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I am submitting herewith a thesis written by Samuel Winton Hixson entitled "Application of High Performance Liquid Chromatography to the Study of Dissolved Organic Phosphorus Compounds Released by Algae." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Environmental Engineering.

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APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE STUDY OF DISSOLVED ORGANIC PHOSPHORUS COMPOUNDS RELEASED BY ALGAE

A Thesis

Presented for the

Master of Science

Degree

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Samuel Winton Hixson

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ABSTRACT

The development of an analytical technique utilizing high performance liquid chromatography (HPLC) for separating the inositol phosphates was studied. Commercial inositol hexaphosphate (IHP) and inositol monophosphate (IMP) were adequately separated from each other on Aminex A-27 resin with ammonium bicarbonate as the eluent. A breakdown of the organic bound phosphorus to orthophosphate occurred while on the HPLC column. This breakdown procedure could be speculated to be due to an alkaline hydrolysis process. Chromatograms of solutions initially containing only IHP yielded four peaks which appear to be best ascribed to residual IHP and the intermediate inositol phosphates containing 5, 4, and 2 phosphate groups per molecule. IMP clearly was not an intermediate of IHP hydrolysis on the LC column.

Dissolved organic phosphorus compounds released by the culture Chlamydomonas reinhardtii were subjected to preliminary separation on Sephadex G-25. Sephadex G-25 fraction, which was equal to the inositol phosphate elution position, was chromatographed on the HPLC and resulted in only the orthophosphate peak being identified. This HPLC chromatogram showed that a preliminary clean-up procedure was needed prior to HPLC separation. Alkaline bromination was chosen as the clean-up procedure since all the non-inositol phosphates would be oxidized. Samples from the culture study were subjected to alkaline bromination and then chromatographed on Sephadex G-25 before being placed on the

HPLC. The results of the HPLC showed three major peaks which did not match any of the inositol phosphate standards. Alkaline bromination followed by re-chromatographing the Sephadex fractions resulted in one peak at the elution position of IMP, thus providing marginal evidence for the presence of this compound in the culture solution.

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CHAPTER I

INTRODUCTION

The significance of phosphorus in the aquatic environment has been well documented. A positive relationship existing between the rate of input of phosphorus and algal productivity has been shown by Vollenweider (1970). The majority of phosphorus input into lakes is due to sediment transport of soil from agricultural areas and from the effluents of municipal and industrial wastes. Consequently, an excess of phosphorus into the aquatic environment system results in an increase in algal blooms and aquatic weed growth. Increasing frequency of algal productivity may cause the introduction of unpleasant tastes and odors into the drinking water supply and may also cause unsightly deterioration of the recreational usefulness of the affected waters. Therefore, emphasis has been placed on the role of phosphorus in the control of eutrophication.

Utilization of phosphorous as a building block for biological activity by algae, bacteria, and other aquatic organisms has been demonstrated. Orthophosphate functions as the primary phosphorus species used by the aquatic organisms. Through metabolic activity or cell death dissolved organophosphorus (DOP) compounds are released to the aquatic environment. The release of the DOP species establishes the cyclic nature of phosphorus in the natural aquatic system. In

focusing on this cycle or possible pathway of phosphorus, the complexity of the natural aquatic system must be realized. Due to the lack of data concerning the chemical identity of the DOP components, little is known about the function of the DOP compounds. The only organic phosphorus compound which has been identified in natural water samples is DNA (Minear, 1971). Minear (1971) found that generally 15 to 30 percent of the total phosphorus, and at times as much as 90 percent, of the total soluble phosphorus may be in the DOP form. Additional information concerning the identity of the low molecular weight component of naturally occurring dissolved organic phosphorus compounds would lead to a better understanding of the phosphorus pathway in the aquatic environment.

Understanding the function and identity of the DOP compounds is a prerequisite for accurate prediction and ultimate control of the eutrophication process. Over 50 percent of the total phosphorus content in the effluent of advance wastewater treatment plants, utilizing chemical coagulation, is DOP compounds (Herbes, 1974). Since chemical coagulation is more efficient in removal of orthophosphate than organic phosphorus (Herbes, 1974), a thorough understanding of the chemical composition and role of the DOP compounds is needed.

Prior soil chemistry investigations (Dyer et al, 1940; Yoshida, 1940) have linked the organic phosphorus content of the soil to a species of the inositol phosphates, inositol hexaphosphate. Recent research has shown that inositol phosphates are present in lake sediments (Weimer, 1973). Consequently, this implies the possibility that

the inositol phosphates may exist in the natural aquatic environment. Herbes (1974), using enzymatic hydrolysis, characterized the soluble organic phosphorus fraction of 12 concentrated lake water samples as naturally occurring substrates which were identical to those of inositol phosphates. Walanski (1974) investigated the presence of inositol phosphates in laboratory cultures of Chlorella pyrenoidosa and showed strong evidence of the presence of inositol monophosphate and inositol diphosphate. McEntyre (1976) utilized thin layer chromatography in characterizing the DOP compounds released by laboratory cultures of Clamydomanas reinhardtii. He presented evidence that the DOP compounds released by the algal cultures had similar chromatographic characteristics to inositol monophosphate.

The objectives of this research investigation were to develop an analytical technique utilizing high performance liquid chromatography (HPLC) for separating the inositol phosphates and to apply the developed experimental technique to aid in characterizing the DOP compounds released by an algal culture. The experimental phase of this investigation was divided into three phases. The first phase involved the development of the analytical technique utilizing HPLC. The second phase involved reviewing and adapting previous separation techniques developed by other researchers (Minear, 1971; Walanski, 1974) for use as preliminary separation steps. The third and final phase involved applying the experimental techniques investigated in the previous two phases to aid in the characteration of the DOP compounds release by a laboratory algal culture.

CHAPTER II

LITERATURE REVIEW

Aquatic Phosphorus Forms

In the natural aquatic environment, phosphorus exists essentially in the pentavalent form as phosphate. Phosphorus is defined by a theoretical classification scheme based on the various physical and chemical forms of phosphorus occurring in the aquatic environment. The chemical forms of the phosphorus are commonly classified as orthophosphates, condensed phosphates, and organic phosphates. Physical forms of phosphorus are defined as dissolved phosphates and particulate phosphates. Dissolved phosphates are operationally defined as the phosphates that will pass through a 0.45 micron membrane filter; where as, the particulate phosphates will not (Standard Methods, APHA, 1971; Strickland and Parsons, 1968). The 0.45 micron membrane filter, which is designed to make gross separation of the particulate forms of phosphate from the dissolved forms, serves as convenient and reproducible analytical technique. In reality, a 0.45 micron membrane filter allows some colloidal particles to pass through while retaining other colloidal particles. Rigler (1964) reported a significant difference in the percent soluble organic phosphorus content of identical samples filtered with a membrane of 0.45 and 0.10 micron pore size.

Operationally, orthophosphate is defined as the phosphorus which reacts directly with the molybdate during analysis. Condensed phosphates are the pyro, meta, and poly phosphates which are operationally

defined as the phosphorus which is acid hydrolyzable. Organic-bound phosphorus is operationally defined as the phosphorus which is released by oxidation. The chemical-physical theoretical classification scheme discussed here is the commonly accepted classification scheme used for the various forms of phosphorus and is shown in Table 1.

In the natural aquatic environment, an interrelationship exists between the various forms of phosphorus. This interrelationship between the dissolved phosphorus forms and the particulate phosphorus compartments occur as complex functions of chemical, physical, and biological parameters and is often referred to as the phosphorus cycle. Figure 1 is a schematic representation of the phosphorus cycle. This interrelationship or cycle between the various phosphorus forms can be considered as a dynamic, steady-state system. Interchange of phosphorus between the various compartments of the cycle can be quite rapid (Rigler, 1964). Although the inorganic forms of the phosphorus cycle are known, the chemical identity of the dissolved organic phosphorus (DOP) compounds of the phosphorus cycle are essentially unknown.

Utilization and Release of DOP by Algae

Phosphorus is classified as a macronutrient since it is utilized as an essential constituent for algal metabolism. Various atomic ratios of Carbon:Nitrogen:Phosphorus (C:N:P) present in algal cells have been reported in the literature (Sawyer, 1973). The ratio of 100C:16N:1P is accepted as the assumed atomic ratio. Rigler (1964) has shown that in the natural aquatic environment an organic:inorganic phosphorus ratio exists. This ratio follows an annual cycle with the organic:inorganic

Table 1. Comparison of Conceptual and Operational Differentiation of the Physical and Chemical Forms of Phosphorus in Natural Waters (McEntyre, 1976)

Conceptual	Operational
Physical	
Dissolved	That which passes through a 0.45μ membrane filter.
Particulate	That which is retained by a 0.45μ membrane filter.
Chemical	
Orthophosphate	That which reacts directly with the molybdate reagent.
Condensed phosphate	Total orthophosphate in sample after acid hydrolysis minus the phosphate measured as orthophosphate.
Organic phosphate	Total orthophosphate in sample after oxidation and hydrolysis minus (orthophosphate + condensed phosphate)

(McEntyre, 1976).

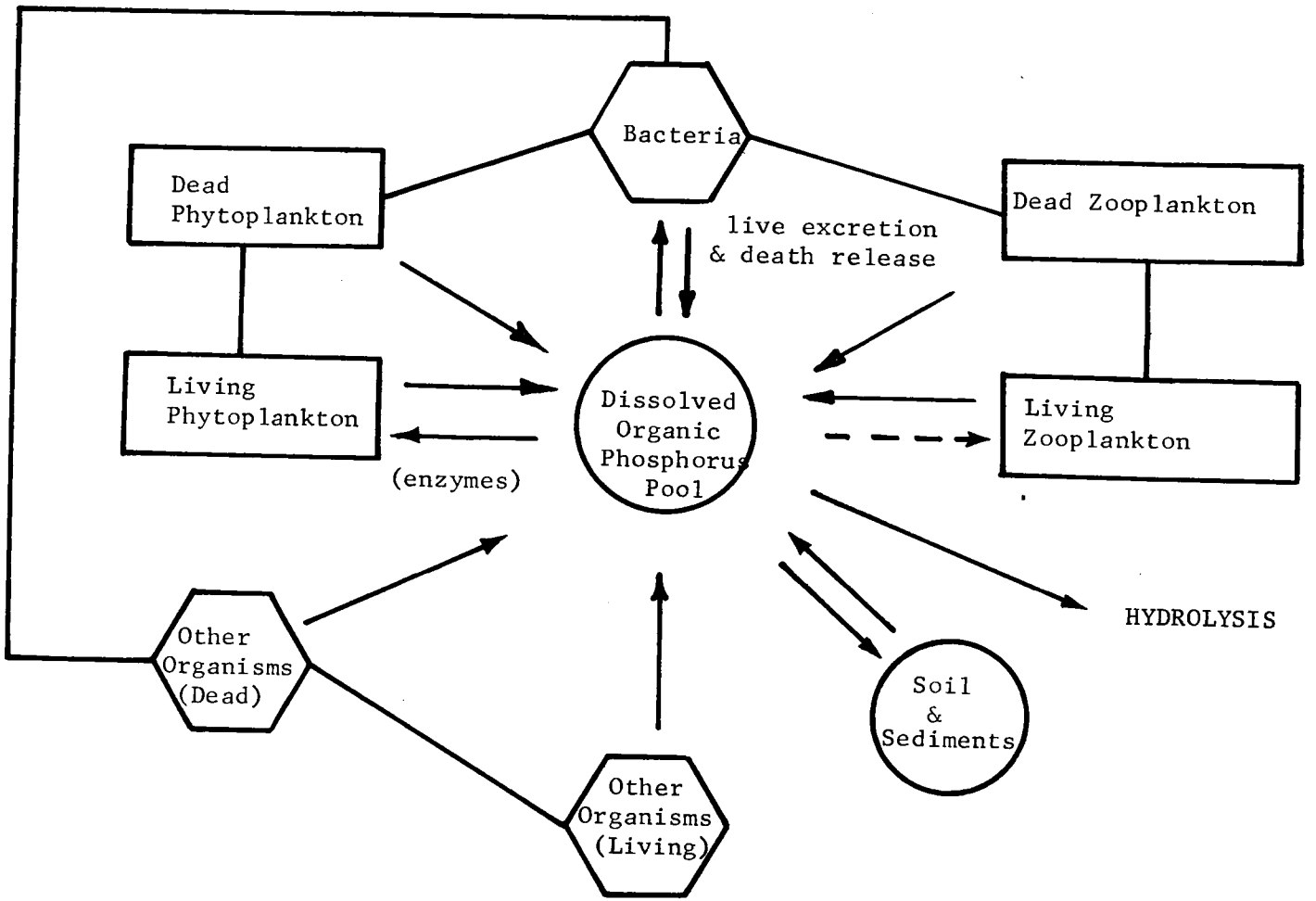


Figure 1. Summary of various possible input and output mechanisms for the dissolved organic phosphorus pool in natural waters (Minear, 1971).

phosphorus ratio the highest during the summer months. Other researchers (Newell, 1962; Rockford, 1963; Strickland and Austin, 1960) have found similar organic:inorganic phosphorus ratios following an annual cycle. Since the DOP constituent in the natural aquatic environment often exceeds the orthophosphate constituent by several orders of magnitude, the possibility of algae utilizing the DOP constituent as a source of phosphorus occurs.

