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PREPARATIVE ISOLATION OF PHOSPHONOLIPIDS BY ASCENDING DRY-COLUMN CHROMATOGRAPHY

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SUMMARY

A simple chromatographic technique to fractionate gram quantities of polar lipids has been described. This enabled the isolation of 200–500-mg quantities of 1,2-diacyl-*sn*-glycero-3-(2-aminoethylphosphonate) and 1-alkyl-2-acyl-*sn*-glycero-3-(2-aminoethylphosphonate) from the total lipids of free living ciliated protozoan *Tetrahymena pyriformis* W.

INTRODUCTION

The concurrent occurrence of glycerol-(2-aminoethylphosphonate) lipids [G-AEP lipids] and glycerol-phosphorylethanolamine lipids [GPE lipids] in the total lipids of the protozoon, *Tetrahymena pyriformis*[1] posed separation problems[2,3], until KAPOULAS[4] and THOMPSON[5] achieved their separation by thin-layer chromatography (TLC), using solvent systems containing substantial amounts of acetic acid and minimal amounts of methanol and/or water. Several attempts[6–8] at the preparative isolation of G-AEP lipids including their further subfractionation into 1-alkyl-2-acyl-*sn*-glycero-3-(2-aminoethylphosphonate) [alkyl acyl-G-AEP] and 1,2-diacyl-*sn*-glycero-3-(2-aminoethylphosphonate) [diacyl-G-AEP] analogs were made. BERGER *et al.*[7] isolated pure alkyl acyl G-AEP at the expense of diacyl G-AEP by selective methanolysis of a mixture of analogs of G-AEP lipids. On the other hand SUGITA AND HORI[8] reported successful chromatographic isolation of diacyl-G-AEP although their yields were very low.

In our laboratory, the ascending dry-column chromatography (ADCC) technique described by JACINI AND FEDELI[9] has been successfully used in the preparative fractionation of the total lipids of *Tetrahymena pyriformis* W. The procedure yielded not only much larger quantities of pure G-AEP lipids and GPE lipids than reported previously, but also yielded substantial amounts of pure alkyl acyl G-AEP and diacyl G-AEP. The present communication describes this fractionation procedure in detail.

EXPERIMENTAL

Materials

2-Aminoethylphosphonic acid (AEP) was a gift of Dr. A. S. ISBELL. Silica Gel G (E. Merck), for TLC use, was used for the ADCC studies. Pre-coated plates—Silica Gel F_{254} and cellulose (E. Merck)—were used for TLC studies. All chemicals used including solvents were of analytical grade and not further purified.

Methods

Maintenance and culture of the organism. Tetrahymena pyriformis W was maintained in 145 × 18 mm screw capped tubes at 20° in a biphasic medium[10] with monthly transfers.

The organism was grown axenically at 28° on a medium containing 2% proteose-peptone (Difco), 0.2% yeast extract (Difco), 0.5% glucose and 0.1 m*M* Fe–EDTA complex[11], in 2-l conical flasks filled to one-third of their normal capacity. After inoculation with a stationary phase culture to a cell count of 10^5 cells/ml, the flasks were shaken on a gyrotory shaker (New Brunswick Scientific Co.) at 160 oscillations/min. At the end of 19 h, the organism attained a late logphase growth ($2 \cdot 10^6$ cells/ml). Cells were harvested by low-speed (1000 × *g*) centrifugation at 3° for 20 min and were once washed with 0.9% saline before lipid extraction.

Lipid extraction. The wet cells were extracted with a mixture of chloroform–methanol (2:1) and the lipid extract freed of non-lipids by the washing procedure of FOLCH *et al.*[12].

Ascending dry-column chromatography. The experimental set up was similar to that described by JACINI AND FEDELI[9]. The experimental details are: column dimension, 50 cm × 3.2 cm; adsorbent, activated (100° for 2 h) Silica Gel G; development solvent, chloroform–acetic acid–methanol–water (75:25:5:1.5); development time, 8 h; amount of lipid chromatographed, 6 g.

After the completion of the chromatographic run under partial vacuum (20 in. mercury), the developing solvent supply was discontinued but the partial vacuum was maintained for another 30 min to remove substantial amounts of solvent vapour. The column was then cooled at 4° for 2 h and the Silica Gel G was extruded and cut into slices 7–8 mm thick; these were extracted in 5 ml of a mixture of chloroform–methanol–water (1:1:0.2) and the lipid extracts from each slice were monitored by TLC using the following systems: Silica Gel F_{254}/chloroform–acetic acid–methanol–water (75:25:5:1.5); Silica Gel F_{254}/chloroform–methanol–water (70:30:5); Silica Gel F_{254}/chloroform–methanol–conc. ammonia (70:30:5).

