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Cycling of Dissolved Organic Phosphorus Compounds in Natural Waters

by

Herbert E. Allen
Thomas D. Brisbin
Kurt O. Thomsen

Pritzker Department of Environmental Engineering
Illinois Institute of Technology
Chicago, Illinois 60616

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UNIVERSITY OF ILLINOIS
WATER RESOURCES CENTER
2535 Hydrosystems Laboratory
Urbana, Illinois 61801

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FOREWORD

Phosphate has been found to be the key element in the eutrophication of natural waters. In the major manifestation of eutrophication, the development of algal blooms, it is only inorganic phosphate which is available to the organisms. Many bodies of water contain significantly more organic than inorganic phosphorus. Little is understood regarding the nature of the organic phosphorus, its biological availability or the means and rate of its reversion to inorganic phosphate.

This study was designed to determine the amount of inorganic phosphorus which could be released from the organic phosphorus by enzyme action. Enzyme, as opposed to chemical, breakdown permits a further characterization of the components of the organic phosphorus. The enzymatic analysis further mimics the breakdown or recycling which takes place in the environment. Therefore, this methodology provides an important tool for further study of phosphorus dynamics in aquatic ecosystems.

Glenn E. Stout,
Director
Water Resources Center

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INTRODUCTION

The key role of phosphorus in the process of eutrophication has been emphasized repeatedly. [1,2] Phosphorus is often the limiting nutrient for algae, whose growth into massive, malodorous blooms is a major manifestation of the eutrophication process. A relationship between phosphorus level and algal productivity has been demonstrated for many natural waters. The subsequent death and decay of the algae may deplete the dissolved oxygen level of lakes producing both changes in the biological species compostiion in the lake and degradation of the lake's value for recreational and municipal water supply purposes.

Present knowledge of phosphorus chemistry and its relationship to algal productivity is incomplete. Although an adequate supply of phosphorus is known to be necessary to support algal growth, the chemical forms of phosphorus which are directly available are not well elucidated and little is known regarding the remineralization of organic phosphorus compounds. Typically, natural waters contain three to four times as much phosphorus which is characterized as "soluble organic phosphorus" as is present as orthophosphate, or more precisely as "soluble reactive phosphorus."

Although orthophosphate is generally regarded as the phosphorus species utilized by organisms, its concentration may decrease to virtually nondetectable levels during the summer.

Berman [3] has shown that the enzyme alkaline phosphatase is induced by the biota during periods of low orthophosphate availability. Recent studies [4] have indicated that virtually none of the organic phosphorus present in samples which have been stored for approximately one hour after collection is capable of being hydrolyzed by the addition of alkaline phosphatase. However, 11 to 51 percent of the phosphorus was hydrolyzable by the enzyme phytase. It would appear the soluble organic phosphorus pool is composed of two distinct fractions -- one having a rapid turnover rate and possibly being hydrolyzable by alkaline phosphatase and the other which is hydrolyzed by phytase being a much more stable material with a long residence time.

This hypothesis would tend to be supported by other relevant studies. Lean [5] studied the phosphorus dynamics in lake water and ascertained that both a high-molecular weight material which was colloidal and a low-molecular weight material were produced by organisms. Mineralization of these components was not further studied. Other investigators [4,6,7] have also noted the organic phosphorus is composed of both high and low-molecular weight components. Approximately equal quantities of the high and the low molecular weight materials are hydrolyzable by phytase. [4] Because phytic acid is a cell wall constituent it may be hypothesized that the high molecular weight, colloidal material is composed of cell wall fragments. Phytase may act on only a portion of the phytic acid which is included in this fraction as a portion may be protected by protein or lipid components.

