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Phospholipids of *Thiobacillus thiooxidans*

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Cells and spent growth media from sulfur- and thiosulfate-grown cultures of *Thiobacillus thiooxidans* were analyzed. The phosphatides were examined by thin-layer chromatography, and the products of their hydrolysis by hydrochloric acid and methanolic potassium hydroxide were separated by paper chromatography. The phospholipids in both cells and spent growth media were identified as phosphatidyl ethanolamine, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl glycerol, and diphosphatidyl glycerol. These comprised about 97% of the total lipid phosphorus. Lyso-phosphatidyl-*N*-monomethylethanolamine and lyso-phosphatidyl glycerol accounted for the remaining 3%. The percentage of the total lipid phosphorus accounted for by each phospholipid depended on the age of the culture.

The phospholipids of *Thiobacillus thiooxidans* have been the subject of several research reports. Schaeffer and Umbreit (21) identified the extracellular lipid necessary for the "wetting" of sulfur as phosphatidyl inositol (PI). Jones and Benson (14), however, were unable to find PI in the cells of the organism and only minor quantities in the spent growth medium. Phosphatidyl glycerol (PG) was the most abundant extra- and intracellular phospholipid. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), diphosphatidyl glycerol (DPG), and phosphatidic acid (PA) were the other phospholipids reported. A preliminary study of *T. thiooxidans* lipids in our laboratory (L. L. Barton, M.S. Thesis, Univ. of Nebraska, Lincoln, 1966) indicated that PE was the major phospholipid and that both PC and PI were absent.

Because of the conflicting reports, an extensive study of the phospholipids of *T. thiooxidans* was undertaken.

MATERIALS AND METHODS

Cultures. A strain of *T. thiooxidans* capable of utilizing thiosulfate as an energy source was isolated from a culture of *T. thiooxidans* ATCC 8085 (not capable of growth in thiosulfate medium). It has not yet been established whether the strain is a contaminant or a direct descendant of the ATCC culture. Cells and spent growth media of the new strain (sulfur or thiosulfate energy source) and the original ATCC strain (sulfur energy source) were analyzed for phospholipids.

Methods of cultivation. The cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of the following media (in grams/liter of distilled water): either $Na_2S_2O_3 \cdot 5H_2O$, 5.0, or sublimed sulfur, 10; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 0.4; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.01; *p*H was adjusted with H_2SO_4 to 4.5 for thiosulfate medium and to 4.0 for sulfur medium. Sulfur was sterilized separately by steaming 0.5-g quantities for 1 hr on 3 successive days and was added aseptically to 50 ml of the sterilized (121 C for 15 min) medium. When the new strain was first isolated, sodium thiosulfate was sterilized separately in concentrated solutions by filtration and was then added aseptically to the medium. It was discovered, however, that sterilization (121 C for 15 min) of the complete medium did not visibly alter the medium or hinder growth of the culture. Therefore, in all phases of this experimentation, the thiosulfate medium was sterilized in its complete form by autoclaving (121 C for 15 min). Inoculations were made with 0.5 ml of cultures (2 and 7 days for thiosulfate- and sulfur-grown cultures, respectively), centrifuged and resuspended in 10^{-4} N H_2SO_4. Incubations were carried out at 30 C on a rotary shaker (New Brunswick Incubator Shaker, model-27) adjusted to 180 rev/min. The sulfur cultures were placed on the shaker after 3 days of stationary incubation.

Growth curves were developed for the thiosulfate-utilizing strain by use of the most probable number technique (1). Samples were taken from growth flasks, diluted in 0.001 M KH_2PO_4 (*p*H 4), and "planted" (five 1.0-ml portions of each dilution) in the described thiosulfate medium. After incubation (14 days at 30 C), a positive tube was indicated by turbidity and a lowered *p*H.

