methods (19), estimates of each analog were calculated by weights of fatty acids recovered from lipid fractions after phospholipase C and A_2 hydrolyses. Alkyl acyl PC and PnE were present in much greater concentrations (approx. 80% and 95% respectively) than their diacyl analogs. The amount of diacyl PsE (approx. 85%)

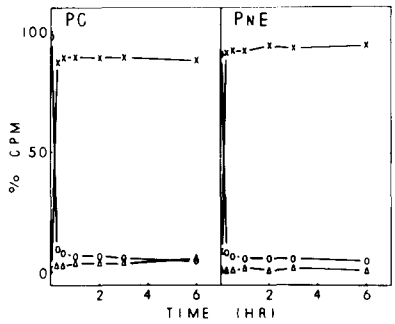

Fig. 3. Hydrolyses of ^{32}P-labeled PC and PnE from *Paramecium* by porcine pancreatic phospholipase A_2. Each reaction mixture contained 100 units (150 μg protein) of the enzyme, deoxycholate, and tricine buffer adjusted to pH 8.0. ○, cpm in the phospholipid; ×, cpm in the lyso derivative; △, cpm in the aqueous phase of the solvent partition (15).

was greater than that of alkyl acyl PsE. The apparent discrepancies using the three methods for estimation of alkyl acyl and diacyl analogs were the results of wide variabilities in the data, particularly in the case of FAME analysis of free fatty acids and lysophospholipids of PsE.

Distribution of fatty acids in PC

Approximately 80% of fatty acids in PC were PUFA (**Table 2**). The major PUFA were $20{:}4^{\Delta 5,8,11,14}$, $18{:}2^{\Delta 9,12}$ (linoleic acid), $18{:}3^{\Delta 6,9,12}$, and $20{:}3^{\Delta 8,11,14}$. The presence of large amounts of $20{:}3^{\Delta 8,11,14}$ in PC was unique to this class.

Saturated fatty acids constituted more than half of the acids of diacyl PC as indicated by a S/U > 1. Palmitic acid (16:0) alone was about half of the total fatty acids in this analog. Most fatty acids had chain lengths shorter than 20 carbons as indicated by a large S/L value.

The fatty acids from all alkyl acyl analogs represent the composition at the C-2 positions of these lipids. Approximately 90% of the acids at this position in this analog of PC were unsaturated. Arachidonate, linoleate, and $20{:}3^{\Delta 8,11,14}$ were mainly in alkyl acyl PC, and γ-linolenate was present in substantial quantities in both analogs.

The C-1 position of PC was mainly occupied by saturated acids. C-1 acids represent those that were esterified at that position of all diacyl phospholipids inasmuch as the 1-alkyl, 2-acyl analogs have no saponifiable acids there. Thus, most of the palmitate in PC was at C-1 of diacyl PC.

Distribution of fatty acids in PsE

The major acids in PsE were palmitate, linoleate, and γ-linolenate (**Table 3**). Diacyl PsE had less saturated fatty acids (S/U < 1) than did diacyl PC, i.e., less than half of the acids in diacyl PsE were saturated. As in diacyl PC, 20-carbon acids were minor components of diacyl PsE. Over half of the acids in this analog were 18-carbon unsaturated acids. Arachidonate and 18-carbon unsaturated acids were the major fatty acids of alkyl acyl PsE as they were in alkyl acyl PC. Most of the palmitate in PsE was at the C-1 position of diacyl PsE. Linoleate was at C-2 of both analogs. γ-Linolenate was present in high concentrations in both positions and comprised about ⅓ of the fatty acids released from C-1 of diacyl PsE. Long chain acids of 20 carbons were present only in low amounts in this class as compared to the other two

TABLE 1. Diacyl and alkyl acyl compositions of *Paramecium tetraurelia* glycerophospholipids

