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# Sensitive assay of phospholipid glycerol in environmental samples

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**Summary**

Measurement of very low levels of microbial biomass was achieved by determining the glycerol content of the phospholipids recovered from environmental samples. Successive application of acid methanolysis and hydrofluoric acid hydrolysis was used to release glycerol from the phospholipids. The glycerol, once released, was acetylated and analyzed by capillary gas-liquid chromatography (GLC). The analysis was sensitive to $10^{-11}$ moles by GLC with flame ionization detection and GLC/mass spectrometry with selective ion monitoring. Estimation of the microbial biomass by the lipid phosphate correlated with the glycerol phosphate measured by the hydrolytic procedures. In addition, indication of the community composition was gained by analysis of the acid labile glycerol. Application of this methodology to the sparse microbiota of the ground water sediments showed agreement with other estimates of microbial biomass.

Key words: *Glycerol – Microbial biomass – Phospholipid measure – Sedimentary biomass*

## Introduction

This paper describes a sensitive method to determine the amount of phospholipid in microorganisms and environmental samples. Extractable lipid phosphate, a measure of the membrane phospholipids, is one of the most useful measures of biomass in sediments [1]. Phospholipids are a part of every cellular membrane and are not endogenous storage products. Phospholipids maintain a relatively fixed proportion of the membranes of various microbes [1, 2] and have a relatively rapid turnover [3, 4]. Thus phospholipids are considered to be an estimate of the active microbial biomass. This includes both the prokaryotes and the microeukaryotes. Extractable lipid phosphate has usually been measured colorimetrically. Unfortunately this method has a maximum sensitivity of $10^{-9}$ moles which corresponds to 20 μg dry wt or $2 \times 10^7$ bacterial cells the size of *Escherichia coli*. Since glycerol is also a component of most phospholipids and can be detected

by gas-liquid chromatography (GLC) at sensitivities of $10^{-11}$ moles by GLC, the assay of the glycerol was used to increase the sensitivity of detection. This sensitivity could be critical in the estimation of the phospholipids in environments containing sparse microbial communities.

Glycerol occurs in a number of lipids that can be extracted from environmental samples. These lipids can be fractionated quantitatively on silicic acid columns into neutral lipids (glycerides and highly acylated glycolipids) that are eluted with chloroform, glycolipids (glycosyl glycerides) that are eluted with acetone, and polar lipids (phospholipids, sphingolipids) that are eluted with methanol [3]. The major phospholipids can be estimated from the glycerol. Phosphatidic acid and monophospholipids with the structure of diacylated glycerol phosphoryl-ethanolamine, -choline, -monomethylethanolamine, -dimethylethanolamine, -inositol, -choline, or -serine have glycerol to phosphate ratios of one. Phosphatidylglycerol (PG) and cardiolipin have glycerol to phosphate ratios of 2:1 and 3:2 respectively. PG is the major phospholipid recovered from the detrital microbiota [3]. Phospholipids like the phytanyl glycerol ethers from the archaebacteria do not yield glycerol on hydrolysis. These lipids generally represent less than 10% of the total phospholipids in sediments [5]. Some sphingophospholipids do not yield glycerol on hydrolysis. These lipids generally form a small proportion of the total sedimentary phospholipids (Nickels, J.S. and White, D.C., unpublished data).

Glycerol from authentic standards and environmental samples was estimated by successive hydrolysis with acid and hydrofluoric acid (HF). Acid methanolysis released glycerol phosphate and glycerol from esters containing glycerol-phosphoryl glycerol moieties. HF hydrolysis released glycerol from the glycerol phosphate. The use of this hydrolysis sequence gave an indication of the PG and cardiolipin content of the lipid in addition to a more sensitive measure of the total phospholipid.

## Materials and Methods

### *Materials*

Glass distilled solvents were used as purchased (Burdick and Jackson, Muskegon, MI) or freshly distilled just prior to use in extractions and derivatizations. Lipid standards and derivatizing reagents were purchased from Pierce Chemical Co., Rockford, IL, Aldrich Chemical Co., Inc., Milwaukee, WI, and PCR Research Chemicals, Inc., Gainesville, FL.