Preparation and analysis of phospholipids. Lipids labeled with ^{32}P were prepared by growing the cultures in 50 ml of media (either sulfur or thiosulfate energy

source) containing 1 mc of carrier-free ^{32}P-phosphoric acid (Isotopes, Inc., Westwood, N.J.). The KH_2PO_4 content of these media was reduced to 0.1 g/liter. Lipids labeled with ^{14}C were prepared by growing the cultures in $^{14}CO_2$ containing environments. The $^{14}CO_2$ was generated from ^{14}C-barium carbonate (Isotopes, Inc., Westwood, N.J.).

The cells were harvested from cultures of different age by centrifugation and were washed with sulfuric acid (hydrogen-ion concentration approximating that of each medium). The spent media were passed through 0.45-μ membrane filters (Millipore Corp., Bedford, Mass.) and were subjected to extraction with chloroform (10 ml of chloroform per 50 ml of medium) for 12 hr at 4 C. The lipids were extracted from the cells with chloroform-methanol (2:1, v/v), by the method of Vorbeck and Marinetti (24). After extraction, the cells were removed by passing the suspension through a 1-cm column of 100-mesh silicic acid (Mallinkrodt Chemical Works, St. Louis, Mo.). The resulting extract was washed with a sodium chloride solution (8). The chloroform extract of the medium was evaporated to dryness, redissolved in chloroform-methanol (2:1, v/v), and washed as above. All washed extracts were evaporated to dryness and redissolved in chloroform-methanol (2:1, v/v).

Samples of the concentrated washed lipids were spotted on activated (110 C for 60 min) silica gel G (E. Merck AG, Darmstadt, Germany) thin-layer plates (250 μ thick). The plates were dried in a desiccator over calcium chloride for 12 hr and were developed in chloroform-methanol-water (66:30:4, v/v).

The phosphatides were deacylated in 0.1 N methanolic potassium hydroxide at 37 C for 20 min (2). After deacylation, neutralization was accomplished by use of ethyl formate (6). The water-soluble phosphate esters were chromatographed on Whatman no. 1 paper with phenol-water [100:38, v/v (2)] and *n*-butanol-propionic acid-water [142:71:100, v/v (2)].

The phosphatides and the glycerophosphoryl esters were located on the chromatograms by exposure to no-screen X-ray film (Eastman Kodak Co., Rochester, N.Y.). Quantitation was accomplished by measuring the radioactivity of the individual spots with a thin-window Geiger tube (Tracerlab, Waltham, Mass.).

The phospholipids were identified by chromatography (as described) of the phosphatides and their glycerolphosphoryl esters with standards [PG and DPG from *Micrococcus lysodeikticus* (19) and *Chlorella* (3); PC, PE, and PI from Pierce Chemical Co., Rockford, Ill.]. Their identification was confirmed by chromatography of the acid hydrolysis (0.1 or 1.0 N HCl) products of ^{14}C-glycerophosphoryl esters with standards (2-aminoethanol, 2-methylaminoethanol, 2-dimenthylaminoethanol, and chloine chloride; Distillation Products Division of Eastman Kodak Co.. Rochester, N.Y.). Chromatography of the ^{14}C-acid hydrolysis products was carried out on Whatman no. 1 paper by use of the previously mentioned solvent systems and in phenol-*n*-butanol-formic acid-water [5:5:3:1, w/v/v/v (5)]. The radioactive spots were located as before.

Standards were located on the thin-layer chromatograms by use of molybdenum blue reagent for phosphorus (7) and ninhydrin for amino groups, and on paper chromatograms by use of perchloric acid-ammonium molybdate-ultraviolet light for phosphorus (6), ninhydrin for amino groups, bromcresol green for basic materials, and alkaline silver nitrate for glycerol (23).