Method	PC		PsE		PnE	
	Diacyl	Alkyl Acyl	Diacyl	Alkyl Acyl	Diacyl	Alkyl Acyl
			%			
Quantitative TLC of diglyceride acetates[a]	17 14–24[b] (4)[c]	83 76–86 (4)	71 60–79 (4)	29 21–40 (4)	4 3–6 (4)	96 94–97 (4)
FAME of diglyceride acetates (fatty acid internal std.)[d]	24 21–26 (3)	76 74–79 (3)	87 76–92 (3)	13 8–24 (3)	15 1–32 (3)	85 68–99 (3)
FAME of free fatty acids and lysophosphatides (fatty acid internal std.)[e]	15 11–21 (5)	85 79–89 (5)	102 76–133 (4)	−2 −33 to +24 (4)	3 1–4 (3)	97 96–99 (3)

[a] Phospholipids of strain 51s cells were digested with phospholipase C and acetylated. The diglyceride acetates were separated into diacyl and alkyl acyl species by TLC, sprayed with phosphomolybdic acid or charred, and quantified by densitometry. Exposure of diglyceride acetate samples on TLC plates to HCl fumes did not change the values, indicating the absence of plasmalogens in these isolated phospholipids.

[b] Range of percentages.

[c] Number of determinations.

[d] Estimated by weights of fatty acids in diacyl and alkyl acyl species. Data presented are for strain 51s. Values obtained from strain $d_4$95 were not strikingly different.

[e] Estimated by weights of fatty acids released by phospholipase A_2 digestion and weights of fatty acids from the lyso compounds. Data presented are for strain 51s. Values obtained from strain $d_4$95 were not strikingly different.

major fatty acids of alkyl acyl PsE as they were in alkyl composition of the whole class and both positions on the glycerol backbone had large S/L values, although the value for C-1 of diacyl PsE was markedly higher. C-1 of diacyl PsE had a S/U value slightly >1 which indicated that almost equal amounts of unsaturated and saturated acids were esterified in that position.

TABLE 2. Fatty acids of *Paramecium tetraurelia* PC[a]