### *Organisms*

*Escherichia coli* strain B was purchased from Sigma Chemical Co., Inc., St. Louis, MO, *Staphylococcus aureus* U-71 was grown in nutrient broth and wild type *Neurospora crassa* FGSC 988 79CA (the gift of Dr. R.M. DeBusk) was grown on Vogel's medium N [6] supplemented with 2% (w/v) sucrose for 3 days. Cultures were washed with 50 mM phosphate buffer pH 7.4 with centrifugation at 4°C and lyophilized.

*Lipid extraction*

Sediments and lyophilized monocultures were extracted by the modified Bligh and Dyer [7] single phase chloroform-methanol method [1]. The phases were separated by adding additional buffer and chloroform and the chloroform phase filtered through fluted folded Whatman 2V filter paper. The lipid was dissolved in chloroform and the solvent removed twice.

*Column chromatography*

The lipid was fractionated on silicic acid (Unisil, 100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA). Columns 0.54 × 29 cm were packed with 1 g silicic acid suspended in chloroform and the lipids dissolved in chloroform were added at less than 0.4 mg dry wt lipid per g of silicic acid. The neutral lipids were recovered by elution with 10 ml of chloroform, the glycolipids by elution with 10 ml acetone, and the phospholipids by elution with 10 ml of methanol. The recovery is quantitative [3].

*Hydrolysis*

Prior to hydrolysis a recovery standard of 0.2 μmole 1,3,4-butanetriol (Aldrich Chemical Co., Milwaukee, WI) was added.

For mild alkaline methanolysis, the phospholipid fraction from the silicic acid column was dissolved in 0.5 ml of methanol-toluene (1:1, v/v) to which an equal volume of freshly prepared 0.2 M methanolic KOH was added, the solution mixed on a vortex mixer and heated for 15 min at 40°C in a test tube with a Teflon lined screw cap. The mixture was partitioned by adding 2 ml each of chloroform and water, mixed for 5 min with a vortex mixer, centrifuged, and the water recovered. The partitioning process was repeated twice. The combined water washes containing the glycerol and glycerol phosphate were neutralized with 10% (v/v) acetic acid and reduced to dryness in vacuo at 40°C.

For acid methanolysis the dried water fraction of the mild alkaline methanolysis or the intact phospholipid eluate from the silicic acid column was dissolved in methanol-chloroform-concentrated HCl (10:1:1, v/v/v) and then heated for 2 h at 60°C. After cooling, 2 ml each of chloroform and water were added and the aqueous portion collected as in the mild alkaline hydrolysis. Half the sample was analyzed for glycerol at this point and half was subjected to HF hydrolysis.

Concentrated HF (48% AR grade, Mallinckrodt, Inc., St. Louis, MO), held at 4°C was added to half the lyophilized aqueous phase of the acid hydrolysis in polypropylene centrifuge tubes, and incubated at 4°C for 3 days with constant mixing. HF was removed in a plastic desiccator in vacuo over $P_2O_5$ and KOH pellets. The vacuum pump was protected by a plastic tube 5 × 35 cm long packed with NaF and a glass trap cooled in dry ice–acetone. The preparations were dried in 24 h at room temperature. The glycerol was transferred to a screw cap test tube with three portions of water and samples dried in vacuo at 40°C.

*Derivatization*

The internal standard of 0.2 μmoles of 1,9-nonanediol (Aldrich Chemical Co.,

Inc., Milwaukee, WI) dissolved in methanol was added to Teflon lined screw cap test tubes containing samples and the solvent removed in a stream of nitrogen at 30–40°C. One ml of acetic anhydride–pyridine (1:1, v/v) was added, the solution mixed with a vortex mixer, and heated to 60°C for 2 h. The test tubes were cooled and 2 ml of chloroform added. The solution was washed three times with an aqueous 20% (w/v) tartaric acid solution with vortex mixing and centrifugation to remove pyridine. The aqueous phase was removed and discarded each time. The chloroform was transferred to a clean dry Teflon capped tube and then removed in a stream of nitrogen. After dissolving in chloroform the sample was analyzed by gas-liquid chromatography.