RESULTS

Phospholipid identification. Thin-layer chromatograms of lipid extracts of both cultures (sulfur- and thiosulfate-grown new strain and the sulfur-grown original strain) yielded six radioactive spots (Fig. 1). Phospholipids 1 to 4 contained 97% of the lipid phosphorus. Phospholipids 1, 3, and 4 cochromatographed with DPG, PE, and PG, respectively. Phospholipid 2 because of its close proximity to PE was tentatively identified as phosphatidyl-*N*-monomethylethanolamine (PME). The only way these two lipids

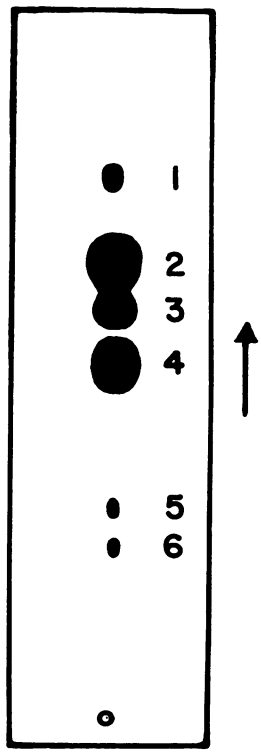

FIG. 1. *Thin-layer chromatogram of ^{32}P-phospholipids of Thiobacillus thiooxidans. Adsorbent: silica gel G. Solvent system: chloroform-methanol-water (66:30:4, v/v). Detection: autoradiography. Key to numbered components: 1, diphosphatidyl glycerol; 2, phosphatidyl-N-monomethylethanolamine; 3, phosphatidylethanolamine; 4, phosphatidyl glycerol; 5, lysophosphatidyl-N-monomethylethanolamine; and 6, lysophosphatidyl glycerol.*

could be consistently separated was to ensure that the thin-layer plate and the sample were devoid of moisture. This was most effectively accomplished by drying the plate in a desiccator after spotting. In most chromatography solvents, these phospholipids will migrate as a single component (18, 22). Phospholipids 5 and 6 did not co-chromatograph with any available phospholipids, including PI and PC. These were tentatively identified as lyso-forms. If the individual phospholipids were scraped from the thin-layer chromatograms, were eluted from the silica gel with chloroform-methanol (2:1, v/v), were deacylated, and the water soluble products were subjected to chromatography, only four different radioactive spots were obtained (Fig. 2). Deacylation of total ^{32}P-lipid extracts and chromatography of the water-soluble products revealed the same four spots. The diesters of phospholipids 5 and 6 migrated with the diesters of 2 and 4, respectively, supporting their tentative identification as lyso-forms. It was realized that phospholipid 6 might be one of the amino acid esters of PG often found in bacteria (11). By co-chromatography with deacylation products of the known phospholipid standards, the glycerolphosphoryl esters (Fig. 2) were identified as glycerophosphoryl glycerophosphoryl glycerol (one) glycerophosphoryl ethanolamine (three), and glycerophosphoryl glycerol (four or six). The position on the chromatogram of the diester of phospholipid 2 or 5 was that expected for glycerophosphoryl-*N*-monomethylethanolamine (16). The water-soluble products were those expected from the tentatively identified phosphatides.

Extraction, deacylation, and paper chromatography of the water-soluble products of ^{14}C-lipids yielded the same four diesters. Amino acids were not released by the deacylation procedure. This was also true when phospholipid 6 (Fig. 1) labeled with ^{14}C was deacylated and chromatographed. Therefore, phospholipid 6 does not appear to be an amino acid ester of phosphatidyl glycerol.

When the four ^{14}C-diesters were eluted from the paper and acid-hydrolyzed (phospholipid 1 for 30 min at 100 C in 0.1 N HCl and phospholipid 2 to 6 for 1 hr at 100 C in 1.0 N HCl), the following compounds were released: glycerol, glycerophosphate, glycerophosphoryl glycerol, and a small quantity of unhydrolyzed material from phospholipid 1; glycerophosphate and methylethanolamine from phospholipids 2 and 5; glycerophosphate and ethanolamine from phospholipid 3; glycerophosphate and glycerol from phospholipids 4 and 6. These products confirmed the identity of the phospholipids as DPG, PME (and its lyso-form), PE, and PG (and its lyso-form).