EXPERIMENTAL DESIGN

The experimental design in Table 1 was developed to determine if a portion of the soluble organic phosphorus in natural water could be enzymatically hydrolyzed by alkaline phosphatase or phytase and if the fraction of the soluble organic phosphorus hydrolyzable by those enzymes could be enhanced by pretreatment of samples with protease and/or lipase. This experiment was conducted to investigate if the organic phosphorus could be degraded to a form which releases orthophosphate or is susceptible to further degradation by either alkaline phosphatase or phytase.

Table 1 summarized the experimental design which included samples, blanks, controls and spikes for all the various incubations. From these data the amount of orthophosphate release from the organic phosphorus was determined. Blanks and controls were run concurrently to establish that neither spontaneous sample hydrolysis nor enzyme autodegradation were responsible for any observed orthophosphate release. Controls consisted of buffer and enzyme with distilled water used as a substitute for natural lake water. Blanks consisted of concentrated lake water, buffer and for the enzyme distilled water was substituted. An additional sample with an added "spike" of known enzyme substrate was also analyzed to ensure that the enzyme was active and that hydrolysis was completed within the incubation period. Control and blank orthophosphate levels were deducted from the orthophosphate release in the lake water sample in order to obtain corrected values for orthophosphate release by enzymatic hydrolysis.

TABLE 1

ENZYME ASSAY EXPERIMENTAL DESIGN

Sample	Concentrated Lake Water	Phytic Acid	Glycero Phosphate	Alkaline Phosphatase	Phytase	Protease	Lipase
1.	1				1		
2.					1		
3.		1			1		
4.	1	1			1		
5.	1			1			
6.				1			
7.			1	1			
8.	1		1	1			
9.	1					1	
10.	1				2	1	
11.	1			2		1	
12.	1					1	2
13.	1				3	1	2
14.	1			3		1	2
15.						1	
16.		1				1	
17.	1	1				1	
18.	1	1			2	1	
19.	1	1		2		1	
20.	1	1				2	1
21.	1	1			3	2	1
22.	1	1		3		2	1
23.	1						1
24.	1				2		1
25.	1			2			1
26.	1						1
27.		1					1
28.	1	1					1
29.	1	1			2		1
30.	1	1		2			1

1. Numbers indicate sequence of incubations.
2. All reaction volumes equal.

The nonspecific acid phosphatase termed "phytase" by the initial investigators [8] functions similary to any general phosphatase. Although the rates of substrate hydrolysis differ for the two enzymes, the primary distinction between the phytase and alkaline phosphatase used in the present study is the ability of phytase to hydrolyze phytic acid (inositol hexaphosphate), which alkaline phosphatase cannot. The difference between the amount of orthophosphate released by the two enzymes, therefore, affords an estimate of the level of phytic acid and "phytic acid-like" material in natural water samples. Phytase is of nonspecific nature; unknown compounds may be present in natural waters which are configurationally similar to phytic acid and are hydrolyzed by phytase but not alkaline phosphatase.

With the addition of protease and/or lipase prior to phytase it was hypothesized that the lake water would show an even greater increase in orthophosphate release. This increase may be due to protease and/or lipase hydrolysis products that are phytase hydrolyzable.

Sample Collection

To conduct this experiment it was necessary to obtain a sample with a high organic phosphorus to orthophosphate ratio. Samples were taken from various surface waters to find a desirable ratio. All samples were filtered through a 0.45 μ membrane filter. This ratio is seasonally as well as source dependent. Table 2 shows the locations and ratios for samples that were considered for the study. Lincoln Park Lagoon located north of Fullerton Avenue approximately one-half mile west of Lake Michigan in Chicago had the highest ratio. It proved to be unsuitable as a sample since after two days storage the Lincoln Park sample ratio decreased from 148 to 0.47. Therefore, this sample was too unstable to be used. The poor stability of the Lincoln Park sample may be attributed to the source of the organic phosphorus. Lincoln Park Zoo was located next to the lagoon and animal excrement is part of the runoff to the lagoon. This organic phosphorus did not have the qualities necessary for the desired enzymatic assays.