*Gas-liquid chromatography*

Samples of triacetylated glycerol were analyzed with a Varian 3700 gas chromatograph with a CDS 111 controller and a model 8000 autosampler with flame ionization detection. Data were processed by a programmable Hewlett-Packard 3502 laboratory data system. One μl of sample was injected on to a 30 m, 0.25 mm internal diameter fused silica capillary column coated with the non-polar methylsilicone Durabond DB-1 (J. and W. Laboratories, Rancho Cordova, CA)

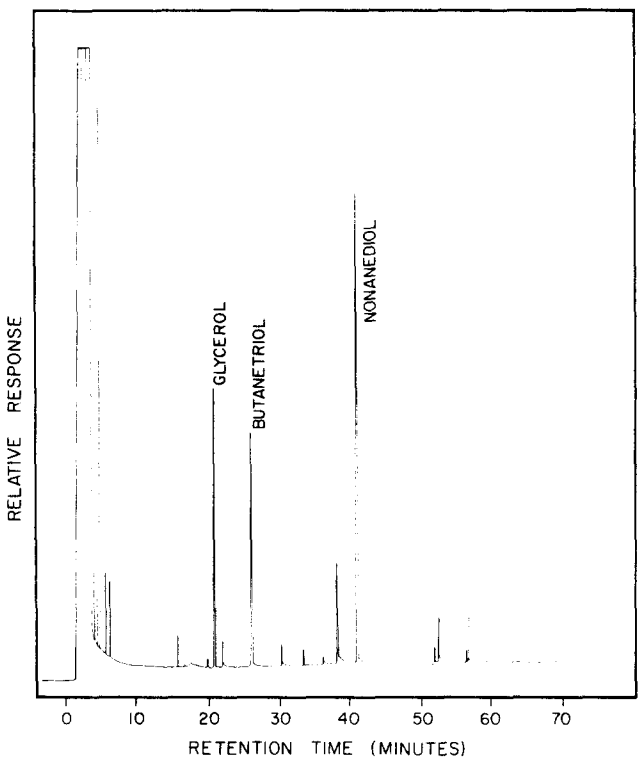


Fig. 1. Chromatogram of triacylglycerol (0.111 nmoles), triacylbutanetriol (0.109 nmoles), and diacylnonanediol (0.20 nmoles) from the phospholipids of an estuarine sediment sample.

operated in the splitless mode with 0.5 min venting time. The temperature program initiated at 45°C and increased to 100°C at 5°/min followed by a 2°C per min rise to 190°C and a 12 min isothermal period. The helium carrier gas flow rate was 1.5 ml/min at 0.92 kg/cm$^2$. The injection port was at 220°C and the detector at 250°C. Under these conditions the response factors of triacetylglycerol to 1,3,4-triacylbutanetriol and diacyl-1,9-nonanediol were 1.4 and 2.0 respectively. A chromatogram is illustrated in Fig. 1.

*Mass spectrometry*

Capillary gas-chromatographic/mass-spectral fragmentography was performed with the Hewlett-Packard 5995A GC/MS using a 50 meter fused silica capillary column with a cross-linked methyl silicone liquid phase (Hewlett-Packard, Avondale, PA), 0.2 mm in internal diameter, 0.11 μm film thickness that was inserted through a Scientific Glass Engineering, Inc., open split interface (Austin, TX) inlet with the restrictor removed and one outlet connected to the auxillary pump. The column was threaded through the device to the inlet valve. The hydrogen carrier gas at a flow rate of 1.5 ml per min gave a pressure in the inlet of 0.02 Torr. The temperature program was the same as that used for GLC. The mass spectrometer was autotuned with decafluorotriphenylphosphine with a 70 meV fragmentation energy. Spectra were recorded at a scan speed of 380 AMU/s (4 samples/0.1 AMU) with a 0.5 s delay between scans of 50 to 350 AMU. The spectrometer was operated in the peakfinder mode at an electron multiplier voltage of 1400 volts. The threshold of detectability was set at 100 linear counts. To achieve maximum sensitivity the mass spectrometer was operated in the selective ion mode with M/z 103 used for triacylglycerol, triacylbutanetriol, and diacylnonanediol.