The release of DOP compounds by algae is well documented (Kuenzler, 1969 and 1970; Minear, 1971; Lean, 1973; Herbes, 1974). Minear (1971) showed the presence of DNA or DNA fragments in pure algal cultures and lake water samples and presented evidence that death and cellular rupture is a mechanism of DOP release from algae. In summary, DOP is known to constitute an appreciable fraction of the total phosphorus in the aquatic environment and has been reported as an appreciable fraction of the total phosphorus released by algae during cellular rupture and metabolic excretion (Lean, 1973; Kuenzler, 1970). Phosphorus deficient algae in natural aquatic environments have shown the ability to utilize certain DOP compounds such as glycerophosphate and phytic acid (Chu, 1946). Natural occurring bacteria has also shown the ability to degrade certain DOP compounds through a formation of phosphatase enzymes which hydrolyze the DOP compounds (Chu, 1946).

Characterization of DOP Compounds

Watt and Hayes (1963) obtained P^{32} labeled DOP by introducing P^{32} inorganic phosphate into sea water containing a common seaweed

(Fucus vesiculusus). The P^{32} labeled DOP produced by the exchange of P^{32} inorganic phosphorus was subject to paper chromatography. Six paper chromatographic fractions were produced using unidimensional descending paper chromatography and detected by autoradiographic scanning. Kuenzler (1969) obtained at least eight different compounds through two-dimensional paper chromatography. Comparison of these compounds' chromatograms with known phosphate ester chromatograms led to no identification of the unknown compounds. Minear (1971, 1972) identified DNA from pure algal cultures using specific chemical and enzymatic procedures. By measuring the ultraviolet absorption spectra of ion exchange chromatography fractions, Koenings and Hooper (1972) characterized the DOP fractions in a northern Michigan bog lake as a mixture of nucleic acids. Goterman (1973) extracted the DOP fraction from a highly colored lake in Holland and proposed that this fraction was bound to humic materials. Krause and Stegmann (1968) concentrated the DOP fraction of lake water and then employed ion exchange and thin layer chromatography to separate the DOP fractions. Five paper chromatographic fractions were produced. Two of the fractions were identified as phosphate esters (sugar phosphates) by comparison with known sugar phosphate compounds and one of the fractions as orthophosphate. The remaining two fractions were unidentified.

Herbes (1974) established strong evidence for the presence of inositol hexaphosphate (IHP) in natural waters by utilizing enzyme hydrolysis. Weimer (1973) and Sommers (1972) presented evidence that inositol phosphates were present in Wisconsin lake sediment. Using

pure laboratory algal cultures, Walanski (1974) presented strong evidence that algal cultures were a potential source of inositol monophosphate (IMP) and inositol diphosphate (IDP). Thin-layer chromatography work by McEntyre (1976) of pure algal cultures supports previous work by Walanski (1974), that is, the lower molecular weight compounds of the DOP fraction were the lower molecular weight inositol phosphates.

Review of Inositol Phosphates

Inositol is the common name for Hexahydroxycyclohexane and has an empirical formula of $C_6H_{12}O_6$ with a molecular weight of 180.16. Several isomers of inositol are known to exist. The chemical structure of the predominant isomer of natural occurring inositol is myo-inositol and is shown in Figure 2. Inositol phosphate esters are formed by the replacement of any of the six available hydroxy groups on the inositol molecule with orthophosphate anions. Since there are six available hydroxy groups per inositol molecule, there are then six possible inositol phosphates: inositol mono, di, tri, tetra, penta, and hexaphosphate. The abbreviations IMP, IDP, ITriP, ITetP, IPP, IHP will be used in this manuscript.

Phosphorus constituents present in soils including inositol phosphates have been the subject of considerable research. Youshida (1940) isolated an organic phosphorus compound which could be linked to IMP. Dyer et al. (1940) utilized an alkaline bromination procedure to separate IHP from other organic phosphorus compounds and then subjected the IHP to precipitation with ferric chloride. The phosphorus to iron ratios were then compared to that of standard inositol

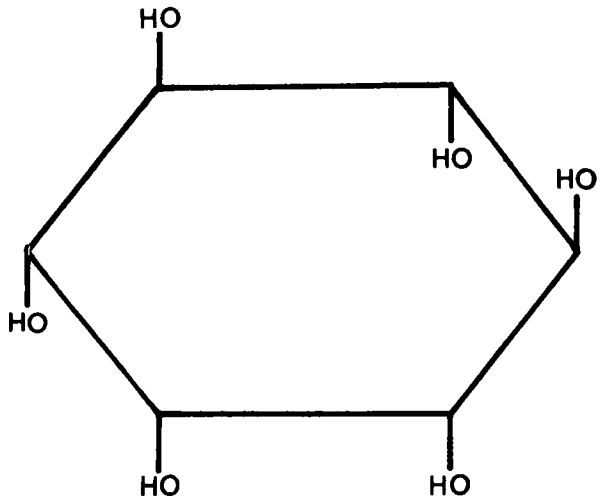


Figure 2. Structure of myo-Inositol (Weimer, 1973).

hexaphosphates. Other researchers (Wrenshall and Dyer, 1941; Brower, 1945; Smith and Clark, 1952; Cosgrove, 1962; Moyer and Thomas, 1970) have utilized various techniques to separate and identify soil inositol phosphates. An extensive review of separation and identification procedures are given by Minear and Walanski (1974) and are summarized in Table 2.

Review of the information concerning the exchange of organic phosphorus in lake sediments with the overlying waters is limited. Lee et al. (1971) found that the adenosine triphosphate (ATP) content in nine Wisconsin lakes to range from 0.34 to 9.5 μg ATP per gram of sediment. Using the ATP content as an approximate indicator of living biomass, the concentration of ATP per gram of sediment indicates that 1 to 21 percent of the sediment organic phosphorus may be associated with living organisms. A smaller percentage of sediment organic carbon, nitrogen, and sulfur was associated with the ATP content. This indicates that in lakes, particularly eutrophic lakes, some sediment organic phosphorus compounds have a higher potential of turnover between the lake sediment and the water above through living organisms. Other specific organic phosphorus compounds that have been found in sediments include IHP and IPP in concentrations ranging from 16 to 34 μg per gram of sediment in four Wisconsin lakes (Sommers et al., 1972). In a study of two Wisconsin lakes, Weimer (1973) measured the quantities of inositol phosphates in the lake sediments and soils of the surrounding watersheds. He found that the inositol phosphates comprised 12 to 63 percent of total sediment organic phosphorus in contrast to soil samples in

Table 2. Summary of Research on Soil Inositol Phosphates (Minear and Walanski, 1974)

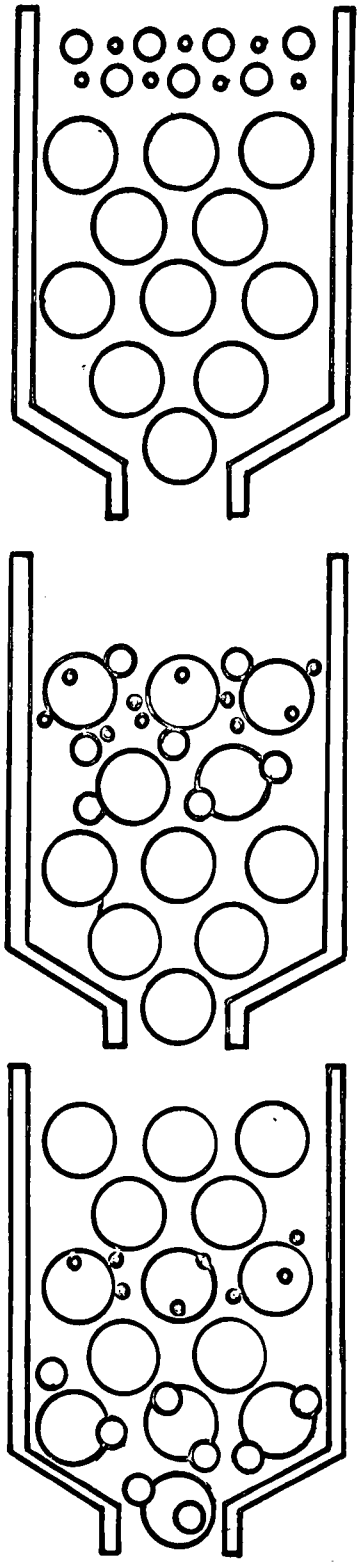
Year	Nature of Investigation	Reference
1914	IMP extracted from wheat bran, purified and identified by elemental analysis.	Anderson[21]
1915	ITriP extracted from wheat bran, purified and identified by elemental analysis.	Anderson[22]
1940	Isolation of inositol from Hawaiian soils and link suggested to organic phosphorus content of soil as IMP.	Yoshida[23]
1940	Verification of IHP resistance to alkaline bromination procedure to isolate IHP from other organics and precipitation with iron. Fe/P ratios and Fischler-Kurten test used to assign identity.	Dyer, et al[24]
1941	Verification of IHP resistance to alkaline bromination. Isolated IHP from soil and studied enzymatic attack.	Wrenshall and Dyer[25]
1945	Investigation of IHP and lower phosphates in two soil groups. Found 50% of total soil P, comprised of these compounds except no IMP found. IHP was 75% of the total inositol phosphates.	Brower[26]
1952	Development of anion exchange technique for separation of inositol phosphates. Lower phosphates produced from IHP by enzymatic breakdown.	Smith and Clark[27]
1958	Specific and quantitative anion exchange and technique for IHP. Improvement on procedure of Smith and Clark and used on soil.	Caldwell and Black[28]
1962	Separation of soil inositol phosphates with anion exchange after alkaline bromination. Seven components obtained, and assigned identities as lower phosphates, myo-IHP and scyllo-IHP by paper chromatography.	Cosgrove[29]
1970	Chromatographed isolated soil organic phosphorus on Sephadex G-75 gel. Found low and high molecular weight inositol phosphates and hypothesized presence of polymeric forms or binding to high molecular weight materials.	Moyer and Thomas[30]

which they constituted 32 to 91 percent of the total soil organic phosphorus. Using pure algal laboratory cultures extracts, Weimer (1973) found IHP to constitute 3 to 9 percent of the total phosphorus.

Separation and Identification Procedures

Gel chromatography has been utilized by several researchers (Minear, 1971, 1972; Lean, 1973; Walanski, 1974; Herbes, 1974) as a separation technique for analysis involving inositol phosphates in pure algal cultures and in natural water samples. Gel chromatography is a chromatographic separation based on exclusion due to molecular size. There are several types of commercial gels available ranging from polystyrene to biological dextran gels. Sephadex is the trade name for a series of polydextran gels which are produced by the chemical reaction between ephichlorohydrin and a carbonhydrate polymer known as dextran. Various commercial gels are produced as dry beads of closely controlled size by Pharmacia Fine Chemicals, Uppsala, Sweden, and are sold under the trade name of Sephadex.