Wherever possible, a highly purified nitrogen atmosphere was maintained through the procedure.

Infrared (IR) spectra were determined as a liquid film with sodium chloride optics on Unicam SP 200 instrument.

Hydrolysis of various lipid fractions was carried out with aqueous 6 *N* HCl at 110° for 48 h (ref. 8) in 100 × 13 mm tubes with PTFE-lined screw caps.

TLC on cellulose plates with butanol–acetic acid–water (60:15:25) was used to detect either ethanolamine, or ethanolamine phosphate or AEP[13].

Specific group reagents used for the detection of lipids on the TLC plates were: amino-lipids: 0.25% ninhydrin in acetone; choline-lipids: 0.25% *cis*-aconitic an-

hydride in acetic anhydride[14]; and phosphorus-lipids: with molybdenum blue reagent of DITTMER AND LESTER[15].

Ester content of various lipid fractions were determined according to the procedure of SNYDER AND STEPHENS[16]. Lipid phosphorus was determined by the procedure of ERNSTER *et al.*[17] after digesting the lipid with 70% perchloric acid for 1 h.

RESULTS

A sample of total lipids of *Tetrahymena pyriformis* W was analysed by quantitative TLC using chloroform–acetic acid–methanol–water (75:25:5:1.5) as the developing solvent system (Table I). After locating the lipids by brief exposure of the TLC plate to iodine vapours, appropriate zones were scraped off, digested with 70% perchloric acid and their phosphorus content determined. A factor of 25 was used to convert the phosphorus content into phospholipid content. Neutral lipids were determined by estimating the phosphorus content of a known weight of total lipids and then subtracting the phospholipid content from the amount of weighed total lipids.

TABLE I

LIPID COMPOSITION OF *Tetrahymena pyriformis* W (TLC ANALYSIS)

Components	*Percent of total lipids*
Non-polar lipids (NL)	60.0
Polar lipids (PL)	
cardiolipin (CL)	1.8
G-AEP lipids	19.5
GPE lipids	11.3
GPC lipids[a] + minor lipids[b] (ML)	7.4

[a] 1-Alkyl-2-acyl-*sn*-glycero-3-phosphorylcholine and 1,2-diacyl-*sn*-glycero-3-phosphorylcholine.

[b] These lipids consist of three components reacting positive to both ninhydrin reagent as well as phosphorus reagent[15], while a fourth component reacting positive to phosphorus reagent alone is also present.

A preparative fractionation of 6 g of total lipids of *Tetrahymena pyriformis* W was carried out by ADCC. TLC monitoring of these fractions together with weight determinations and estimation of ester to phosphorus ratios in different fractions yielded the results presented in Table II.

Fig. 1 shows a TLC separation between G-AEP lipids and GPE lipids in the system Silica Gel F_{254}/chloroform–acetic acid–methanol–water (75:25:5:1.5). The material spotted at A represents pooled material from fractions 45–48 (Table II) and that spotted at C represents fractions 26–36 (Table II). The material spotted at B is a mixture of fractions 45–48 and 26–36. At A one sees two spots partially separated, the top one representing alkyl acyl G-AEP and the bottom one representing diacyl G-AEP.

Fig. 2 is a tracing of the IR spectra of GPE lipids and G-AEP lipids. BAER AND STANACEV[18] reported an absorption at 1030 cm^{-1} due to P–O–C stretching

TABLE II

PREPARATIVE FRACTIONATION OF TOTAL LIPIDS OF *Tetrahymena pyriformis* W BY ASCENDING DRY-COLUMN CHROMATOGRAPHY

The figures in the last two columns represent percent distribution of individual class of lipids from one or more fractions. CL = cardiolipin; NL = non-polar lipids; ML: minor lipids. Please see footnote under Table I.