*Phospholipid analysis*

The lipids were digested in 23.3% perchloric acid at 180°C for 2 h and the phosphate determined colorimetrically as described [1].

## Results and Discussion

*Sensitivity and identification*

It proved possible to detect $10^{-11}$ moles of triacylglycerol derived from the triglycerides in the neutral lipid fraction extracted from the estuarine animals, fungi and the adherent detrital microbiota with a response twice the background. This was done with GLC with flame ionization detection operating at $4 \times 10^{-12}$ A/mV [7]. The triacylglycerol cochromatographed with authentic material and had the same mass spectra with major components at M/z 103 ($HOCHCH_2OCO^+CH_3$) = 100%; M/z 145 ($CH_3OCOCH_2CHOCO^+CH_3$) = 76%; M/z 116 (M-59-73) = 45%; M/z 115 (M-103) = 32%; M/z 86 ($CHCH_2OCO^+CH_3$) = 19%; and M/z 73 ($CH_2OC^+CH_3$) = 15%. The same sensitivity in the analysis can be achieved by using GLC/mass spectrometry with the selective ion monitoring mode using M/z 103 for triacylglycerol, triacylbutanetriol, and diacylnonanediol internal standards.

*Hydrolysis*

Phospholipids can be quantitatively separated from the neutral and glycolipids in environmental samples by silicic acid chromatography [3, 8]. The phospholipid fraction can be quantitatively deacylated by mild alkaline methanolysis and the acyl fatty acids used for analysis [1, 2]. The water soluble glycerol phosphate esters from a mild alkaline methanolysis or the intact phospholipids if the diacyl linked phospholipids are not required for analysis were treated by acid methanolysis. Acid methanolysis released the fatty components linked by ester, amide and vinyl ether bonds. The water from the acid methanolysis contained glycerol, glycerol phosphate and other water soluble compounds. There are two major problems in the analysis of phospholipid by the glycerol: some lipids like PG and cardiolipin contain more glycerol than phosphate and glycerol phosphate is remarkably resistant to acid or basic hydrolysis. Hydrolysis of glycerol phosphate at 100°C with 6 M HCl in sealed glass ampules released 26.4 ± 2.1% ($X$ ± S.D., $n$ = 5) of the glycerol and phosphate after 72 h. Increasing the strength of the HCl to 12 M resulted in only a 21.7 ± 8.8% recovery. However, exposure of glycerol phosphate to HF at 4°C resulted in complete hydrolysis. Kinetic experiments with authentic disodium glycerol phosphate (Sigma Chemical Co., Inc., St. Louis, MO) showed the hydrolysis with HF to be complete in 3 days at 4°C. After 5 days at 4°C, 20% of the glycerol was lost when compared to the phosphate analysis. HF hydrolysis at higher temperatures resulted in decreased yields of glycerol.

The problem of phospholipids with more glycerol than phosphate was solved by a combined hydrolysis. An acid methanolysis of the lipid released the fatty components, glycerol, glycerol phosphate and other water soluble components. If half the acid methanolysate was analyzed by GLC or GLC/mass spectrometry and the other half subsequently treated with HF then the glycerol phosphate would also be hydrolyzed for estimation by the sensitive GLC methods. To test this glycerol-phosphorylglycerol (GPG), 490 nmoles were derived from authentic PG by mild alkaline methanolysis (recovery of phosphate 102 ± 5%). Acid methanolysis of GPG should yield glycerol plus glycerol phosphate. Acid methanolysis of the GPG yielded 510 ± 80 nmoles glycerol (104% of the expected recovery of free glycerol). The combined acid methanolysis and HF hydrolysis should yield 2 moles of glycerol per mole of phosphate. The actual yield was 740 ± 180 nmoles of glycerol or 74% of the 980 nmoles expected. The combined acid methanolysis and HF hydrolysis did not yield quantitative recovery of glycerol.