Figure 3 illustrates the theory employed in exclusion chromatography as a schematic representation of the separation of two different size molecules on a dextran gel column. Altgelt (1968) and Determann (1968, 1969) provided an extensive review of gel chromatography separation mechanisms. The elution behavior of the solute molecules can be described in several parameters. Table 3 lists the various parameters describing the elution behavior in gel chromatography. In this study, the term elution profile refers to a plot of



(a) Sample at top of column, large dots represent large molecules, the small dots are small molecules and the open circles are gel particles.

(b) As the solution passes down the column, the large, excluded molecules pass around the gel particles while the small particles equilibrate between the solvent inside the gel and outside the gel.

(c) The two components are separated as they come off the column.

Figure 3. Schematic representation of three phases of gel chromatography (Minear, 1972).

Table 3. Parameters for the Description of the Elution Behavior in Gel Chromatography (Determann, 1968)

Parameter	Calculation	Remarks
V_e	direct measurement	only the results obtained on the same gel bed can be compared
$\frac{V_e}{V_t}$	division of the elution volume (V_e) by the total volume of the gel bed (V_t)	independent of the geometry of the column; sensitive to differences in packing density; greatest accuracy for small V_e (large molecules)
$\frac{V_e}{V_o}$	division of the elution volume (V_e) by the elution volume of an excluded substance (V_o)	
K_d	$K_d = (V_e - V_o)/V_t$	independent of the geometry and packing density of the column; uncertainty in the determination of V_i; greatest accuracy for large V_e (small molecules)
	V_i (volume inside of the gel grains); dependent on the amount of dry gel and its solvent regain upon swelling (S_t)	
K_{av}	$K_{av} = \frac{V_e - V_o}{V_t - V_o}$	independent of the geometry and packing density of the column; all columns are easily measured; greatest accuracy for large V_e (small molecules)

solute concentration parameter as a function of Ve/V which has been designated as R in this study.

Ion Exchange

Inositol phosphates have been separated from other phosphorus compounds in the soil by anion-exchange chromatography as early as 1951 by Smith and Clark (1951). Caldwell and Black (1958) simplified the original method developed by Smith and Clark (1951) by removing all phosphates other than inositol phosphates as a single fraction. The extract of the soil was loaded on a De-Acidite weak anion exchange resin and then eluted from the column with three separate increasing concentrations of HCl solutions. Cosgrove (1962) used a Dowex AGI-X8 anion exchange resin with a gradient elution of HCl to separate the inositol phosphates. Weimer (1973) isolated specific groups of inositol phosphates on a Bio-Rad AGI-X2, strong-base anion exchange resin. The majority of the research involving anion exchange resins were developed for soil analysis. Herbes (1974) attempted to utilize the anion-exchange properties of zirconium oxide to remove orthophosphate from lake water but was unable to successfully remove the naturally occurring levels of orthophosphate from the lake water.

CHAPTER III

EXPERIMENTAL DESIGN

Algal Culture

The algal species used in the culture study was Chlamydomonas reinhardtii. This particular species was selected since considerable research work with this species has showed that DOP are produced and released into the culture media with time (Minear, 1971; McEntyre, 1976). The algal species was grown in a culture apparatus which is shown in Figure 4. The culture media chosen for this study is given in Table 4.

The culture media was prepared and placed in the culture flask, 250 ml of culture media, in a one liter Erlenmeyer flask. All nutrients except the trace metal nutrients were autoclaved with the entire culture apparatus at 15 psig for one-half hour. The trace metal nutrients (which had been filter sterilized through a 0.45 micron membrane filter) were added to the culture media after the media had cooled to room temperature. The culture media was then innoculated from stock cultures of Chlamydomonas reinhardtii.

Isolation of Soluble Culture Material

During the growth of the culture, all samples were withdrawn by means of a sterile syringe through a septum cap and then centrifuged at 1000 rpm for 30 minutes. The supernatant was then filtered through a 0.45 micron membrane filter under a low vacuum of 2.0-3.0 psig. The

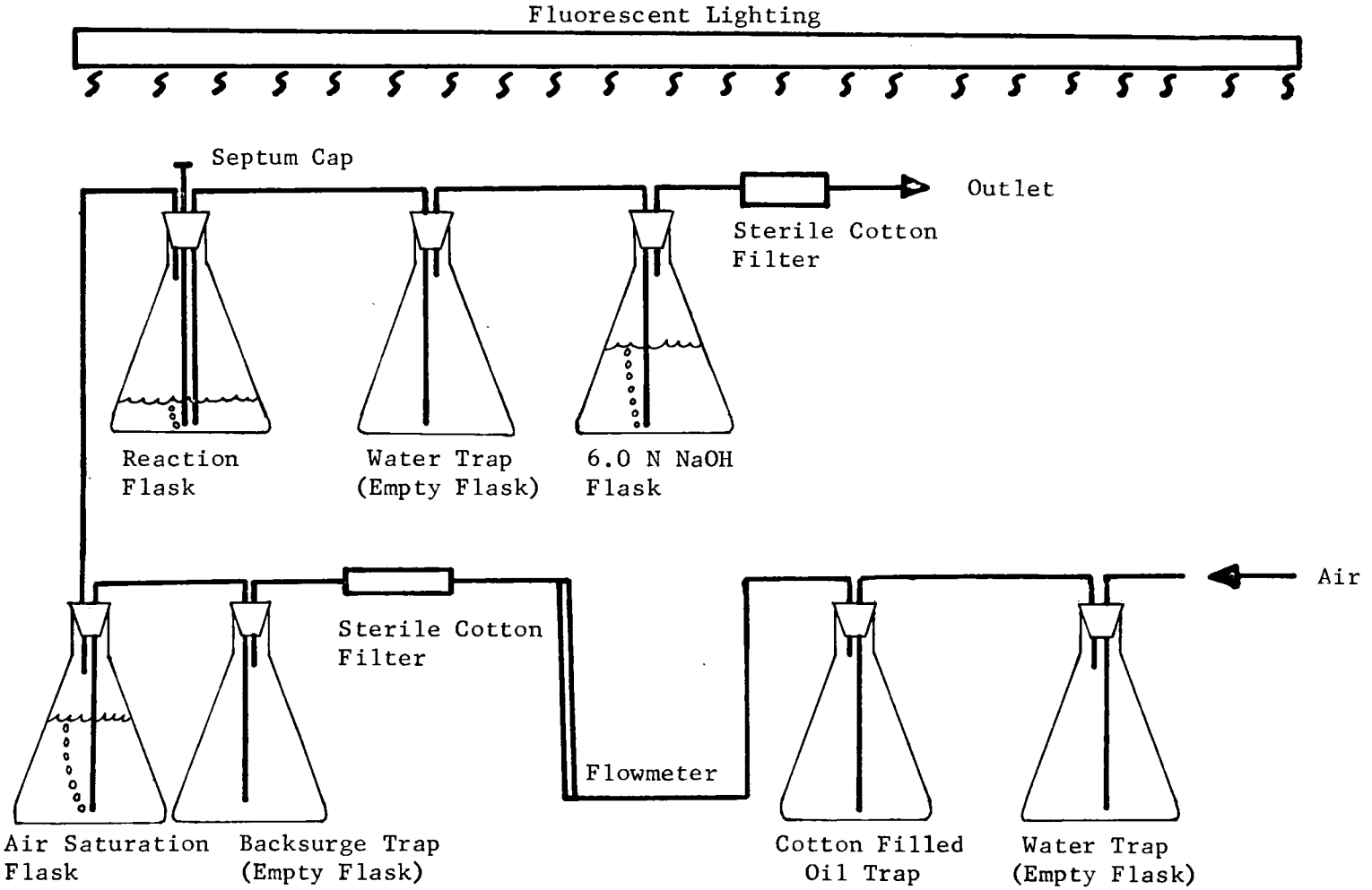


Figure 4. Schematic of the algal culture growth apparatus.

Table 4. Basic Growth Medium Used for the Algal Cultures (Minear, 1971)

Master Solutions (MS)					
Solution	Salt	Concentration		ml MS per liter	Final Solution Conc., mM
		g/l	Molar.		
A	$NaNO_3$	8.5	0.1	5.0	0.5
B	$MgSO_4 7H_2O$	24.65	0.1	1.0	0.1
C	KH_2PO_4	13.6	0.1	1.12	0.112
	NaOH	3.2	0.035		0.039
D	NaCl	5.84	0.1	30.0	3.0
K	$FeSO_4.7H_2O$	6.2	0.0224	1.0	0.0224
	Na_2 EDTA	6.5	0.0224		0.0224
	H_3BO_3	1.854	0.03		0.03
	$ZnSO_4.7H_2O$	0.287	0.001		0.001
	$MnCl_2.9H_2O$	1.98	0.01		0.01
	$Na_2MoO_4.2H_2O$	0.242	0.001		0.001
	$CuSO_4.5H_2O$	0.0499	0.0002		0.0002
	$Co(NO_3)_2.6H_2O$	0.291	0.0001		0.0001

filtrate was analyzed or used immediately, if not the filtrate was stored in a closed glass container at 5°C until used.

Phosphorus Analyses

Orthophosphate was determined by the colorimetric ascorbic acid method of Murphey and Riley (1962) as outlined by Strickland and Parsons (1968). Total dissolved phosphorus was determined as orthophosphate present after the persulfate oxidation method of Menzel and Corwin (1965). Organic phosphorus was measured as the difference between the total phosphorus and orthophosphate values. Samples were read at 885 nm on a Beckman Model B spectrophotometer with a one centimeter light path. All glassware used during this study was washed with 6.0 N HCl as described by Standard Methods (APHA, 1971).

Gel Chromatography Studies

Sephadex G-25 (medium) gel and a Pharmacia Fine Chemicals K 26/100 column were used in this study. The Sephadex gel was swollen in 0.05 N NH_4HCO_3 for 24 hours and decanted to remove fines prior to pouring the column. The K 26/100 column with A-26 adjustable length upward flow adaptors was aligned vertically with a level prior to pouring the column. The decanted gel was poured into the column as a single operation to prevent layering. Compaction of the gel was prevented during the pouring operations by reducing the flow rate and head pressure.

Column dimensions after filling were 2.5 X 82 cm. The column was maintained in a constant temperature room at 20°C $\pm$ 1°C. Throughout this study, the column was operated in an upward flow condition

with 0.05 N NH_4HCO_3 as the eluent. The flow rate through the column was maintained at approximately 0.50 ml/min by utilizing a two liter reservoir with constant head stand pipe (Mariotte's principle). A six milliliter sample was applied to the column through a three-way valve system and four milliliter fractions were collected from the column on an automatic fraction collector. Figure 5 shows a schematic of the Sephadex column system.

The exclusion volume (V_o) and uniformity of the gel packing were determined with Blue Dextran 2000 (BD-2000), a dyed dextran of molecular weight in excess of 2×10^6.