The same phospholipids were found in the spent growth medium of both cultures regardless of the energy source.

Relative concentrations of the phosphatides. The new isolate, growing in the thiosulfate medium, reached the maximal stationary phase of growth in about 45 hr with a generation time of about 4 hr (Fig. 3). The *p*H of the medium decreased to about 1.8 by the 45th hr. Slightly slower growth rates were recorded when the phosphate content of the medium was reduced. In the logarithmic growth phase, the total lipid phosphorus was accounted for as: PG, 37%; DPG, 7%; PE, 20%; and PME, 36% (Table 1). As the culture aged.

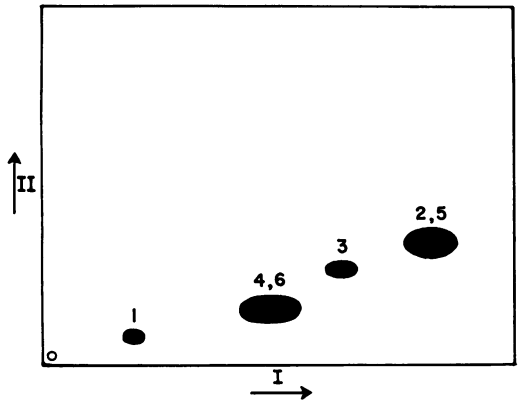

FIG. 2. *Paper chromatogram of deacylated ^{32}P-phospholipids of Thiobacillus thiooxidans. Solvent system: direction I, phenol-water (100:30, v/v); direction II, n-butanol-propionic acid-water (142:71:100, v/v). Detection: autoradiography. Key to numbered components: 1, glycerophosphorylglycerophosphoryl glycerol; 2 and 5, glycerophosphoryl-N-monomethylethanolamine; 3, glycerophosphoryl ethanolamine; 4 and 6, glycerophosphoryl glycerol.*

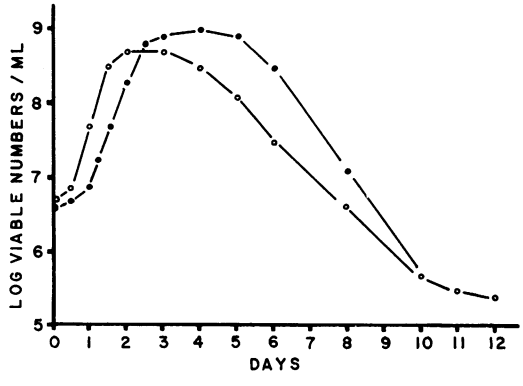

FIG. 3. *Growth curves of Thiobacillus thiooxidans in thiosulfate medium. Symbols: ○, 3.0 g of KH_2PO_4 per liter; ●, 0.1 g of KH_2PO_4 per liter, present in the medium.*

TABLE 1. *Phospholipids of Thiobacillus thiooxidans*[a]

Lipid	Percentage of total lipid phosphorus at culture age (days) of					
	1	1.5	2	3	5	8
Phosphatidyl glycerol	37	37	31	27	27	22
Diphosphatidyl glycerol	7	8	9	14	16	21
Phosphatidyl ethanolamine	20	14	8	4	4	3
Phosphatidyl-*N*-monomethyl-ethanolamine	36	41	52	55	53	54

[a] Data calculated from paper chromatograms of water soluble deacylation products.

there appeared to be a quantitative conversion of PG to DPG and PE to PME. This seemed to be a result of nutrient depletion and was not triggered by the accumulation of acid, since lowering the *p*H after 24 hr of growth did not alter the phospholipid pattern.

Quantitation of the phosphatides on thin-layer chromatograms yielded essentially the same results. The relative concentration of the lyso-forms increased slightly as the cultures aged. Other lyso-forms may have been present, but only at a level below the sensitivity of our methods.