Fatty Acid	Total	1,2-Diacyl	1-Alkyl, 2-Acyl[b]	C-1[c]	C-2
			Weight %		
14:0	0.3 (0.2–0.5)[d]	2.4 (1.9–3.0)	0.2 (0.2–0.3)	2.1 (1.7–2.8)	0.3 (0.3–0.6)
14:1	trace	0.7 (0.0–1.9)	0.7 (0.0–1.8)		
15:0	0.2 (0.2–0.3)	1.0 (0.7–1.7)		1.3 (0.8–1.7)	0.1 (0.1–0.2)
16:0	8.0 (5.9–11.5)	43.7 (32.0–55.4)	1.2 (1.1–1.3)	55.5 (49.9–61.0)	6.4 (2.3–12.0)
$16:1^{\Delta 7}$ + $16:1^{\Delta 9}$	trace	1.4 (0.9–2.1)	0.2 (0.1–0.3)	2.0 (0.0–3.4)	0.5 (0.4–0.9)
17:0	0.1 (0.0–0.1)	0.6 (0.3–1.0)		0.8 (0.7–1.0)	0.2 (0.0–0.8)
$17:1^{\Delta 8}$ + $17:1^{\Delta 9}$	trace	0.1 (0.0–0.3)	0.1 (0.0–0.1)	0.2 (0.0–0.3)	0.1 (0.1–0.2)
18:0	0.8 (0.5–1.3)	9.9 (6.5–13.3)	0.8 (0.3–1.8)	11.7 (6.7–18.6)	0.9 (0.4–1.5)
$18:1^{\Delta 9}$ + $18:1^{\Delta 11}$	9.1 (6.0–11.4)	11.3 (9.1–13.2)	10.8 (6.5–13.7)	4.1 (3.4–4.6)	12.1 (7.1–19.7)
$18:2^{\Delta 9,12}$	25.4 (24.1–26.6)	13.7 (6.2–17.9)	19.6 (16.4–21.5)	2.0 (0.3–3.2)	19.3 (12.8–26.2)
$18:3^{\Delta 6,9,12}$	15.4 (14.2–16.6)	8.8 (6.4–10.9)	15.2 (10.9–17.9)	17.6 (10.2–23.1)	19.3 (13.1–34.6)
$20:1^{\Delta 11}$	0.4 (0.3–0.6)	0.6 (0.4–0.7)	0.3 (0.1–0.6)	0.2 (0.0–0.4)	0.3 (0.0–0.5)
$20:2^{\Delta 8,11}$	0.1 (0.0–0.2)		0.1 (0.0–0.3)	0.3 (0.1–0.6)	0.1 (0.1–0.2)
$20:3^{\Delta 8,11,14}$	11.7 (8.7–14.6)	1.2 (0.6–2.2)	6.6 (5.6–8.3)	0.3 (0.0–0.5)	7.5 (2.9–13.6)
$20:4^{\Delta 5,8,11,14}$	26.4 (23.7–28.2)	3.9 (2.9–5.1)	38.9 (36.8–41.1)	0.8 (0.4–1.3)	28.5 (24.0–30.3)
$20:5^{\Delta 5,8,11,14,17}$	1.7 (1.6–2.0)	0.3 (0.0–0.5)	4.0 (3.3–5.1)	0.3 (0.2–0.5)	3.4 (1.7–6.1)
Others	0.3 (0.2–0.4)	0.1 (0.0–0.2)	1.3 (0.5–1.9)	1.1 (0.5–2.5)	0.7 (0.3–0.9)
% Sat'd. FAME	9.4	57.0	2.2	71.4	7.9
% PUFA	80.8	28.5	84.8	21.4	78.2
S/U	0.10	1.35	0.02	2.56	0.09
S/L	1.47	14.18	0.97	48.65	1.49
# determinations	3	3	3	5	5

[a] Data are from strain 51s. Values obtained from strain $d_4$95 were not different.
[b] Fatty acids at C-2 of alkyl acyl PC. Data of FAME from alkyl acyl and diacyl species were from the same lipid samples.
[c] Fatty acids at C-1 of diacyl PC. Data of FAME from C-1 and C-2 were from the same lipid samples.
[d] Ranges of values are in parentheses.

Distribution of fatty acids in PnE

The fatty acids of PnE were almost exclusively 20-carbon PUFA (**Table 4**). Arachidonate constituted about 90% of the total fatty acids in this class. Both the S/U and S/L values for fatty acids of PnE were strikingly low. The 20-carbon PUFA were at C-2 of alkyl acyl PnE. Over half of the fatty acids from C-1 of diacyl PnE was palmitate and, as in diacyl PsE and

TABLE 3. Fatty acids of *Paramecium tetraurelia* PsE[a]