Alkaline Bromination

The alkaline bromination procedure used in this study was a method which previous researchers (Dyer et al., 1940; Wrenshall and Dyer, 1941; Bower, 1945; Cosgrove, 1962; Walanski, 1974) had shown as a procedure for destroying extraneous organic matter while leaving the inositol phosphates relatively unaffected. The alkaline bromination procedure used was similar to the method used by Minear and Walanski (1974) with minor modification due to the samples being radioactive. To a sample volume (30 to 60 ml), 10 ml of 5.0 N NaOH and 10 ml of saturated bromine water were added. The mixture was then boiled for one hour in a 250 ml erlenmeyer flask with groundglass joints connected to a 300 mm water jacket condenser with a plug of cotton at the top to trap any aerosols. After cooling, the solution was neutralized by dropwise addition of 6.0 N HCl. The neutralized

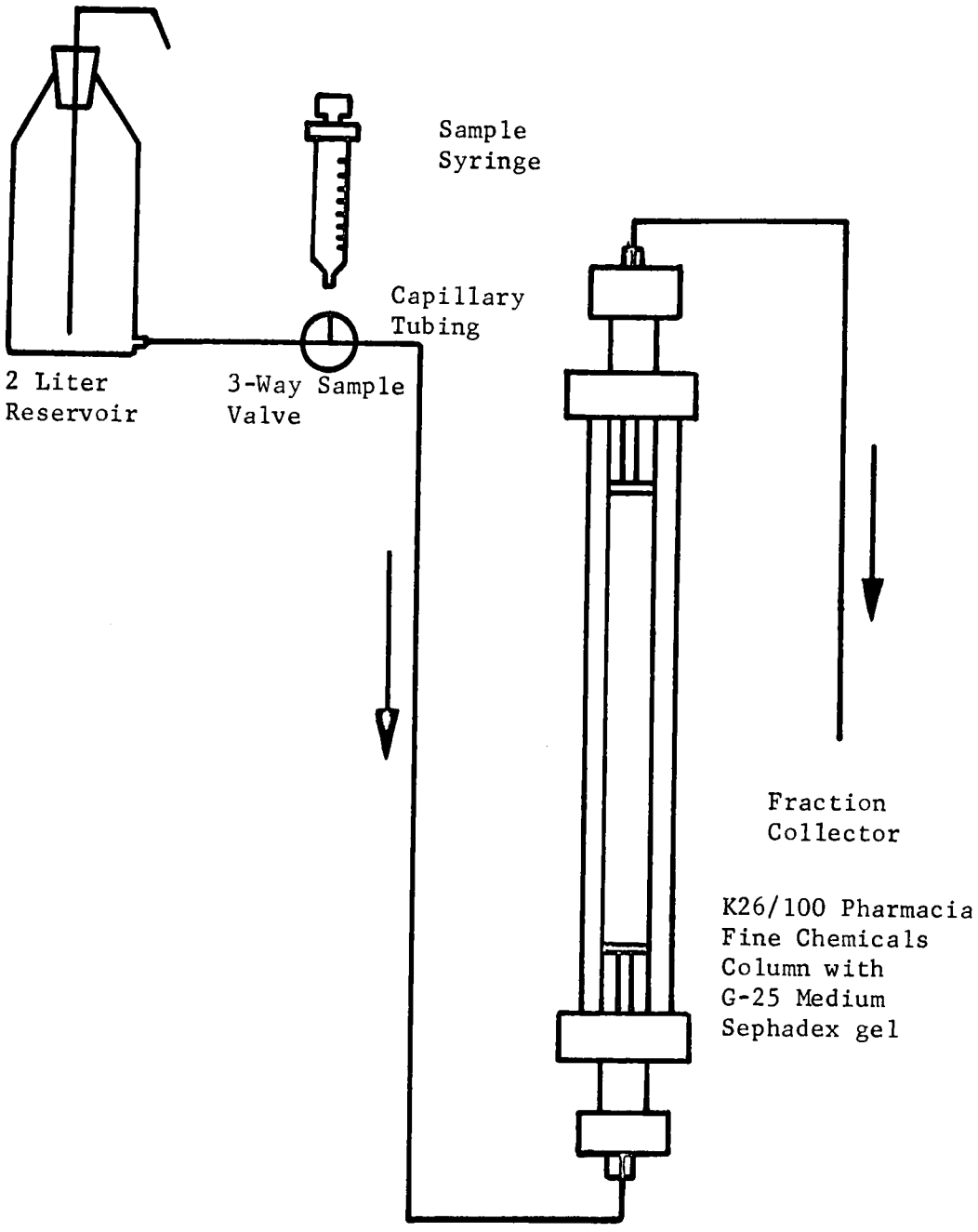


Figure 5. Schematic of the Sephadex Column system.

mixture was then filtered through a 0.45 micron membrane filter before further analyses or use.

Sample Concentration

Due to the dilution which occurred on the Sephadex column, selected fractions collected from this procedure were concentrated before further analysis. Vacuum evaporation was chosen as the method for sample concentration since only relatively low temperatures (less than 25°C) were needed to obtain desired sample concentrations. Concentration factors of 10 to 20 were obtained.

Cation Exchange

All samples were passed through a 2.5 X 26 cm Dowex 50W-X8 cation exchange column (NH^+_4 form) at a flow rate of 5 to 10 ml per minute before being placed on the liquid chromatographic column. To facilitate separation techniques on the liquid chromatographic column, the samples were passed through a cation exchange resin in order to maintain a fixed cation composition. The cation exchange column was flushed with two liters of deionized-distilled water before regeneration with one liter of NH_4Cl (100 g/l).

High Performance Liquid Chromatography (HPLC)

A Spectra-Physics high performance liquid chromatograph, Model 3500-B, was used as the pumping system in the isocratic mode. The pumping system used dual reciprocating pistons with some overlap in the pressure stroke of the piston to compensate for pulsating flow.

The pumping system was designed for pressures up to 7000 psi; however, during this study the pressure ranged 570 to 650 psi.

A high pressure rotary sample injection valve was used to inject the sample into the pressurized eluent stream just before the chromatographic column. Sample volume to be injected on the column depended on the volume of the sample loop used. In this study, two sizes of sample loops were used. For the preliminary standardization of the column, a 0.10 milliliter sample loop was used. For all culture samples, a 1.0 milliliter sample loop was used. Both rotary sample injection valves and loops were designed to operate within the pressure ranges used in this study.

The ion exchange resin used in this study was Aminex A-27 produced by Bio-Rad Laboratories. This anion exchange resin used was a copolymer with quaternary amine active sites. The average resin particle size was 8 to 16 microns. The resin was packed under 2500 psi into a 0.455 X 150 cm column. The column is constructed out of 316 stainless steel and was surrounded by a one inch o.d. stainless steel heating jacket connected to a controlled temperature water bath. The eluent used in this study was 0.50 N NH_4HCO_3 at pH 8.8. Table 5 summarizes the physical characteristics and operating parameters used in this study. The Aminex A-27 column was placed in the NH_4HCO_3 mode for 30 minutes prior to sample injection to ensure a single solvent system on the column. After each sample run, the column was regenerated with deionized-distilled water for at least 12 hours before preparation for another sample run. The column, resin, and sample

Table 5. Physical Characteristics and Operating Parameters

Parameters	Description
Column	0.455 x 150 cm.
Resin	Aminex A-27, Lot No. 9425
Resin particle size	8-16 microns
Sample size	0.10 or 1.0 ml.
Column temperature	45^{o} C
Flow rate	0.20 ml/min.
Eluent	0.50 N NH_4HCO_3, pH 8.8
	Isocratic mode
Fraction volume*	4 ml.
Operating pressure	500-650 psi

*Automatic fraction collector--time mode.

injector used in this study were provided by Oak Ridge National Laboratory.

Scintillation Counting

The Sephadex and HPLC profiles of the radioactive phosphorus-32 (^{32}P) labeled cultures were obtained by direct counting of the fractions on a Beckman LS-200-B liquid scintillation counter. A plug-in discriminator which was factory preset to represent the optimal pulse-height window settings for ^{32}P was used. Counting times ranged from 1.0 to 5.0 minutes depending upon the source strength of the samples. Borosilicate glass scintillation vials of low potassium-40 content were used. Background counts were obtained on all sciltillation vials before use in this study.

By utilizing the Cerenkov radiation produced by the beta particles of ^{32}P, a 90 percent counting efficiency was obtained on the direct counting of the fractions collected from the Sephadex and HPLC studies. Cerenkov radiation occurs only when the particle velocity exceeds the velocity of light in the medium and the source is free of associated gamma radiation (Jelley, 1958). Phosphorus-32 met these requirements since it was a very energetic beta particle (1.71 MeV) and was also a pure beta emitter free of associated alpha and gamma particles. Direct counting utilizing Cerenkov radiation was chosen since the sample is not diluted or mixed with a scintillation solution and thereby can be utilized in further analysis.

CHAPTER IV

RESULTS AND DISCUSSION

Overview

The experimental phase of this study on the release of inositol phosphates by algae was divided into three phases. The first phase involved the development of an analytical technique utilizing high performance liquid chromatography (HPLC) for separating the inositol phosphates. Three solvent systems and a combination of many physical parameters were investigated before attempting investigation into the other phases.

The second phase involved utilizing previous techniques developed by other researchers (Minear, 1971; Walanski, 1974). Sephadex gel chromatography of standard inositol phosphates and alkaline bromination of standard inositol phosphates followed by Sephadex gel chromatography were investigated.

The third phase involved applying the techniques investigated in the previous two phases to the DOP compounds released by laboratory algal cultures to determine the presence or absence of any inositol phosphates.

HPLC Studies

Aminex A-27 anion exchange resin (Bio-Rad Laboratories) was chosen as the resin used to separate the inositol phosphates due to

preliminary work with this resin. Other resins (Partisil Sax K158J and Vydac AX107) were tested in the preliminary study but failed to yield adequate results. The Aminex A-27 anion exchange resin used was a copolymer with quaternary amine active sites with an average particle size of 8 to 16 microns.

Preliminary work with the Aminex A-27 resin involved use of ammonium acetate ($NH_4C_2H_3O_2$) as the eluent on a gradient system starting with distilled water and finishing with 6 M $NH_4C_2H_3O_2$. Initial preliminary work involved the use of ^{32}P labeled algal culture as the sample. Well defined single peaks were obtained from the ^{32}P labeled culture samples. Efforts to standardize the Aminex A-27 column with nonradioactive inositol phosphates standards failed due to interference from the 6 M $NH_4C_2H_3O_2$ eluent with the potassium persulfate digestion method for total phosphorus. Due to the presence of oxidizable acetate in the eluent, two to three times the normal amount of potassium persulfate was required to release the phosphorus from combination with organic material. In addition to the excess amount of potassium persulfate, larger volumes of NaOH were necessary to neutralize the sample. Upon addition of the ascorbic acid-ammonium molybdate mixture, some standard samples, which were less than 100 percent digested, produced a yellow color instead of the blue phosphomolybdenum color. The inability to standardize the inositol phosphates when prepared in 6 M $NH_4C_2H_3O_2$ pointed out the need for a different eluent.

Ammonium nitrate (NH_4NO_3) at pH 4.8 was tried as the eluent in an isocratic mode. In evaluating the 0.50 N NH_4NO_3 as the eluent,

adenosine monophosphate (AMP) was used as the standard phosphate compound. AMP was used since it can be detected at 258 nm on a UV detector. Efforts to standardize the Aminex A-27 column with AMP resulted in 100 percent conversion of the organic bound phosphate in AMP to orthosphate upon passage through the column. The conversion of the organic bound phosphate can be speculated to occur due to possible acid hydrolysis conditions created by the 0.50 N NH_4NO_3 at pH 4.8 and the high pressure of operation. Actual hydrolysis mechanisms and the phosphorus chemistry involved is beyond the scope of this study. No other standards were evaluated with the 0.50 N NH_4NO_3 crystallizing out and damaging the liquid chromatograph's pumping system.

In preliminary evaluation of ammonium bicarbonate (NH_4HCO_3) as the eluent, a gradient system was used starting with distilled water and finishing with 1 N NH_4HCO_3. This resulted in a group of peaks for an IMP standard which eluted at a concentration of 0.50 N NH_4HCO_3. The results of the gradient work with NH_4HCO_3 showed that 100 percent of the total phosphorus from the IMP standard came off the column midway through the gradient run. Ammonium bicarbonate at pH 8.8 was chosen as the eluent after an evaluation of 0.50 N NH_4HCO_3 in the isocratic mode resulted in a well defined single peak for IMP with less than 50 percent conversion to orthophosphate upon passage through the column. Various operating parameters were investigated including column temperature, flow rate, and concentration of NH_4HCO_3. After many preliminary trial runs, the operating parameters listed previously in Table 5 were chosen and were used throughout the rest of this study.