Only minor quantities of phospholipid were found in the medium until the culture was in the later stages of the logarithmic growth phase. When the cells were grown in the presence of ^{32}P for 1 day, the total chloroform extract from 50 ml of spent medium contained only 2,000 counts/min. The total chloroform extract of a 2-day-old culture contained 35,000 counts/min; 3-day culture, 50,000 counts/min; 5- and 8-day cultures, 60,000 counts/min. The proportion of each phospholipid in the medium was about the same as in the cells. PME was in slightly higher quantities, and DPG was in considerably lower quantities. The relative concentration of the lyso-forms was greater in the medium of older cultures. The same general results were obtained regardless of the energy source supplied to the cells.

DISCUSSION

The phospholipid analysis reported here differs in two respects from that reported by Jones and Benson (14). First, the ratios of the phospholipids differ markedly. In the present experiments, the cells were labeled as growth occurred. Previously, the labeling was accomplished by inoculating large quantities of sulfur-grown cells into a thiosulfate medium devoid of unlabeled phosphate. Growth probably did not occur. Therefore, different labeling patterns would be expected. Second, the phospholipid identified as PC was in all probability PME. The diesters derived from these two lipids migrate almost as a single component in phenol-water and *n*-butanol-propionic acid-water and are easily confused when further information is not obtained. However, strain differences might account for the presence or absence of PME or PC. Although the strain used by Jones and Benson and the ones used in these experiments had the same origin (25), the cultures have been carried separately for some time.

Methylated forms of PE have been demonstrated in a number of bacteria (9, 10, 13, 15, 17, 20, 27), but the significance of their presence has not been elucidated. Hagen, Goldfine, and Le B. Williams (10) suggest that these phospholipids are associated with the elaboration of intracytoplasmic membranes. Ikawa (13) hypothesizes that PC plays a role in the development of highly efficient electron transfer systems.

Lyso-forms were noted in the phospholipid extracts, but it was not resolved whether they were part of the normal phospholipid composition or were artifacts resulting from either the cultural environment or the method of extraction. Increased proportions in older cells and media suggest that their formation is the result of the external environment.

The phospholipid content of the spent growth media increased as the cultures aged, but the proportion of each phospholipid was about the same as in the cells. Therefore, the phospholipids appeared to be released into the medium principally as cell decomposition products. This seems logical since the phospholipids are principally components of the membrane-cell wall complex (12, 15).

D. S. Herson (Bacteriol Proc., p. 108, 1967) recently reported the presence of PI in *T. thiooxidans*. We are unable to confirm this finding. PI is either absent in the organisms analyzed or is in insufficient quantities to be detected by our methods of analysis. In this regard, other methods of extraction (4, 21, 26) did not reveal any PI.

Nonphosphorus-containing lipids have been discovered in both cultures and are currently under investigation. These contaminate the phospholipids and are especially troublesome when interpreting thin-layer chromatograms of unlabeled and ^{14}C-labeled lipids. It is possible that one of these lipids acts as the sulfur "wetting agent."

Phospholipid analysis of the ATCC strain and the thiosulfate-utilizing isolate yielded comparable

results. This suggests that the new isolate is in fact a direct descendant of the ATCC strain. A more detailed description of the isolation and growth characteristics of the culture will be the subject of a future communication.