Fatty Acid	Total	1,2-Diacyl	1-Alkyl, 2-Acyl[b]	C-1[c]	C-2
			Weight %		
14:0	0.6 (0.6, 0.6)[d]	1.2 (0.5–1.6)	2.1 (0.3–3.5)	1.3 (0.4–2.7)	0.6 (0.4–0.9)
14:1	trace	0.1 (0.1–0.2)	2.6 (0.6–6.4)		0.1 (0.0–0.1)
15:0	0.7 (0.7, 0.7)	0.9 (0.7–1.3)	0.5 (0.2–1.0)	1.2 (0.7–1.5)	0.2 (0.1–0.2)
16:0	26.5 (26.2, 26.7)	30.8 (26.2–33.7)	11.0 (4.6–20.6)	52.5 (47.3–58.9)	3.8 (2.1–5.0)
$16:1^{\Delta 7}$ + $16:1^{\Delta 9}$	0.5 (0.2, 0.8)	1.4 (0.9–1.9)	1.9 (0.4–4.3)	1.3 (0.8–1.9)	4.7 (1.2–14.4)
17:0	0.3 (0.2, 0.3)	0.2 (0.1–0.3)	0.1 (0.0–0.1)	0.3 (0.3–0.4)	0.2 (0.2–0.3)
$17:1^{\Delta 7}$ + $17:1^{\Delta 9}$	0.3 (0.3, 0.3)	0.3 (0.2–0.5)	0.2 (0.0–0.6)	0.2 (0.1–0.2)	0.5 (0.3–0.8)
18:0	0.6 (0.6, 0.7)	1.4 (1.1–1.8)	1.5 (0.8–2.4)	1.7 (1.6–1.9)	1.6 (0.4–3.0)
$18:1^{\Delta 9}$ + $18:1^{\Delta 11}$	9.3 (9.3, 9.4)	10.4 (8.8–11.5)	10.7 (7.1–13.3)	4.9 (2.1–13.3)	20.0 (15.9–26.5)
$18:2^{\Delta 9,12}$	30.6 (30.5, 30.7)	27.2 (26.3–27.8)	12.3 (11.1–13.7)	1.4 (0.7–2.1)	36.2 (26.5–48.9)
$18:3^{\Delta 6,9,12}$	20.4 (20.3, 20.4)	21.3 (17.8–26.3)	17.6 (14.0–19.7)	32.8 (26.1–39.9)	18.2 (11.2–34.3)
$20:1^{\Delta 11}$	0.1 (0.0, 0.2)	0.9 (0.9–0.9)	1.3 (0.7–2.1)		0.7 (0.5–0.8)
$20:2^{\Delta 8,11}$	0.6 (0.5, 0.6)	0.1 (0.0–0.3)	0.3 (0.0–0.9)	0.1 (0.1–0.2)	1.0 (0.9–1.2)
$20:3^{\Delta 8,11,14}$	1.5 (1.5, 1.5)	0.9 (0.8–1.0)	2.4 (1.9–2.4)	0.2 (0.0–0.5)	2.3 (1.1–2.8)
$20:4^{\Delta 5,8,11,14}$	7.3 (7.3, 7.4)	2.1 (1.2–2.8)	33.0 (18.7–40.2)	0.2 (0.2–0.4)	7.4 (3.9–11.6)
$20:5^{\Delta 5,8,11,14,17}$	0.4 (0.4, 0.4)	0.2 (0.1–0.4)	2.1 (0.3–3.8)	0.4 (0.3–0.6)	0.9 (0.4–1.7)
Others	0.5 (0.4, 0.6)	0.6 (0.3–1.2)	0.2 (0.0–0.6)	1.6 (1.3–1.9)	1.6 (1.3–1.9)
% Sat'd. FAME	28.7	34.3	15.1	56.9	6.4
% PUFA	61.0	52.1	67.7	35.5	66.1
S/U	0.40	0.53	0.18	1.36	0.07
S/L	8.89	21.16	1.55	75.08	6.94
# determinations	2	3	3	4	4

[a] Data are from strain 51s. Values obtained from strain $d_4 95$ were not different.
[b] Fatty acids at C-2 of alkyl acyl PsE. Data of FAME from alkyl acyl and diacyl species were from the same lipid samples.
[c] Fatty acids at C-1 of diacyl PsE. Data of FAME from C-1 and C-2 were from the same lipid samples.
[d] Ranges of values are in parentheses.

diacyl PC, γ-linolenate was a major acid esterified at that position.

Distribution of fatty acids in pawn mutants

Analyses of the pawn mutant strain indicated that the fatty acid distributions in the three major glycerophospholipids were not different from those of parental type cells. One possible exception was an apparent greater concentration of 20-carbon PUFA in diacyl PnE from the mutant (S/U = 0.57, S/L = 1.85).