*Quantitative recovery of glycerol*

In a reconstruction experiment the loss of glycerol from the combined acid methanolysis and HF hydrolysis was traced to the free glycerol in the HF hydrolysis. Glycerol phosphate (1000 nmoles) yielded 960 ± 50 nmoles glycerol after HF hydrolysis. Glycerol (2000 nmoles) after HF hydrolysis resulted in recovery of 1400 ± 100 nmoles of glycerol or 77 ± 7% recovery.

To correct for the loss of glycerol from the HF hydrolysis a 'recovery' internal standard of butanetriol which has similar volatility to glycerol was added to the hydrolysis mixture. The calculations can be readily visualized in a series of equations:

*A. After acid methanolysis*

1. Area glycerol/area nonanediol × response factor glycerol/nonanediol = $G_a$ = 'acid labile or free glycerol'.
2. Area butanetriol/area nonanediol × response factor butanetriol/nonanediol = $B_a$ = 'free butanetriol'.
3. $G_a/B_a$ = glycerol/butanetriol after acid methanolysis.

*B. After HF hydrolysis*

4. Area glycerol/area nonanediol × response factor glycerol/nonanediol = $G_c$ = glycerol from combined hydrolysis.
5. Area butanetriol/area nonanediol × response factor butanetriol/nonanediol = $B_r$ = 'remaining butanetriol'.
6. $G_a/B_a \times B_r = G_r$ = remaining glycerol.
7. $G_c - G_r = G_h$ = 'glycerol released by HF'.
8. $G_h + G_a = G_t$ = total phospholipid glycerol.

A reconstruction experiment with 1000 nmoles of glycerol, glycerol phosphate and butanetriol gave: $G_a = 1010 \pm 20$ nmoles; $B_a = 980 \pm 70$ nmoles; $G_c = 1100 \pm 200$ nmoles; $B_r = 164 \pm 67$ nmoles. $G_r$ thus = $196 \pm 62$ nmoles; $G_h = 931 \pm 140$ nmoles; $G_t = 1941 \pm 130$ or $95 \pm 9\%$ of the expected 2000 nmoles of glycerol.

*Acid labile glycerol*

An advantage of the dual hydrolysis was that it gives an estimate of the phospholipids containing more glycerol than phosphate in the sample. For the lipids from *N. crassa*, *E. coli*, and *S. aureus* the 'acid' labile glycerol was $12 \pm 1.2\%$, $19 \pm 2\%$, and $52 \pm 4.5\%$ of the total lipid glycerol respectively. The lipids of *N. crassa* have been reported to contain less than 10% phospholipid with acid labile glycerol [9, 10]. *E. coli* was reported to contain 19% phosphatidylglycerol [11]. *S. aureus* contained about 55% lysylphosphatidylglycerol and phosphatidylglycerol in the mid-stationary growth phase [12]. Authentic phosphatidylethanolamine yielded $< 0.01$ nmoles acid labile glycerol and the expected glycerol after combined acid and HF hydrolysis.

*Analysis of estuarine sediment*

Using the internal standard of butanetriol to correct for losses of acid labile glycerol and nonanediol to estimate the errors in injection, the estimate of phospholipid recovered from estuarine sediments by analysis of the lipid phosphate was compared to the estimate of glycerol phosphate after combined acid and HF hydrolysis. Estuarine sediment was extracted and the lipid recovered and fractionated by silicic acid chromatography. Mild alkaline methanolysis of the neutral and phospholipid fractions yielded water soluble esters which were analyzed for acid labile glycerol and glycerol phosphate. The extractable lipid phosphate measured colorimetrically was $330 \pm 90$ nmoles lipid phosphate/g dry wt. The acid labile glycerol ($G_a$) was $330 \pm 60$ nmoles with $B_a = 100 \pm 20$ nmoles and $B_r = 42 \pm 8$ nmoles. Thus $G_a/B_a \times B_r = 330/100 \times 42 = 140$ nmoles = $G_r$; $G_c - G_r = 480 - 140 = 340$ nmoles = $G_h$; $G_h + G_a = 340 + 330 = 670$ nmoles = $G_t$. $G_h = 340$