With a ratio of 29, Slough II was the next location to be further investigated. The organic phosphorus in the sample was stable for over six weeks when stored at 5°C. The ratio was favorable but the total organic phosphorus concentration of 29 μg/l necessitated concentration to permit the organic phosphorus in this sample to be used as a substrate for enzyme assays.

TABLE 2

PRELIMINARY SAMPLES

Sample	Location*	Total P (µg/l)	PO_4 (µg/l)	Org-P (µg/l)	Org-P/PO_4
1.	North Branch of the Chicago River (In Chicago: approx. 5100 N & 3800 W)	552.0	466.0	86.0	0.185
2.	Lincoln Park Lagoon (In Chicago: North of Fullerton Ave. & ½ mile West of Lake Michigan)	148.0	<1.0	148.0	148.000
3.	Slough I (In Spears Woods: Rt. 45 & 95th St.)	17.2	<1.0	17.2	17.200
4.	Slough II (In John J. Duffy Forest Preserve: Will-Cook Rd. & 131st St.)	29.2	<1.0	29.0	29.000
5.	Papose Lake (In White Oak Woods: Rt. 45 & 87th St.)	54.4	27.4	27.0	0.985
6.	Lake Tampier (In John J. Duffy Forest Preserve: along Rt. 45)	24.8	<1.0	24.0	24.000
7.	Golden Leaf Stables Pond (Parker & 131st St.)	27.6	<1.0	27.0	27.000

*All sampling locations were in the immediate Chicago area.

*Abbreviations: Total Soluble Phosphorus (Total P) Orthophosphate (PO_4) Organic Phosphorus (Org-P)

Sample Preparation

A means of concentrating the soluble organic phosphorus fraction was necessary. Several qualifications are essential for a suitable concentration procedure:

1. Losses of phosphorus should be minimal and nonspecific.
2. Mild conditions should be employed to minimize organic phosphorus alteration.
3. A concentration factor of at least one order of magnitude should be attainable.
4. A large sample volume should be easily concentrated.

The concentration procedure which most nearly satisfied the requirements outlined is freeze concentration, or "freezing-out". In this method the water sample is held slightly below freezing and is stirred rapidly. The ice that forms at the container wall is extremely pure; the solutes are excluded from the ice lattice and concentrated in the unfrozen central portion. Essentially complete recoveries of organic solutes from distilled water after concentrations up to 20-fold have been reported.[9,10] Increased dissolved solids concentrations have been reported by Baker to reduce the recovery efficiency; however, losses appear to be nonspecific.[11] Figure 1 shows how the freeze-concentration apparatus was constructed.

A sample from Slough II which had an organic phosphorus concentration of 175 µg/l and an organic to orthophosphate ratio of 4.55 was concentrated 4.73 fold. During concentration, contamination of orthophosphate reduced the ratio significantly, but it was still a suitable sample for use in the experiment. Having obtained a sample the experiment (Table 1) could be conducted.