DISCUSSION

Phospholipase digestions

Phospholipase C from *B. cereus* hydrolyzed all three glycerophospholipids, but the enzyme from *C. welchii* hydrolyzed only PC. Hence, this study confirmed the findings of other workers (23), i.e., pure samples of PC, but not of PsE, are substrates for phospholipase C from *C. welchii*. This enzyme can hydrolyze PsE when it is mixed with other phospholipids. The present study demonstrated that pure samples of the phosphonyl analog of PsE, PnE, are also not hydrolyzed by this enzyme. This suggests that the enzyme from *C. welchii* is similar to that from *C. perfringens*, which is inhibited by phosphonolipids (24). This study, therefore, did not confirm the findings of Andrews and Nelson (10) who reported that digestion of PnE from *P. tetraurelia* by *C. welchii* phospholipase C yields a water-soluble product that comigrates with aminoethylphosphonate. Since both PnE and PC contain substantial amounts of ether linkages at C-1, the greater rate of hydrolysis of PC compared to that of PnE indicates that the ether linkage does not affect the function of the *B. cereus* enzyme but that the phosphonyl linkage at C-3 may partially hinder its action.

All three glycerophospholipids were digested by the four phospholipase A_2 preparations tested. The loss of lyso compounds, particularly that of lyso PsE, indicated that further deacylation occurred during hydrolysis. The enzyme from *N. naja* supplied by Sigma Chemical Co. has been reported to contain three major components as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (25). Hence it was possible that the enzyme preparation either contained phospholipase A_1 and/or lysophospholipase activities, or that acyl migration occurred.

The enzymatic digestion with the enzyme from *C. adamanteus* was done under conditions slightly different from those used by Pieringer and Conner (1) for hydrolyses of *Tetrahymena* phospholipids. These workers employed a glycylglycine buffer and a probe sonifier to prepare their lipid substrate mixtures. The times required for complete hydrolyses of lipids were similar in both studies, i.e., several more hours were required for the complete hydrolysis of PnE compared to the digestions of PC or PsE. In the present study, hydrolysis of ^{32}P-labeled PsE was accompanied by loss of lyso PsE and accumulation of water-soluble radioactivity. This enzyme preparation was reported to contain only one major component when analyzed by SDS-PAGE (25) and thus likely to be free of other lipase activities.

Hydrolysis of PsE by the enzymes from *C. atrox* and porcine pancreas also led to further hydrolysis of lyso PsE. The presence of phospholipase A_1 or lysophospholipase activity, however, was ruled out as the cause of the loss of lyso PsE in the case of hydrolysis by the *C. atrox* enzyme since this preparation was one of known purity. Furthermore, the composition of all phospholipase A_2 preparations used in the present study was analyzed by SDS-PAGE employing the discontinuous buffer system of Laemmli (26). Enzyme preparations from *C. atrox, C. adamanteus,* and porcine pancreas each contained a single major band having approximate molecular weights of 19,500, 20,000, and 17,500, respectively. The phospholipase preparation from *N. naja* contained two major bands having approximate molecular weights of 12,000 and 16,000.[3] Hence it was concluded that digestion of PsE by all of the four preparations was accompanied by acyl migration, but the possibility that there was phospholipase A_1 or lysophospholipase activity in the *N. naja* preparation was not ruled out.

Prevention of acyl migration in PsE was achieved by addition of $Na_2B_4O_7$ to the reaction mixture (27, 28). The mechanism of action of borate on the prevention of acyl migration was probably by intermolecular complexation with the hydroxyl groups which resulted from cleavage of C-2 ester linkages in the glycerolipids. That site was then unable to receive acyl groups from C-1 and was therefore not susceptible to further action of phospholipase A_2.

With the possible exception of the *N. naja* enzyme digestions, the apparent absence of acyl migration in PC and PnE is not obvious. According to our estimations by three methods, PC has about 20% and PnE has about 5–10% diacyl forms. If rates of acyl migration in PC and PnE were similar to that of PsE, the loss of at least lyso PC (with concurrent rise in the water-soluble product) with reaction time would have been obvious.