Separation of IHP, IMP and Orthophosphate Standards. A sample of each of the three standards was individually chromatographed on the Aminex A-27 column. As shown in Figure 6, the chromatograms of duplicate orthophosphate standards revealed that the orthophosphate peak had considerable tailing. The tailing could imply adsorption of solute molecules at a few active sites which release the molecules after the peak due to poor adsorption-desorption kinetics. The chromatogram of duplicate IMP standards is shown in Figure 7, and the chromatogram of of duplicate IHP standard is shown in Figure 8. These chromatograms, when compared to the chromatogram of orthophosphate (Figure 6), shows the ability of the column and eluent to separate these three standards from each other. Less than 50 percent of IMP and IHP are broken down to orthophosphate during the time on the column. Possible intermediate organic phosphorus compounds occur due to the breakdown of IHP on the column. Since intermediate inositol phosphates are not available commercially, comparison of the elution position with standards could not be obtained. Considering that the inositol phosphates are a family of a homologous series of compounds, then a linear relationship between the log of the elution position and the log of the number of units in the homologous series should occur (Whitaker, 1963). The plot of the log of the elution position for IMP and IHP versus their respective log of the number of phosphate groups in this homologous series of compounds is shown in Figure 9. A summary of the data used to plot Figure 9 is shown in Table 6. Inspection of Figure 8 and Figure 7 indicates that IHP does not breakdown to IMP on the column and that a

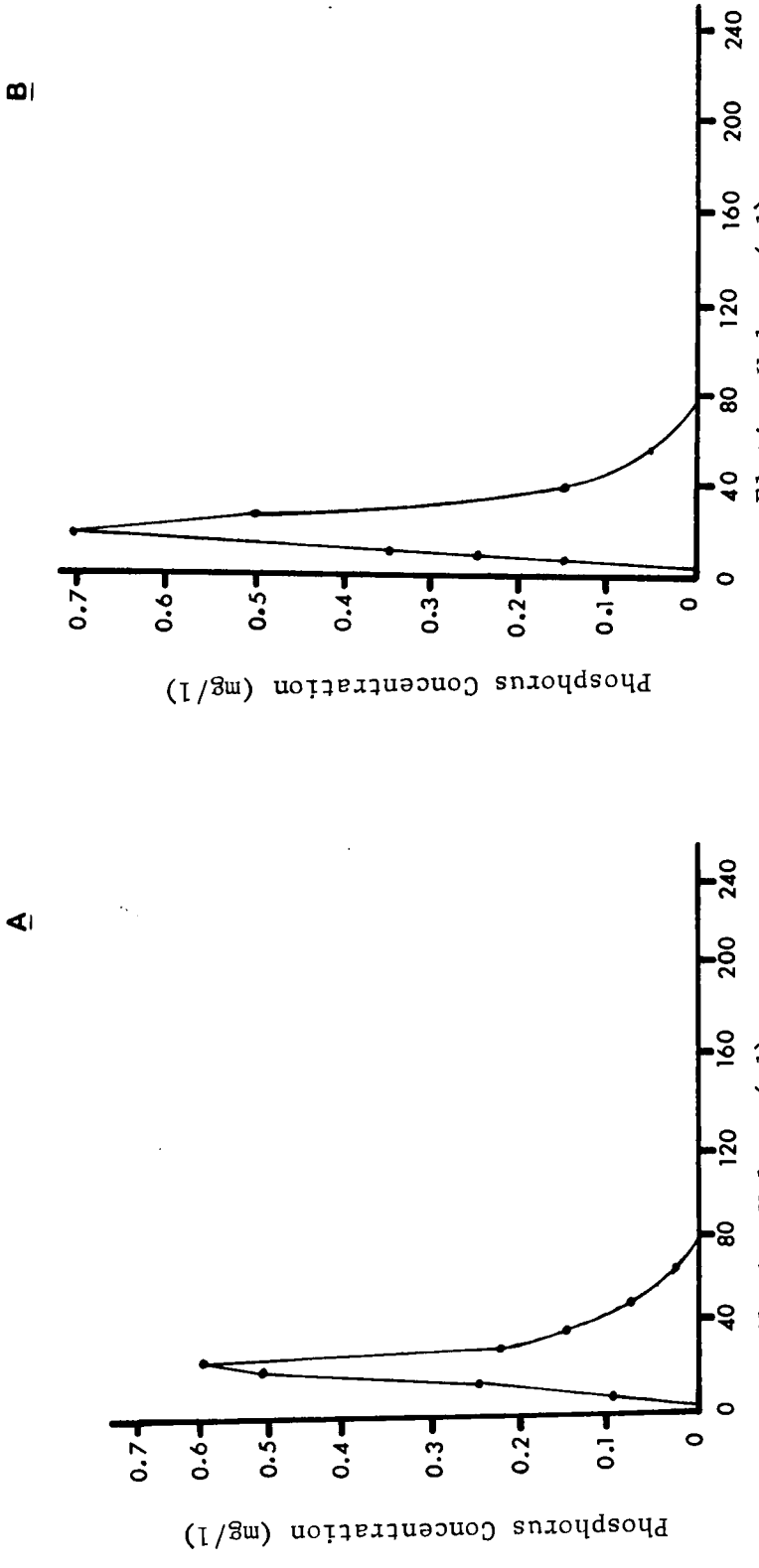


Figure 6. Duplicate Chromatograms of Orthophosphate Standards on Aminex A-27.

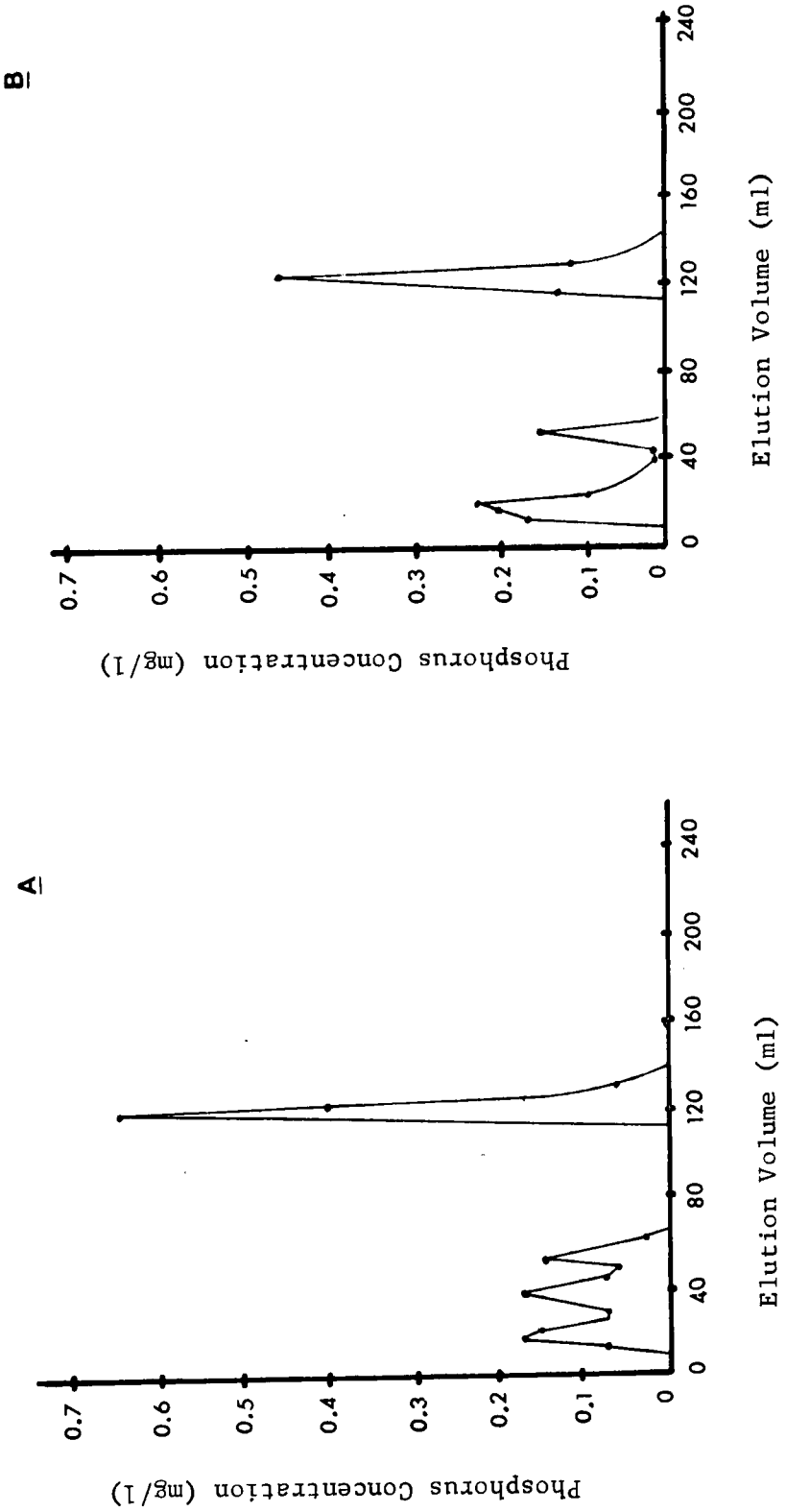


Figure 7. Duplicate Chromatograms of IMP Standards on Aminex A-27.

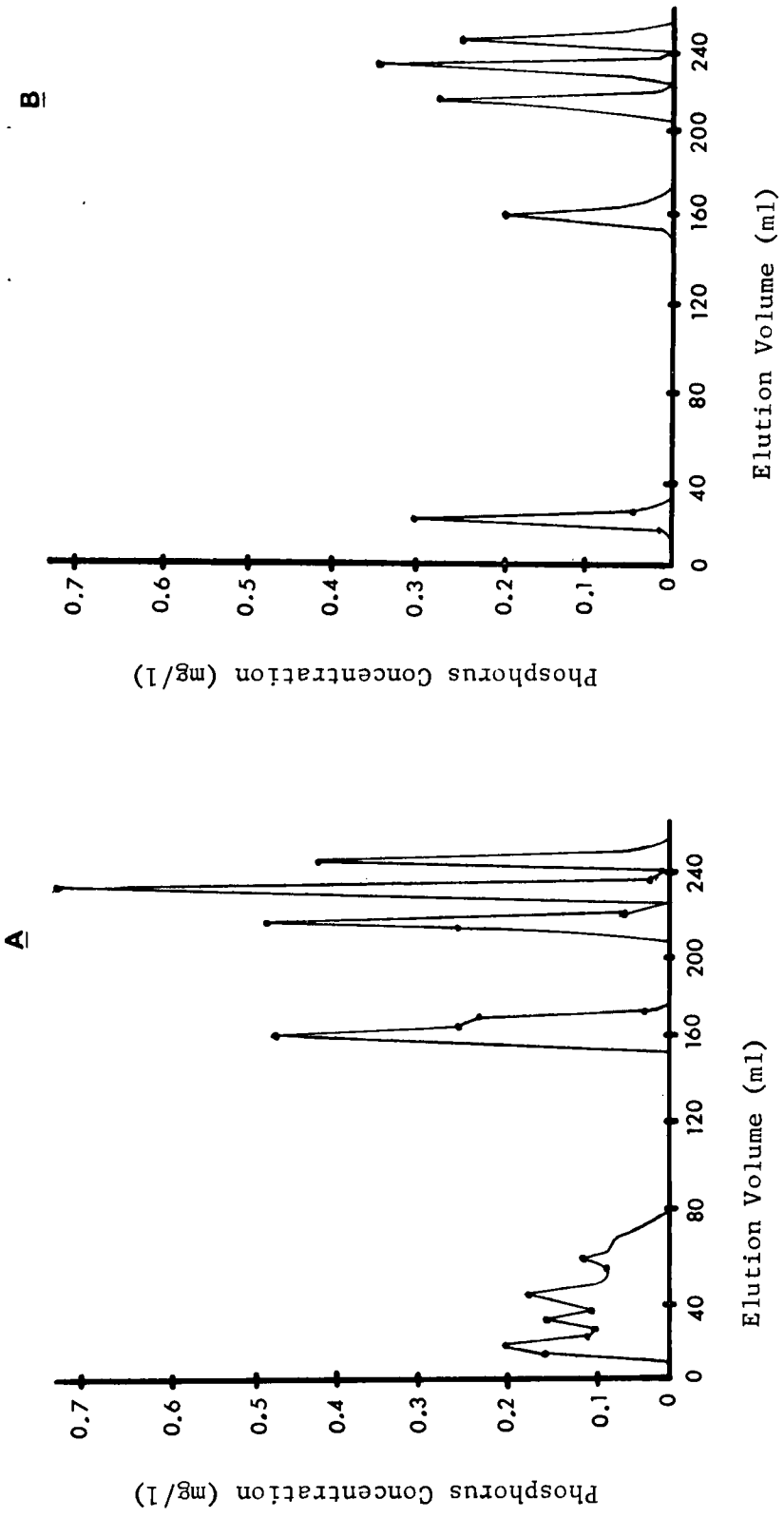


Figure 8. Duplicate Chromatograms of IHP Standards on Aminex A-27.