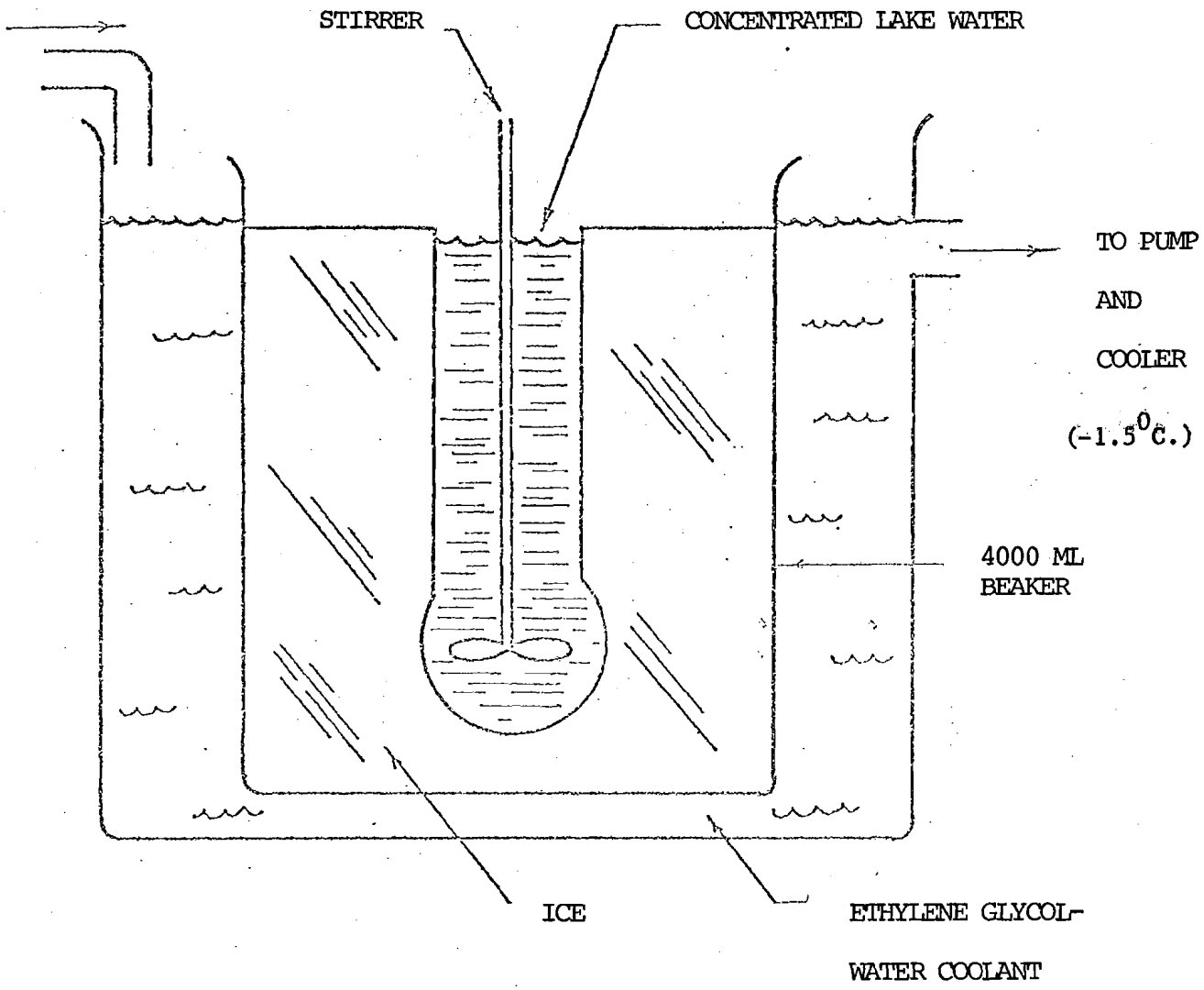

Figure 1. Freeze-concentration apparatus.

Methods and Materials

Triplicate samples were incubated at 20^0C in 125 ml erlenmeyer flasks. All samples and controls contained the appropriate enzyme activators and were adjusted to the pH of optimum enzymatic activity. All incubation times were two hours and after each enzyme incubation the enzyme present was inactivated by boiling prior to adding the next enzyme. Previous experiments conducted on the concentrated lake water have shown that boiling does not release orthophosphate from the soluble organic phosphorus. To insure complete enzyme inactivation, the last incubation for 11 all samples was terminated by trichloroacetic acid.

Aliquots were withdrawn from the incubated samples for determinating the amount of orthophosphate. The method of phosphate analysis used was the modified single solution ascorbic acid reduction method. [12] A Perkin - Elmer spectrophotometer Model 120 with a 10 cm cell was used.