TABLE 1

ESTIMATES OF THE MICROBIAL BIOMASS OF THE GROUND WATER SEDIMENTARY MICROBIOTA

| Analysis | nmoles/g dry wt.[a] | Estimated number as *E. coli* |
|---|---|---|
| Muramic acid | 2.1 ± 1.2 | $1.4 \times 10^{8}$ [b] |
| Total extractable fatty acids | 7.1 ± 4.4 | $7 \times 10^{7}$ [c] |
| LPS bound fatty acids | 0.18 ± 0.09 | $1.5 \times 10^{7}$ [d] |
| Phospholipid glycerol | 0.98 ± 0.18 | $2 \times 10^{7}$ [e] |

[a] Expressed as $X \pm$ S.D., $n = 4$.
[b] Estimated as $1.4 \times 10^{-5}$ moles muramic acid/g dry wt. [13], assuming $10^{12}$ cells/g dry wt.
[c] Estimated as $1 \times 10^{-4}$ moles fatty acids/g dry wt. [14].
[d] Estimated as $1.2 \times 10^{-5}$ moles hydroxy fatty acids/g dry wt. [15].
[e] Estimated as $5 \times 10^{-5}$ moles glycerol phosphate/g dry wt. [1].

nmoles compared to the lipid phosphate of 330 nmoles for 103% of the expected amount. The $G_t$ value that equals twice $G_h$ suggests a major phospholipid in this sediment was PG with minor amounts of cardiolipin and the monophosphate phospholipids. This type of lipid composition has been detected in the detrital phospholipids [3]. The neutral lipid of this sediment contained 245 ± 8 nmoles of glycerol per g dry wt.

*Use in the analysis of ground water sediment*

If the sediments from the ground water aquifer are recovered with utmost care to prevent contamination with surface microbes, the native aquifer microbes can be examined. Because of the sparseness of this microbiota, sensitive methods of biomass measurement were utilized. The results of estimations from a core taken 12 ft below the surface at Ft. Polk, LA are given in Table 1. The muramic acid was measured by a modification of the GLC method [13], the total extractable fatty acids by a summation of the responses of the acyl and hydroxy fatty acid esters after GLC [14]; the acid labile hydroxy fatty acids from the lipid A of the lipopolysaccharide were estimated by GLC [15]; the phospholipid was determined as the glycerol phosphate after acid methanolysis and HF hydrolysis as described in this paper. The different measures show reasonable agreement when estimated in terms of a bacteria with about $10^{12}$ cells/g dry wt like *E. coli* 'equivalents'.

## Conclusions

The methods described in this paper make possible the estimation of phospholipid in terms of the glycerol phosphate and glycerol in environmental samples. The glycerol content of both the neutral lipid and glycolipid fractions of the extractable lipid could also be determined. In the analysis of the phospholipid the acid labile glycerol to glycerol phosphate ratio can give an indication of the proportions of prokaryotes as the PG type lipids are more common in bacteria than in

the microeukaryotes [16]. Both the glycerol phosphate glycerol and acid labile glycerol can be estimated with great sensitivity by use of GLC or GLC/mass spectrometry. In this analysis the typical sample would be extracted with the one-phase chloroform-methanol method, the chloroform phase fractionated on silicic acid columns and the phospholipid fraction recovered. The phospholipid would then be subjected to acid methanolysis after the addition of butanetriol. Half of the water-soluble components would be analyzed after addition of nonanediol and derivatization. The other half of the water soluble components would be hydrolyzed by HF, acylated after addition of nonanediol, and the hydrolysates from the acid methanolysis and the combined acid methanolysis and HF hydrolysis analyzed by GLC or GLC/mass spectrometry. The acid labile glycerol ($G_a$), free butanetriol ($B_a$), glycerol released from glycerol phosphate ($G_h$) and total glycerol phospholipid glycerol ($G_t$) would then be calculated.

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