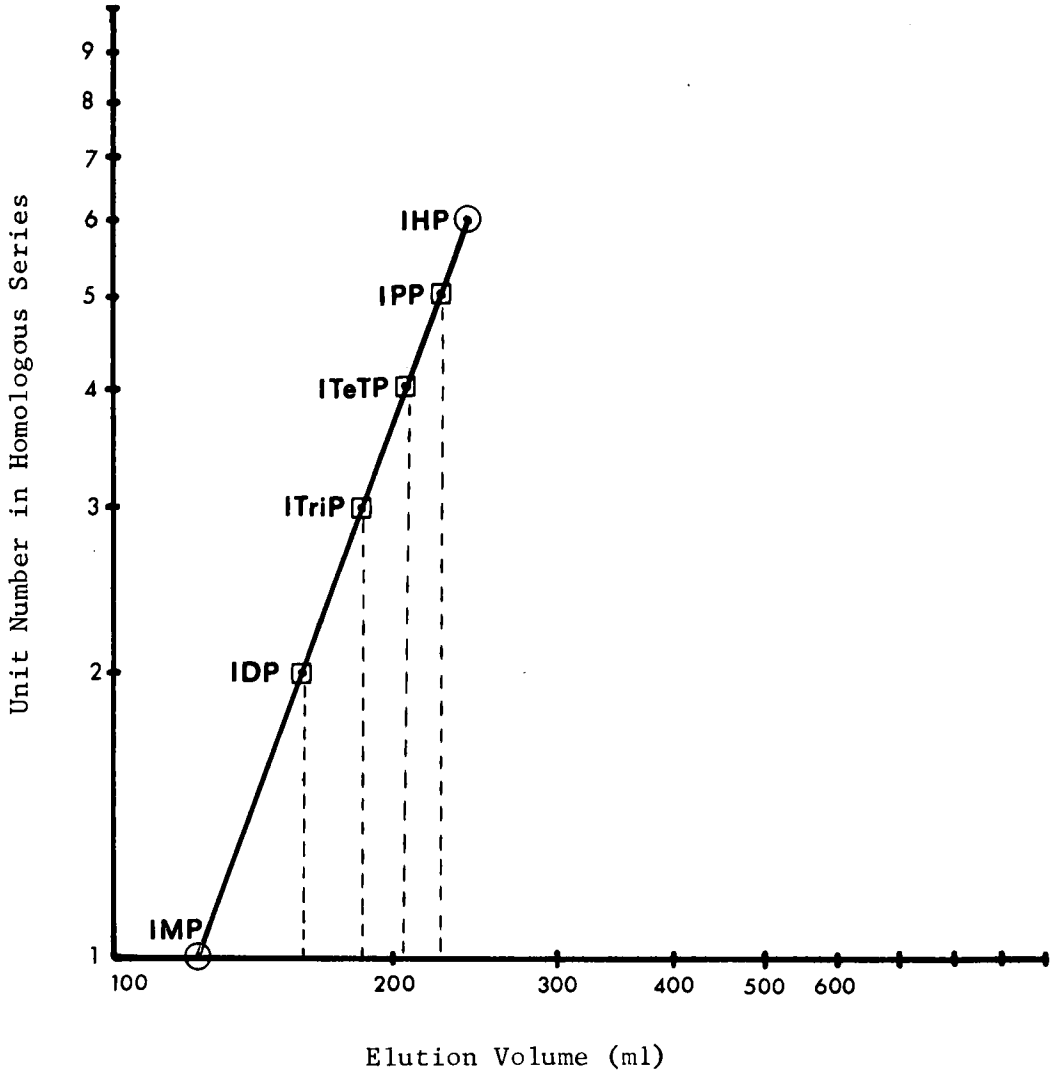


Figure 9. Log-Log Plot of Unit Number in Homologous Series Versus Elution Volume on Aminex A-27.

Table 6. Measured and Calculated Elution Positions for IHP, IMP, Intermediate Inositol Phosphates and Orthophosphate on Aminex A-27

Compound	Measured Elution Volume (ml)	Calculated Elution Volume (ml)	Unit Number in Homologous Series
Orthophosphate	20	--	--
IMP	124	124	1
IDP	160	160*	2
ITriP	--	185*	3
ITetP	212	210*	4
IPP	228	225*	5
IHP	240	240	6

*Calculated values from Figure 9.

separation of all three standards could be obtained. Breakdown of the IHP and IMP standards to orthophosphate while on the column can be speculated to occur due to alkaline hydrolysis conditions created by the NH_4HCO_3 eluent at pH 8.8 and to the high pressure of operation. Non-inositol standards (AMP and B glycerophosphoric acid), when chromatographed, resulted in complete breakdown of the organic bound phosphate to orthophosphate.

Assuming that IHP does not breakdown to IMP while on the column, then a mass balance calculation of the μg mole as IHP could be calculated for each organic peak. This mass balance calculation is summarized in Table 7. By assuming complete recovery of the total phosphorus, the mass balance calculations are within two percent of the calculated μg mole as IHP. The identity of the peaks, based upon the mass balance, are also within good agreement with those identities presented in Figure 9 and Table 6.

The effective separation of IMP and IHP from each other, as well as from orthophosphate, indicate that operation parameters, eluent, and Aminex A-27 resin were suitable for separation work involving the DOP fraction produced from an algal culture. Mass balances on each of the standards used, along with percent recovery and percent breakdown to orthophosphate (PO_4), are summarized in Table 8. No percent recovery or percent breakdown is given for the duplicate chromatograms labeled as A in Figures 6, 7, and 8.

Table 7. Summary of Mass Balance Calculations for Duplicate Samples of IHP on Aminex A-27.

For Chromatogram A

Calculated μg mole as IHP = 0.141

Organic Peak	Conc. μg	Conc. μg mole as P	Conc. μg mole as IHP
1 = IDP	4.90	0.158	0.079
2 = IT_e + P	3.05	0.098	0.024
3 = IPP	5.15	0.166	0.033
4 = IHP	2.25	0.073	0.012

actual measured μg mole as IHP = 0.148

For Chromatogram B

Calculated μg mole as IHP = 0.051

Organic Peak	Conc. μg	Conc. μg mole as P	Conc. μg mole as IHP
1 = IDP	1.32	0.043	0.021
2 = IT_e + P	1.60	0.052	0.013
3 = IPP	1.68	0.054	0.010
4 = IHP	1.24	0.040	0.007

actual measured μg mole as IHP = 0.051

Table 8. Summary of Mass Balances on Standards

Component	Initial DOP µg	Initial PO_4 µg	Initial % DOP	Final DOP µg	Final PO_4 µg	Final % DOP	% Total P Recovery	% Breakdown to PO_4
Orthophosphate	--	22.0	--	--	21.5	--	97.7	--
IMP	10.8	0	100	5.0	4.3	53.8	86.1	46.2
IHP	9.5	0	100	5.8	1.4	80.1	76.2	19.9

G-25 Chromatographic Studies

Previous work done by Minear and Walanski (1974) indicated that Sephadex G-25 medium was the appropriate gel for separating the inositol phosphates. Sephadex G-25 medium has a fractionation range for dextrans of 100 to 5,000 molecular weight and a dry bead diameter of 50 to 150 microns with a 2.5 ml per gram water regain. In this study, the G-25 column was used for preliminary separation of the DOP fraction from orthophosphate.

Separation of IHP, IMP, Orthophosphate and BD-2000 Standards. The exclusion volume (V_o) of the G-25 column was determined using a 1 percent (w/v) solution of Blue Dextran 2000. The absorbance of the eluted fractions was read at 620 nm. The exclusion volume of the G-25 column was observed to be 190 ml (thereby defined as R = 1.00). To avoid interference from the BD-2000 blue color, the phosphate standards were individually chromatographed on the G-25 column. As shown in Figure 10, the composite chromatograms of the phosphate standards and the superimposed BD-2000 standard are represented. The elution behavior of these compounds is summarized in Table 9.

As shown in Figure 10, the IHP and orthophosphate were completely separated from each other, while IMP and orthophosphate were not completely resolved from one another. Any fractions taken from the Sephadex profile in the area of IMP (R = 1.50) would probably result in a mixture of IMP and orthophosphate. This does not rule out the usefulness of G-25 for preliminary separation work with inositol

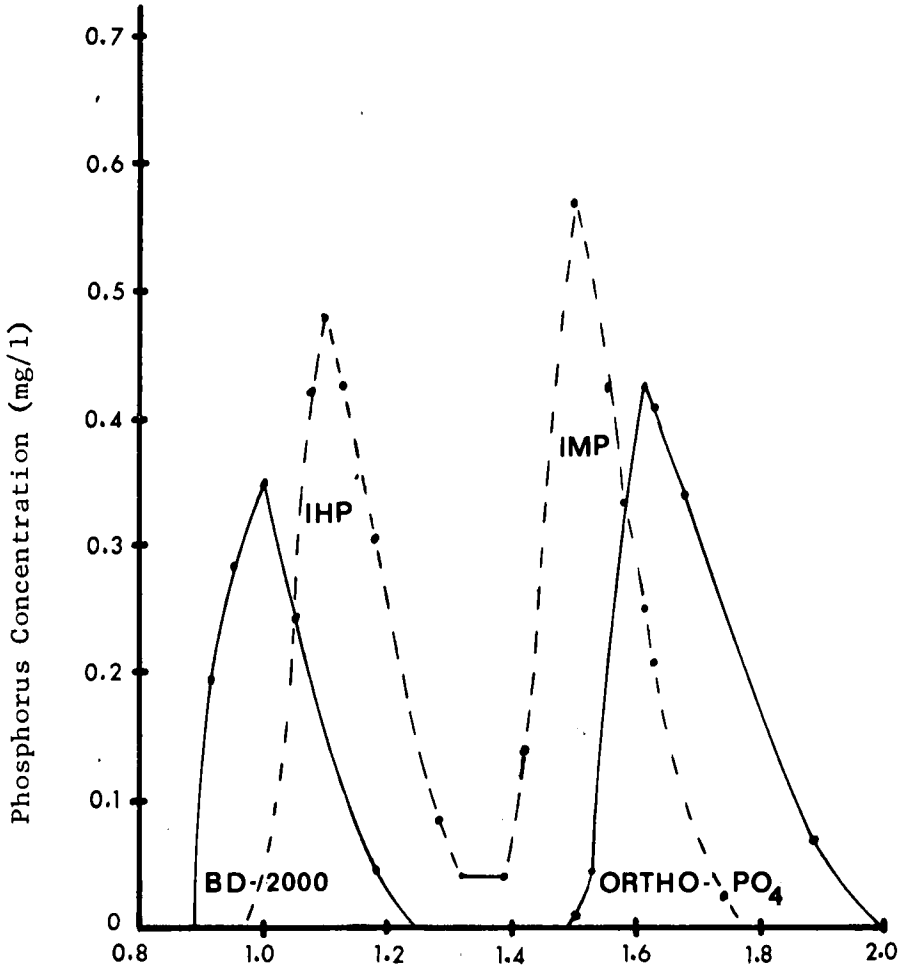


Figure 10. Composite Chromatogram of BD-2000, IHP, IMP, and Orthophosphate.