Phytase (myoinositolhexaphosphate phosphohydrolase) was prepared according to the procedure of Nagai and Funahashi. [13] An outline of the isolation and purification procedure employed is presented in Table 3. The phytase solution thus prepared maintained a high level of activity for up to six months while frozen, and up to one month at 5^0C. Protease (Type I: Pancreatic), lipase (Type VII: Candida cylindracea) and alkaline phosphatase (Type I: Calf Intestinal Mucosa) were obtained from the Sigma Chemical Company, St. Louis, Missouri.

TABLE 3

PREPARATION OF PHYTASE FROM WHEAT BRAN*

1. 670 g. wheat bran extracted with 2 l H_2O overnight at 5°C.
2. Supernatant obtained by squeezing bran in two layers of cheesecloth and centrifuging for ten minutes at 5,000 rpm; precipitate discarded.
3. 30 g $(NH_4)_2SO_4$/100 ml supernatant added and stirred two hours at 5°C; centrifuged ten minutes at 5,000 rpm and precipitate discarded.
4. Supernatant saturated with $(NH_4)_2SO_4$ and stirred for 45 minutes at 5°C; centrifuged and supernatant discarded.
5. Precipitate dissolved in 700 ml cold distilled water and dialyzed overnight against cold running tap water.
6. 380 ml acetone added with stirring at 0°C and allowed to precipitate one hour at -20°C.
7. Mixture centrifuged and precipitate discarded; supernatant brought to 50 percent v/v acetone and held at 0°C for ten minutes with stirring.
8. Mixture centrifuged and supernatant discarded; precipitate dissolved in 170 ml distilled water and dialyzed overnight at 5°C against running tap water.

*Nagai and Funahashi (1962).

9. Solution brought to 75 percent v/v MeOH and held at -20°C for one hour; centrifuged.
10. Precipitate dissolved in 170 ml distilled water and dialyzed overnight at 5°C.
11. Dialyzate applied to 2 x 12 cm DEAE-cellulose column OH^- form, and eluted with 0.02 M Tris (pH 7.3); 10 ml fractions collected.
12. Phytase activity in fractions tested by incubation of aliquot with 15 mg PO_4/l phytic acid at 5.0 and analysis or orthophosphate released after one hour incubation at 20°C; tubes #17 - 25 combined (90 ml).
13. Phytase preparation frozen in 6 ml volumes and stored at -20°C until use.

RESULTS

Data from the enzymatic hydrolysis of concentrated lake water are presented in Table 4. All commercial preparations of lipase that were tested contained unacceptably high orthophosphate concentrations. Contamination in the samples containing lipase made it impossible to determine small enzymatic releases of phosphate.

Sample 1, containing concentrated lake water and phytase, released 88 µg/l of orthophosphate. Concentrated lake water and alkaline phosphatase in Sample 2 released 21 µg/l of orthophosphate. The results indicate that 67 µg/l or approximately 4 times as much organic phosphorus is phytase hydrolyzable than is alkaline phosphatase hydrolyzable. From Herbes [4] investigation it appears likely that in the two hour incubation period phytase and alkaline phosphatase would have extensively hydrolyzed all the same materials with the exception of phytic acid. The phytase would hydrolyze the same material as alkaline phosphatase and in addition would have hydrolyzed phytic acid. It would, therefore, appear that the 67 µg/l difference between phytase and alkaline phosphatase hydrolyzed phosphate was due to phytic acid in concentrated lake water.