Since much of the PC and PnE molecules from *Paramecium* have ether bonds at C-1, and since hy-

[3] Merkel, S. J., and E. S. Kaneshiro. Unpublished data.

TABLE 4. Fatty acids of *Paramecium tetraurelia* PnE[a]

Fatty Acid	Total	1,2-Diacyl	1-Alkyl, 2-Acyl[b]	C-1[c]	C-2
			Weight %		
14:0	0.2 (0.1, 0.2)[d]	6.2 (1.0–11.0)	0.8 (0.1–1.6)	1.1 (0.0–2.1)	0.5 (0.4–0.5)
14:1	trace	0.9 (0.7–1.3)	0.7 (0.5–1.1)		
15:0	0.1 (0.0, 0.1)	1.4 (0.6–2.9)	0.1 (0.1–0.2)	0.7 (0.0–1.1)	0.1 (0.0–0.2)
16:0	1.6 (1.4, 1.8)	30.4 (26.9–35.4)	1.7 (0.4–2.6)	56.7 (46.9–71.5)	2.9 (1.9–4.3)
$16:1^{\Delta 7}$ + $16:1^{\Delta 9}$	trace	2.9 (1.2–6.1)	0.4 (0.0–1.0)	1.1 (0.0–2.0)	0.3 (0.2–0.4)
17:0	trace	0.7 (0.0–1.6)		0.1 (0.0–0.4)	
$17:1^{\Delta 8}$ + $17:1^{\Delta 9}$	trace	0.2 (0.0–0.6)			
18:0	trace	13.1 (6.0–20.0)	0.6 (0.1–1.1)	5.0 (3.2–8.1)	1.7 (1.2–2.6)
$18:1^{\Delta 9}$ + $18:1^{\Delta 11}$	0.4 (0.4, 0.5)	11.4 (9.5–13.7)	1.5 (0.7–2.3)	4.0 (1.1–5.5)	1.7 (1.3–2.1)
$18:2^{\Delta 9,12}$	1.5 (1.4, 1.5)	7.7 (3.2–15.2)	0.4 (0.4–0.5)	1.7 (0.0–2.5)	1.7 (1.3–2.1)
$18:3^{\Delta 6,9,12}$	1.3 (1.1, 1.5)	8.8 (3.3–17.5)	1.4 (0.3–3.3)	18.6 (15.7–21.4)	1.0 (0.7–1.4)
$20:1^{\Delta 11}$	trace	0.3 (0.0–0.5)		0.6 (0.0–1.1)	
$20:2^{\Delta 8,11}$	trace	0.3 (0.0–1.0)			
$20:3^{\Delta 8,11,14}$	0.4 (0.2, 0.6)	0.3 (0.0–0.9)	0.1 (0.0–0.2)	0.1 (0.0–0.3)	0.5 (0.3–0.8)
$20:4^{\Delta 5,8,11,14}$	89.6 (89.5, 89.7)	11.4 (6.0–15.6)	82.8 (80.0–87.9)	8.1 (0.5–11.9)	82.4 (79.7–87.0)
$20:5^{\Delta 5,8,11,14,17}$	4.5 (4.3, 4.6)	1.9 (0.0–4.0)	7.8 (5.1–9.7)	0.9 (0.0–2.2)	5.8 (3.4–10.5)
Others	0.8 (0.7, 1.0)	1.3 (0.0–3.9)	1.5 (0.3–3.4)	1.4 (0.1–3.3)	0.8 (0.3–1.4)
% Sat'd. FAME	1.9	51.1	3.2	63.6	5.1
% PUFA	97.5	30.4	93.2	29.5	92.0
S/U	0.02	1.12	0.03	1.81	0.05
S/L	0.05	5.89	0.08	9.08	0.11
# determinations	2	3	3	3	3

[a] Data are from strain 51s. Values from strain $d_4 95$ were not strikingly different, but there were apparently greater concentrations of 20 C PUFA in the mutant diacyl PnE (S/U = 0.57; S/L = 1.85).