Table 9. Elution Behavior of IHP, IMP, Orthophosphate, and BD-2000 on G-25

Component	Elution Volume (Ve)	R (Ve/Vo)
Blue Dextran	190 ml	1.00
IHP	210 ml	1.11
IMP	285 ml	1.50
Orthophosphate	304 ml	1.60

phosphates, since when using a culture sample the ratio of DOP to orthophosphate can be controlled.

Intermediate inositol phosphates are not available commercially, but their elution positions are of interest. Previous researchers (Whitaker, 1963) have reported a linear relationship between R and the log molecular weight for a family of compounds. If the inositol phosphates follow this observed relationship, then it would be possible to predict the elution volume of the intermediate inositol phosphates from the known R values of IMP and IHP. The plot of the R value for IHP and IMP versus their respective log molecular weights are shown in Figure 11. A summary of the data used to plot Figure 11 and the resulting R values for the intermediate inositol phosphates are shown in Table 10.

Alkaline Bromination Studies of Inositol Phosphates. Walanski (1974) utilized alkaline bromination as a means of isolating the inositol phosphates from extraneous organic matter. In this study, alkaline bromination followed by Sephadex chromatography was used as a secondary separation step for the DOP fraction from extraneous organic matter and orthophosphate. Individual samples of IMP and IHP were subjected to alkaline bromination and then chromatographed on Sephadex G-25. In order to obtain percent breakdown and recovery of each sample, phosphorus analyses were performed before and after alkaline bromination. The chromatogram of the brominated IHP is shown in Figure 12 and the chromatogram of the brominated IMP is shown in Figure 13. Mass balances on each of the brominated inositol phosphate

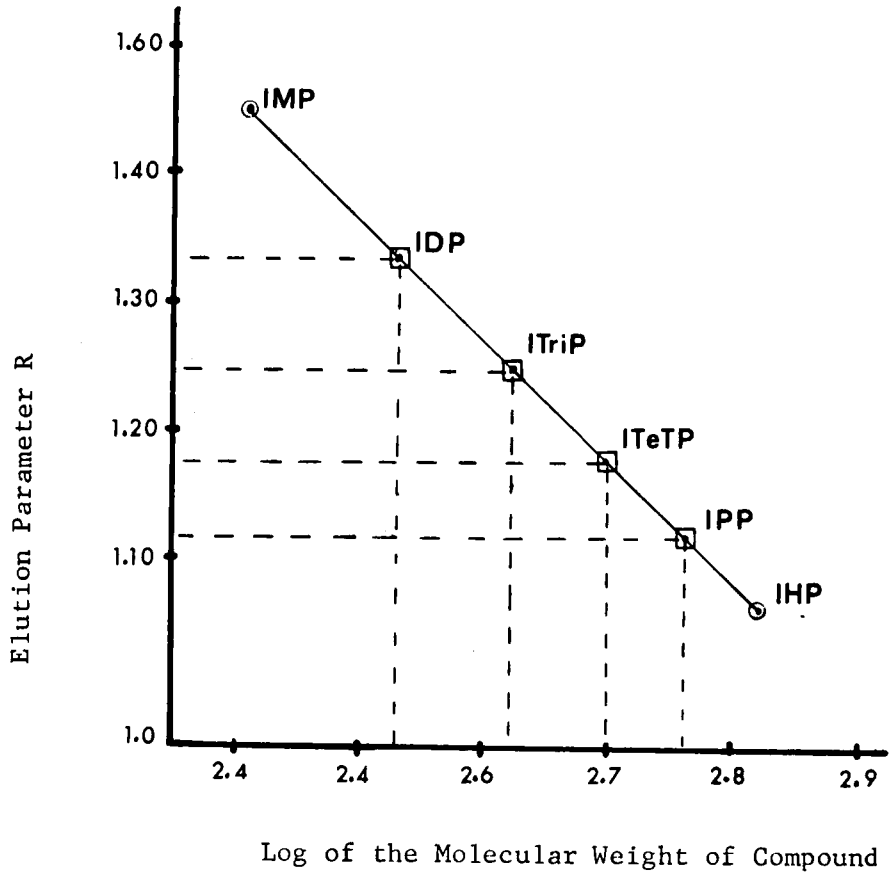


Figure 11. Plot of Log Molecular Weight Versus Elution Parameter R for Inositol Phosphates on Sephadex G-25.

Table 10. Measured and Calculated R Values, Elution Volumes, and Log Molecular Weights for the Inositol Phosphates on Sephadex G-25

Compound	Molecular Weight	Log MW	R	Elution Volume (ml)
IHP	660	2.819	1.11	210
IPP	580	2.763	1.165*	221*
ITetP	500	2.699	1.22*	232*
ITriP	420	2.623	1.29*	245*
IDP	340	2.531	1.385*	263*
IMP	260	2.412	1.50	285

*Calculated values from Figure 11.

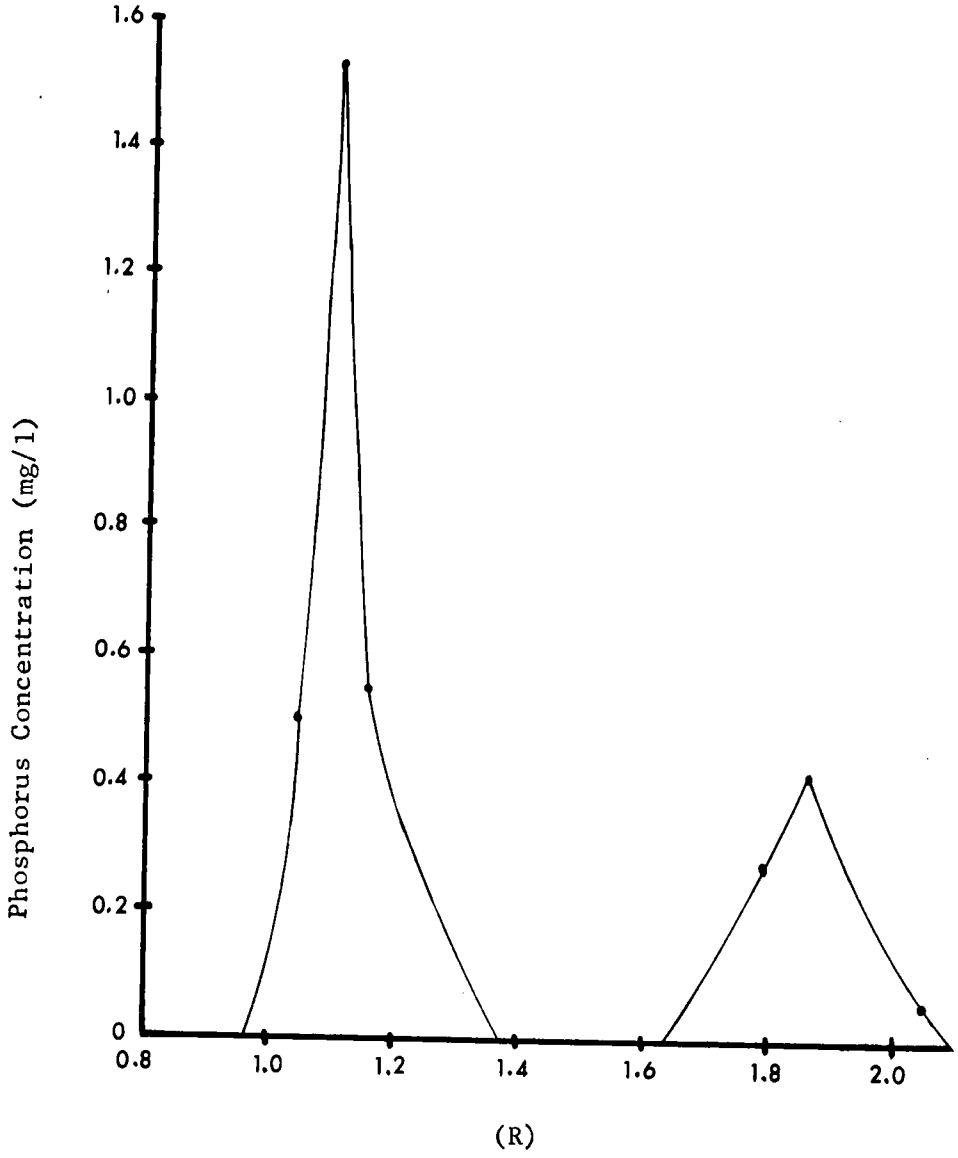


Figure 12. Chromatogram of Brominated IHP on Sephadex G-25.

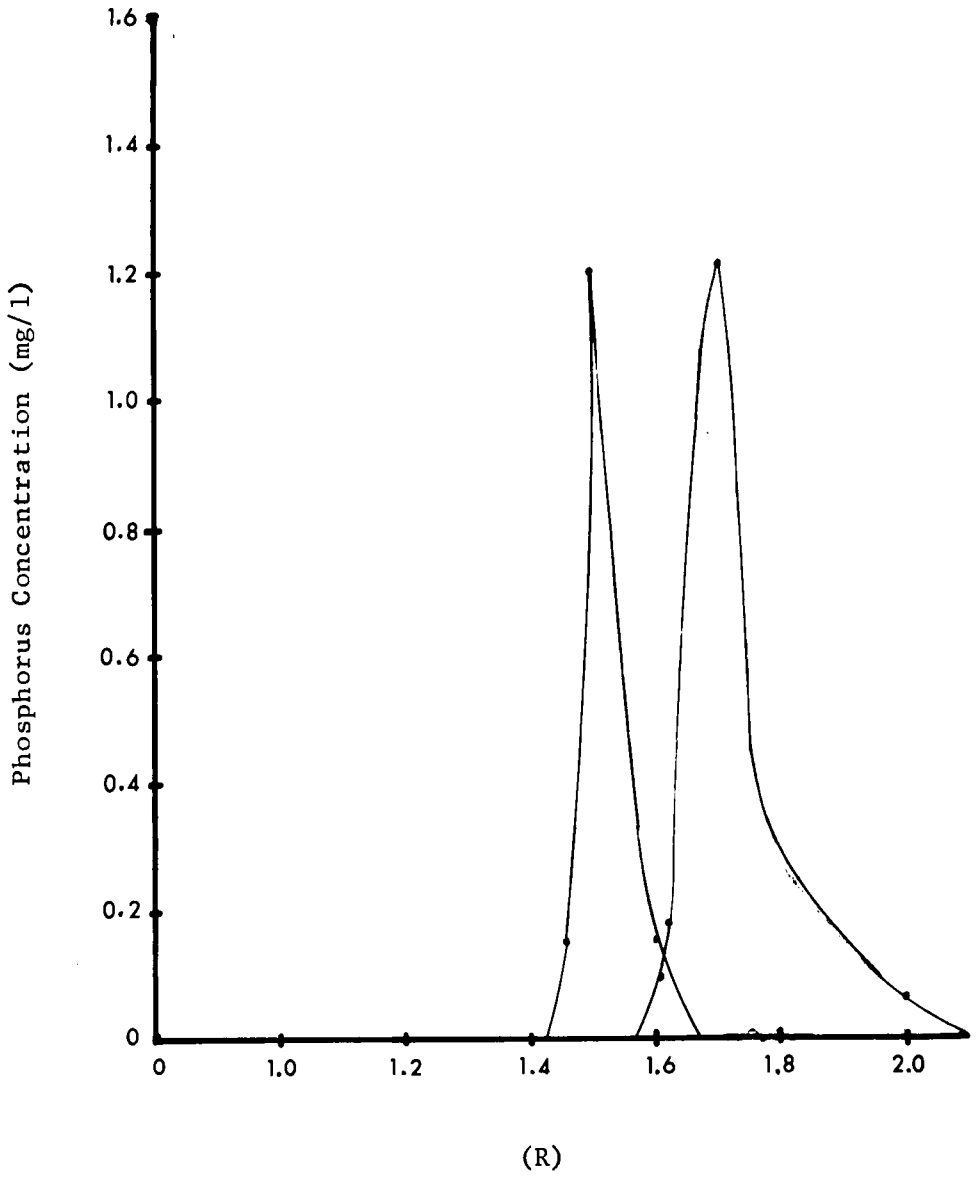


Figure 13. Chromatogram of Brominated IMP on Sephadex G-25.

standards used with percent recovery and breakdown data are summarized in Table 11.