Fraction numbers (from origin)	*Lipid component*	*Amount of lipid G*	*Percent distribution on*	
			Recovered polar lipids	*Recovered total lipids*
0–5	—	—		
6–10	GPC-lipid	0.296	15.9	6.6
11–14	GPC-lipid plus ML (6:1)	0.080		
15–25	ML	0.256	11.7	4.9
26–36	GPE-lipid	0.412	23.0	9.6
37–40	GPE-lipid plus diacyl-G-AEP (1:1)	0.229		
41–44	Diacyl-G-AEP	0.280	22.5	9.3
45–48	Diacyl-G-AEP plus alkyl acyl-G-AEP (3:2)	0.202		
49–60	Alkyl acyl-G-AEP	0.538	27.0	11.2
61–70	CL + NL	3.222		58.4

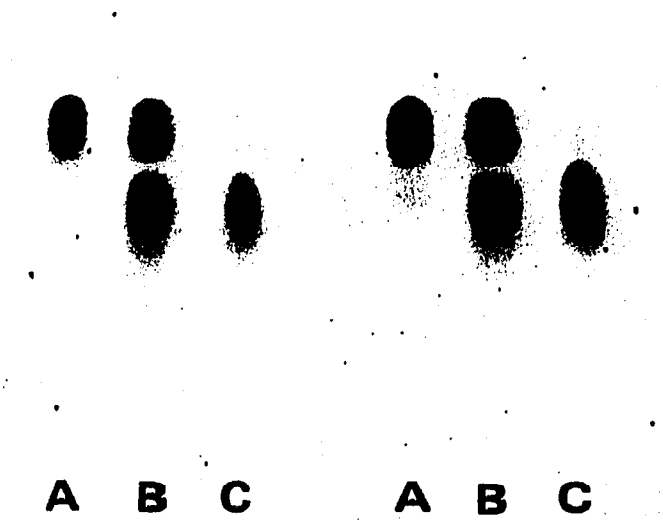

Fig. 1. TLC separation of G-AEP and GPE lipids. Adsorbent: Kieselgel F_{254}; solvent: chloroform–acetic acid–methanol–water (70:25:5:1.5); spray reagent: ninhydrin; material spotted at: A: G-AEP lipids; C: GPE lipids, B: A + C.

vibrational frequency to be characteristic of GPE lipids which was absent in the corresponding G-AEP lipids. In Fig. 2 one observes an additional distinguishing feature between GPE lipids and G-AEP lipids. The stretching vibrational frequency due to P=O around 1230 cm^{-1} in GPE lipids (Fig. 2A) is shifted to a lower frequency

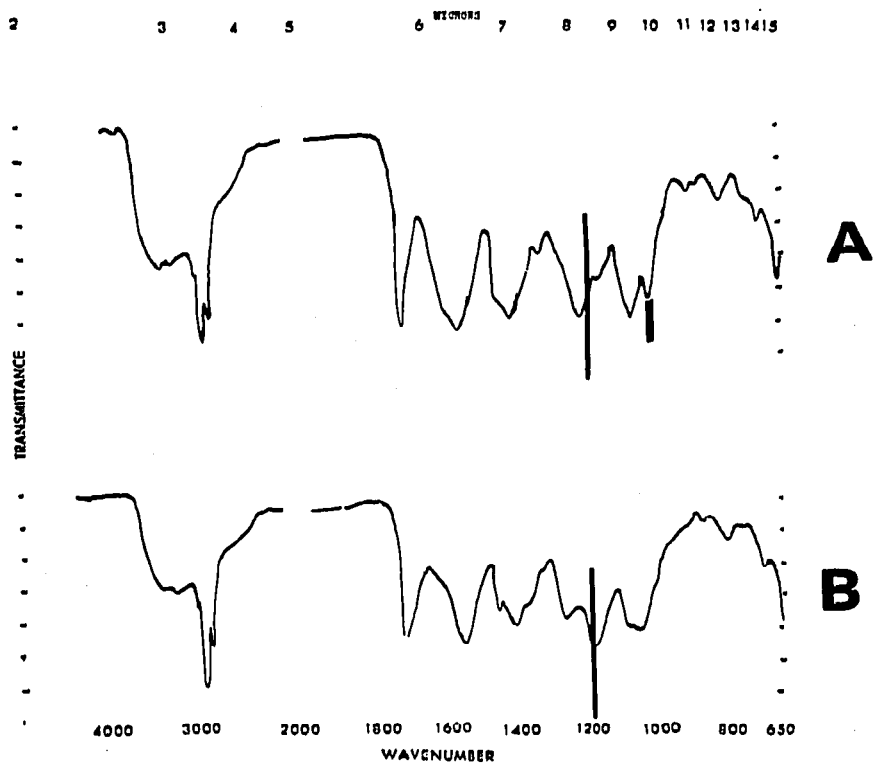

Fig. 2. IR spectra of GPE lipids (A) and G-AEP lipids (B) determined as a liquid film with sodium chloride optics.

around 1200 cm^{-1} in G-AEP lipids (Fig. 2B). This observation receives substantial support in the work of THOMAS[10], who noted an increase in frequency of P=O stretching vibrations when an electronegative substituent appeared on phosphorus but a decrease in frequency when the phosphorus was directly linked to either carbon or hydrogen.