[b] Fatty acids at C-2 of alkyl acyl PnE. Data of FAME from alkyl acyl and diacyl species were from the same lipid samples.

[c] Fatty acids at C-1 of diacyl PC. Data of FAME from C-1 and C-2 were from the same lipid samples.

[d] Ranges of values are in parentheses.

drolysis of PnE by both of the *Crotalus* enzymes was markedly slower than hydrolysis of either PC or PsE, it was concluded that the ether function at C-1 apparently does not hinder the action of these enzymes. The direct C to P bond in PnE, however, does hinder the action of all phospholipase A_2 preparations tested.

The reason for the decreased rates of hydrolysis of PnE and PC in the presence of tetraborate is not readily apparent. The reason for the decreased rate of hydrolysis of PC with Triton X-100, compared to rates achieved with deoxycholate, is also not entirely clear. The different rates of hydrolysis of PC employing different detergents may be due to the effective concentrations of the detergents relative to those of the enzyme and substrate, factors which are known to affect the nature of micellar and vesicular structures (29).

Estimation of diacyl and alkyl acyl analogs

Values obtained by three different methods of estimating the relative percents of diacyl and alkyl acyl forms present in each phospholipid were in general agreement with one another. Although some ranges of values were broad, the combined data permitted the conclusion that about 90–95% of PnE, about 80% of PC, and about 15% of PsE contained alkyl acyl species. In *Tetrahymena*, about 60% of PC and PnE and 2% of PsE have chimyl alcohol at the C-1 position. In *Paramecium*, PC and PnE contain substantially more glyceryl ethers. In fact, these are among the highest in glyceryl ether content of glycerophospholipids reported from various cell types (30).

Fatty acid distributions

Saturated fatty acids were located mainly at C-1 in the three major glycerolipids of *Paramecium*. The C-2 position of these lipids was occupied by large amounts of PUFA. An exception was the substantial amount of palmitate at C-2 in alkyl acyl PsE. With regard to the bulk of lipids in the organism, however, alkyl acyl PsE constituted only about 15% of total PsE. Two features of the fatty acid distributions of *Paramecium* major glycerophospholipids were striking: *a*) γ-linolenate was a major acid at C-1 in the three phosphatides and *b*) C-2 of PnE was occupied almost exclusively by arachidonate. The localization of arachidonate and other 20-carbon PUFA at C-2 of PnE suggests that an acyltransferase which is highly specific for both *a*) the P-C and/or C-O-C bond and *b*) 20-carbon PUFA is present in this ciliate.

The presence of γ-linolenate as the major component of C-1 acids in *Tetrahymena* glycerolipids was attributed to either *a*) the presence of an acyltransferase which was specific for γ-linolenate and which catalyzed the esterification of that acid to either the C-1 or the C-2 position, or *b*) the presence of a desaturase which acted on esterified fatty acid residues in these phospholipids (1). These data from *Paramecium* glycerophospholipid fatty acids do not rule out either of the two possibilities. It is, however, interesting that in *Tetrahymena*, γ-linolenate is the most abundant end product of fatty acid biosynthesis. In *Paramecium*, arachidonate is the major end product, but in both ciliates, γ-linolenate is clearly the major PUFA at C-1 in these phospholipids. The presence of a large percentage of glycerolipids with PUFA at C-1 in both these ciliates is unlike the situation observed in most phosphoglycerides isolated from various animal tissues (23). Phospholipids with PUFA rather than saturated acids at C-1 would be different in their interactions with proteins and other lipids and hence influence the nature of packing of lipid bilayers in the membranes of these ciliates.

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