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LITERATURE CITED

1. AMERICAN PUBLIC HEALTH ASSOCIATION. 1965. Standard methods for the examination of water and waste-water, 12 ed. American Public Health Association, Inc., New York.
2. BENSON, A. A., AND B. MARUO. 1958. Plant phospholipids. I. Identification of the phosphatidyl glycerols. Biochim. Biophys. Acta **27**:189–195.
3. BENSON, A. A., AND E. H. STRICKLAND. 1960. Plant phospholipids. III. Identification of diphosphatidyl glycerol. Biochim. Biophys. Acta **41**:328–333.
4. BLIGH, E. G., AND W. J. DYER. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **27**:911–917.
5. BREMER, J., AND D. M. GREENBERG. 1959. Mono- and dimethylethanolamine isolated from rat-liver phospholipids. Biochim. Biophys. Acta **35**:287–288.
6. DAWSON, R. M. C. 1960. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. **75**:45–53.
7. DITTMER, J. C., AND R. L. LESTER. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**:126–127.
8. FOLCH, J., M. LEES, AND G. H. SLOANE STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497–509.
9. GOLDFINE, H., AND M. E. ELLIS. 1964. *N*-methyl groups in bacterial lipids. J. Bacteriol. **87**: 8–15.
10. HAGEN, P. P., H. GOLDFINE, AND P. J. LE B. WILLIAMS. 1966. Phospholipids of bacteria with extensive intracytoplasmic membranes. Science **151**:1543–1544.
11. HOUTSMULLER, U. M. T., AND L. L. M. VAN DEENEN. 1965. On the amino acid esters of phosphatidyl glycerol from bacteria. Biochim. Biophys. Acta **106**:564–576.
12. HUGHES, D. E. 1962. The bacterial cytoplasmic membrane. J. Gen. Microbiol. **29**:39–46.
13. IKAWA, M. 1967. Bacterial phosphatides and natural relationships. Bacteriol. Rev. **31**:54–64.
14. JONES, G. E., AND A. A. BENSON. 1965. Phosphatidyl glycerol in *Thiobacillus thiooxidans*. J. Bacteriol. **89**:260–261.
15. KATES, M. 1964. Bacterial lipids, p. 17–90. *In* R. Paoletti and D. Kritchevsky [ed.], Advances in lipid research, vol. 2. Academic Press, Inc., New York.
16. KATES, M., AND B. E. VOLCANI. 1966. Lipid components of diatoms. Biochim. Biophys. Acta **116**:264–278.
17. LASCELLES, J., AND J. F. SZILAGYI. 1965. Phospholipid synthesis by *Rhodopseudomonas spheroides* in relation to the formation of photosynthetic pigments. J. Gen. Microbiol. **38**:55–64.
18. LETTERS, R., AND B. BROWN. 1966. Phospholipids of yeast. I. Improvement in analysis by two-dimensional strip-transfer paper chromatography. Biochim. Biophys. Acta **116**:482–488.
19. MACFARLANE, M. G. 1961. Isolation of phosphatidylglycerol and a glycolipid from *Micrococcus lysodeikticus* cells. Biochem. J. **80**:45P.
20. PARK C., AND L. R. BERGER. 1967. Complex lipids of *Rhodomicrobium vannielii*. J. Bacteriol. **93**:221–229.
21. SCHAEFFER, W. I., AND W. W. UMBREIT. 1963. Phosphotidylinositol as a wetting agent in sulfur oxidation by *Thiobacillus thiooxidans*. J. Bacteriol. **85**:492–493.
22. SHAPIRO, D., AND Y. RABINSOHN. 1964. Synthesis of *N*-monomethyl- and *N*,*N*-dimethylcephalins. Biochemistry **3**:603–605.
23. TREVELYAN, W. E., D. P. PROCTER, AND J. S. HARRISON. 1950. Detection of sugars on paper chromatograms. Nature **166**:444–445.
24. VORBECK, M. L., AND G. V. MARINETTI. 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res. **6**:3–6.
25. WAKSMAN, S. A., AND J. S. JOFFE. 1922. Microorganisms concerned in the oxidation of sulfur in the soil. II. Thiobacillus thiooxidans, a new sulfur-oxidizing organism isolated from the soil. J. Bacteriol. **7**:239–256.
26. WELLS, M. A., AND J. C. DITTMER. 1965. The quantitative extraction and analysis of brain polyphosphoinositides. Biochemistry **4**:2459–2468.
27. WOOD, J. B., B. W. NICHOLS, AND A. T. JAMES. 1965. The lipids and fatty acid metabolism of photosynthetic bacteria. Biochim. Biophys. Acta **106**:261–273.