For Sample 3 concentrated lake water was incubated with protease. A release of 58 µg/l orthophosphate was determined. Sample 4 was identical to Sample 3 except after the incubation with protease the sample was incubated with phytase. Sample 4 released 171 µg/l of orthophosphate. Of this 171 µg/l orthophosphate release, 58 µg/l can be attributed to protease hydrolysis

Table 4

DATA SUMMARY

Sample	PO_4 µg/l	TSP µg/l	Org-P µg/l	µg PO_4 released/l
Initial CLW	38.5	214.0	175.0	-
CLW & Phytase	-	-	-	88.1
CLW & Alk-P	-	-	-	20.5
CLW & Protease	-	-	-	58.4
CLW, Protease & Phytase	-	-	-	171.0
CLW, Protease & Alk-P	-	-	-	96.7

Abbreviations:

Concentrated lake water (CLW)

Alkaline Phosphatase (Alk-P)

Total Soluble Phosphorus (TSP)

and 88 µg/l to phytase hydrolysis. The additional 35 µg/l release could be an organic phosphorus form that has been converted to a phytase hydrolyzable form by the action of protease. The total orthophosphate that was released by protease plus phytase was approximately equal to the total initial 175 µg/l of organic phosphorus. From this data one can conclude that 98 percent of the organic phosphorus in this sample can be hydrolyzed by protease plus phytase.

Sample 5 contained concentrated lake water, protease and alkaline phosphatase. A 97 µg/l orthophosphate release was determined. Of this release, 58 µg/l of orthophosphate could be attributed to protease hydrolysis of organic phosphorus in concentrated lake water and 21 µg/l to alkaline phosphatase hydrolysis. The remaining 18 µg/l could have resulted from alkaline phosphate hydrolysis of an organic phosphate that was converted to a phytase reactive form by protease hydrolysis. Data from Sample 5 indicates that 55 percent of the organic phosphorus in the concentrated lake water is protease plus alkaline phosphatase hydrolyzable.

CONCLUSIONS AND RECOMMENDATIONS

It was determined that 88 µg/l of the initial total 175 µg/l organic phosphorus in the concentrated lake water sample was phytase hydrolyzable. It was also determined that 21 µg/l of the organic phosphorus was alkaline phosphatase hydrolyzable. The difference of 67 µg/l between phytase and alkaline phosphatase hydrolyzable material is due to phytic acid present in the concentrated lake water. The 67 µg/l of phytic acid represents 38 percent of the total organic phosphorus in the concentrated lake water. Protease released 58 µg/l orthophosphate from the organic phosphorus in the concentrated lake water. This release indicates that 38 percent of the organic phosphorus is protease hydrolyzable.

It is believed that the orthophosphate release in concentrated lake water by protease needs further investigation in order to confirm the experimental results. Three possible explanations for the orthophosphate release by protease which should be further investigated are: (1) contamination of the commercial protease extract with phosphate hydrolyzing enzymes, (2) the release of polyphosphates from "metachromatic bodies" or "volutin granules"[14] that have been protease hydrolyzed, and (3) the effect of trichloroacetic acid extraction of polyphosphates from protease hydrolyzed and non-protease hydrolyzed materials in the concentrated lake water.

Concentrated lake water that was incubated with protease, prior to phytase incubation, released 171 µg/l orthophosphate from the organic phosphorus. This orthophosphate release represents 98 percent

of the total organic phosphorus material present in the concentrated lake water. By subtracting the release due to separate incubations of protease and phytase, an additional release of 35 µg/l orthophosphate was found when the sample was incubated with protease prior to incubation with phytase.

Concentrated lake water that was incubated with protease prior to incubation with alkaline phosphatase released 97 µg/l orthophosphate from the organic phosphorus. By subtracting the release due to separate incubations with protease and alkaline phosphatase, an additional release of 18 µg/l orthophosphate was determined to have taken place when the sample was incubated by protease prior to alkaline phosphatase.

The difference between the additional release by protease followed by phytase (35 µg/l) and protease followed by alkaline phosphatase (18 µg/l) is 17 µg/l. This difference of 17 µg/l is phytic acid or phytic acid-like material.

These results indicate that organic phosphorus can be characterized by enzymatic assays. The enzymatic procedure determines both the phytase available and the initially phytase unavailable phytic acid component of the organic phosphorus pool. This methodology should permit better definitions of the components in both the rapidly and the slowing cycling organic phosphorus.

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