A determination of the ester[16] to phosphorus[17] ratio indicated GPE lipid and G-AEP lipid isolated by preparative TLC system of THOMPSON[5] to be a diester and a mixture of 45 parts of diester and 55 parts of ether ester, respectively (Table III).

TABLE III

CHEMICAL CHARACTERIZATION OF GPE LIPID AND G-AEP LIPID ISOLATED BY TLC AND ASCENDING DRY-COLUMN CHROMATOGRAPHY (ADCC)

Origin of lipid	*Ester : phosphorus molar ratio*	*Base*[13]	*Nature of lipid*
Preparative TLC	2.07	Ethanolamine	Diacyl-GPE
Preparative TLC	1.45	AEP	Alkyl acyl G-AEP + diacyl G-AEP
ADCC (Fr. 41–44) from Table II	2.10	AEP	Diacyl G-AEP
ADCC (Fr. 49–60) from Table II	1.03	AEP	Alkyl acyl G-AEP

Similar ester:phosphorus ratio determination in the case of column fractions 41–44 and 49–60 (Table II) indicated them to be pure diacyl G-AEP and alkyl acyl G-AEP, respectively (Table III).

The IR spectra tracings (Figs. 3A and B) of the compounds representing fractions 49–60 and 41–44 (Table II) did not indicate any difference but resembled that of mixed G-AEP lipids (Fig. 2B).

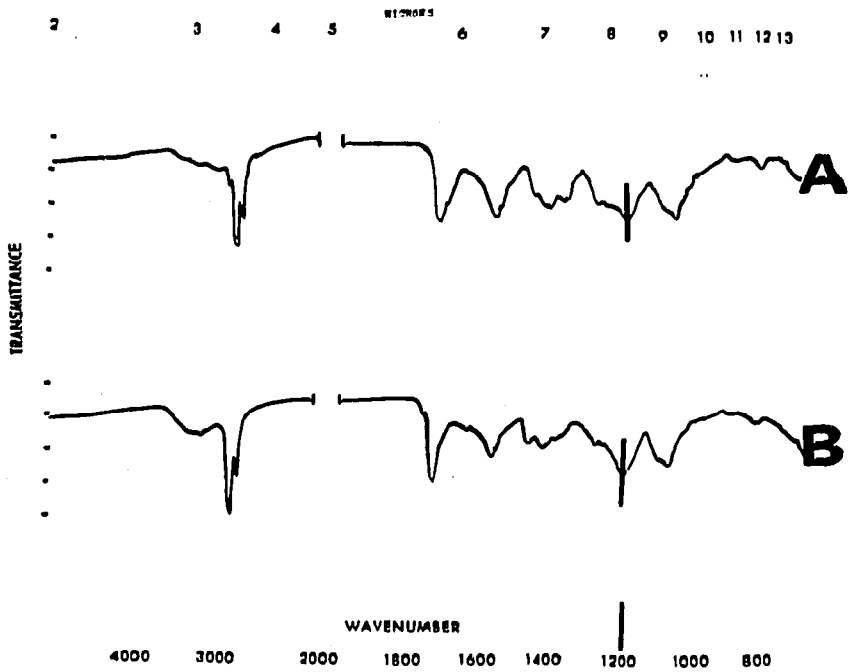

Fig. 3. IR spectra of alkyl acyl G-AEP lipid (A) and diacyl G-AEP lipid (B) determined as a liquid film with sodium chloride optics.

DISCUSSION

Ascending preparative dry column chromatography was successfully used in the fractionation of non-polar lipids, isolation of triglycerides from natural oils and fats, argentation fractionation of molecular species of triglycerides and fatty acid methyl esters and partition chromatography of 2,4-dinitrophenylhydrazone of normal aldehydes[9]. The present work has not only indicated further application of this technique in the large scale fractionation of polar lipids but also enabled us to separate the chromatographically difficult separable analogs of G-AEP lipids in substantial amounts. Further it helped concentration of minor lipids whose chemical characterization will be reported in a later communication.

The present technique not only saved larger amounts of solvents required in the conventional procedure of column chromatography used in the fractionation of lipids but also achieved the fractionation in one step instead of the multi-step fractionation procedure used by other workers[6,7]. The use of Silica Gel G, normally

used for TLC fractionation, led to better resolution between different lipid components and use of partial vacuum during solvent development allowed use of longer columns and thus improved the separation efficiency.

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