The results of the bromination of IMP and IHP agreed with previous work by Walanski (1974). Recovery of total phosphorus for IMP was nearly 100 percent, but over half of the recovered total phosphorus was orthophosphate. Results of the IMP chromatogram (Figure 13) showed that the elution volume of IMP on G-25 was not affected by the bromination step. Examination of the chromatogram indicated that the elution volume of orthophosphate on G-25 was affected by the bromination step. Walanski (1974) observed that the change in elution volume for orthophosphate was due to a salting effect from the 5 N NaOH used in the bromination procedure. The change in elution volume for orthophosphate enabled a greater resolution between IMP and orthophosphate.

Since recovery of total phosphorus for IHP was only 78 percent with 91.6 percent of the recovered phosphorus as organic phosphorus. The percent breakdown was based upon the actual amounts of ortho and organic phosphorus measured in solution, assuming equal recovery of both forms. Lower recovery rates for IHP could be accounted for due to the brown percipitate formed during the bromination procedure. The chromatogram (Figure 11) of the brominated IHP standard indicated that the elution volume of IHP on G-25 was not affected by the bromination step. As observed previously in the brominated IMP chromatogram, the orthophosphate peak was affected by this procedure. Results of the high salt effect on the orthophosphate was expected not to cause interference in the separation of the inositol phosphates. Evaluation of

Table 11. Summary of Alkaline Bromination of IHP and IMP

Component	Initial DOP mg	Initial PO_4 mg	Initial % DOP	Final DOP mg	Final PO_4 mg	Final % DOP	% Total P Recovery	% Breakdown to PO_4
IHP	0.31	0	100	0.20	0.02	90.9	71.0	9.1
IHP	30.0	0	100	24.0	2.0	92.3	86.7	7.7
IMP	30.0	0	100	14.6	14.5	50.2	97.0	49.8
IMP	3.0	0	100	1.30	1.6	44.8	96.7	55.2

the brominated IMP and IHP peaks revealed that these peaks were more symmetrical after bromination. This could be due to possible destruction of extraneous organic matter even though both standards were reagent grade.

Algal Culture Studies

Chlamydomonas reinhardtii was chosen as the algal species to be used in this study since Minear (1971), Walanski (1974), and McEntyre (1976) had previously investigated the orthophosphate uptake and the DOP release of this species. The initial orthophosphate concentration used in this study for the culture work was 3.13 mg/1. Approximately a 14 to 18 day growth period was necessary to achieve a high DOP build-up concentration and a low residual orthophosphate concentration. Optimization of the initial orthophosphate concentration of the culture media enhanced the use of radioactive phosphorus-32 to be used as a tracer since only one half-life would occur. Radioactive phosphorus-32 has a half-life of 14.3 days, thus during a two-week growth period almost half of the radioactivity originally present in the culture was lost due to decay. The orthophosphate uptake and DOP release from the culture study is shown in Figure 14.

The soluble culture material was withdrawn from the reaction flask after 15 days of growth by procedures described in the experimental design section. The DOP and orthophosphate concentrations were determined at this time and were found to be 0.97 and 0.45 mg/1, respectively. The soluble culture material was then divided up into two aliquots as shown in the flow chart in Figure 15.

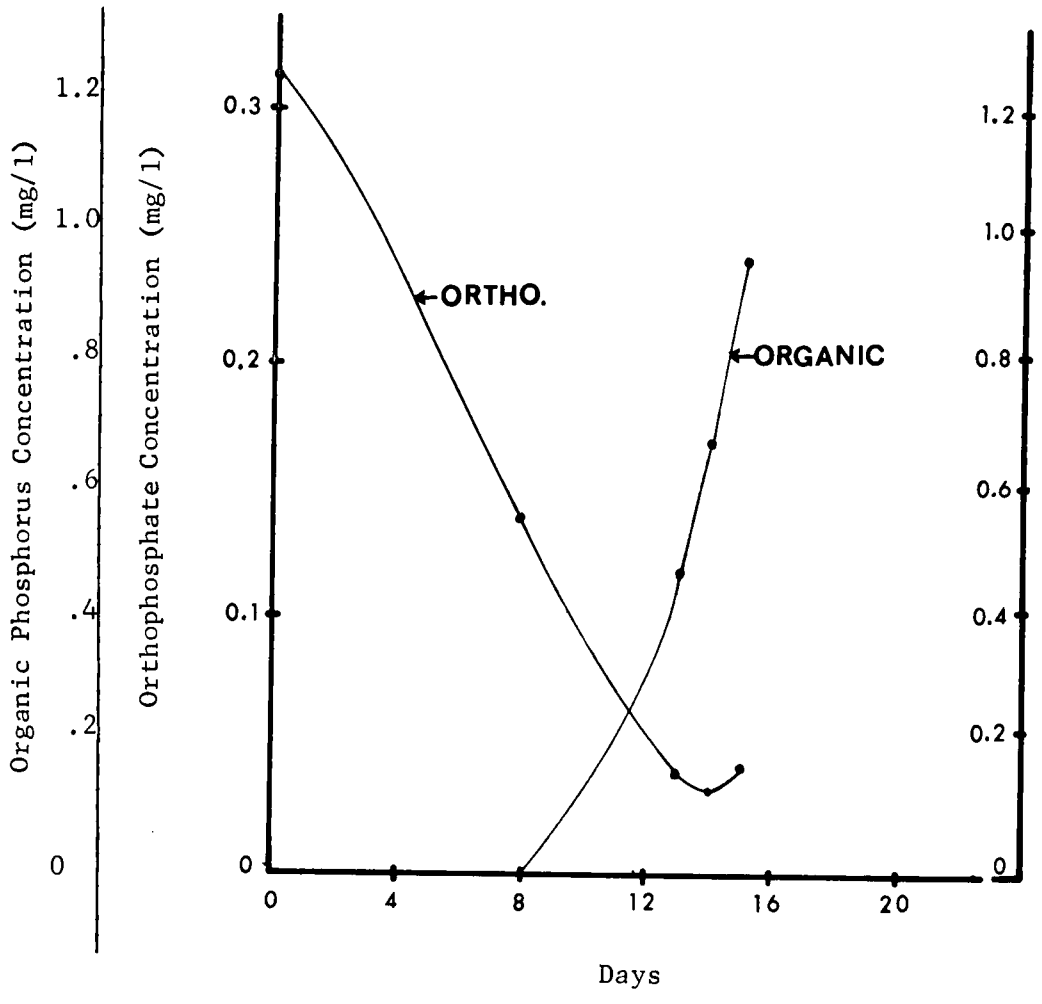


Figure 14. Orthophosphate Uptake and DOP Release from Chlamydomas reinhardtii.

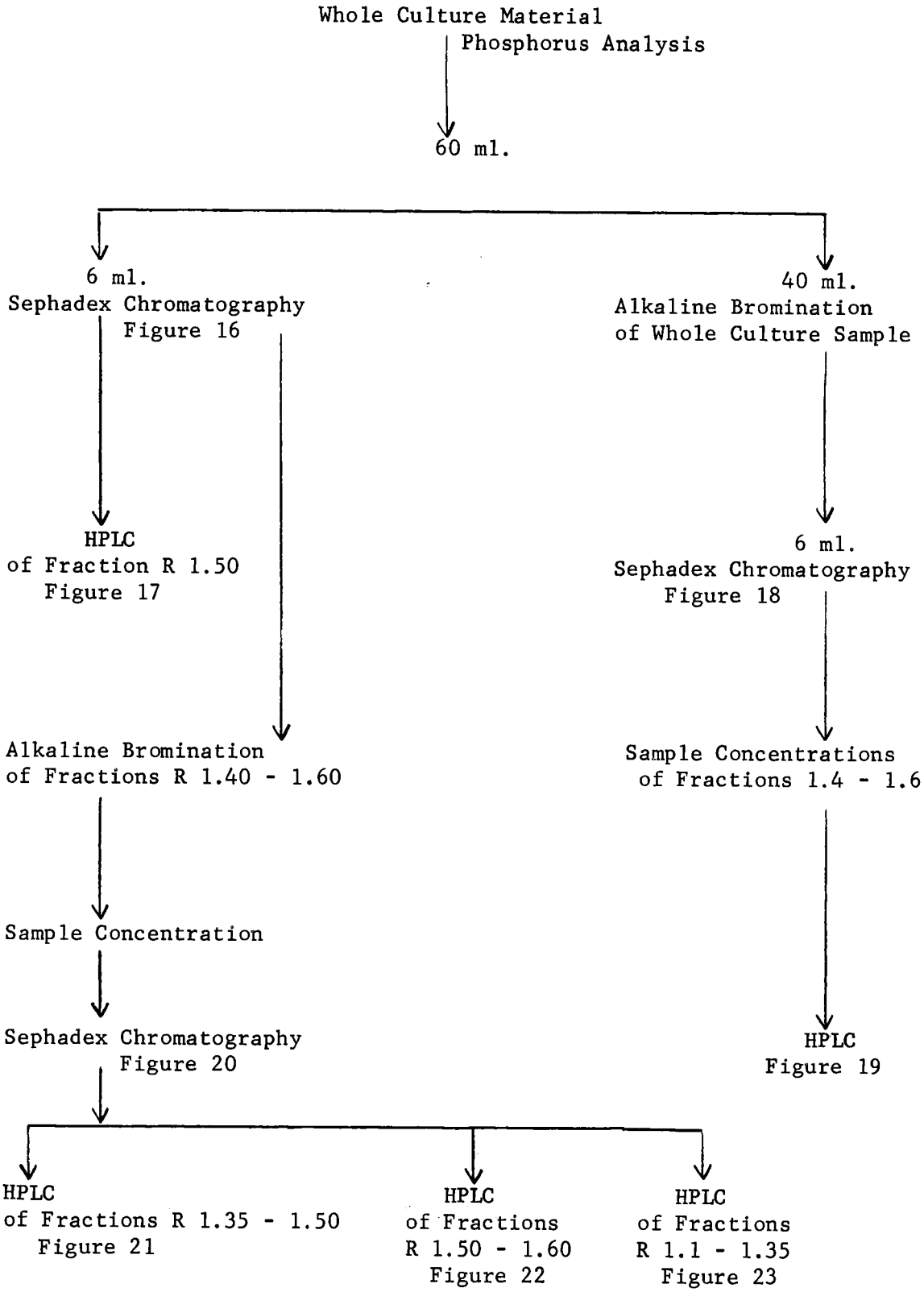


Figure 15. Flow Chart of Culture Experiment.

Whole Culture Sample on G-25. The whole culture sample was chromatographed on the G-25 column and the resulting chromatogram is shown in Figure 16. The large peak at the exclusion volume can be associated, in part, with DNA and its fragments (Minear, 1971). The fraction at R = 1.50 was chromatographed on the A-27 column and is shown in Figure 17. Investigation of this chromatogram revealed that the orthophosphate concentration was high enough to cause tailing and masking of other peaks. Results from this first whole culture chromatogram indicated that additional preliminary separation techniques would be needed to reduce the orthophosphate and extraneous organic material present.

Alkaline Bromination of Whole Culture Sample. Alkaline bromination, followed by Sephadex G-25 chromatography, was employed as a secondary separation technique in which the bulk of organic matter and non-inositol organic phosphorus compounds material present in the whole culture sample could be destroyed. Presence of any organic phorphorus peak after alkaline bromination did not offer conclusive proof for the presence of inositol phosphates, but did provide a sample free of interfering organic material. The alkaline bromination recovery for the whole culture is summarized in Table 12. Following bromination, an aliquot of the brominated whole culture (ABWC) sample was chromatographed on Sephadex G-25 as shown in Figure 18. Examination of this chromatogram revealed that not all of the extraneous organic material was destroyed by the bromination procedure. Insufficient bromination time could be responsible for incomplete reduction of the

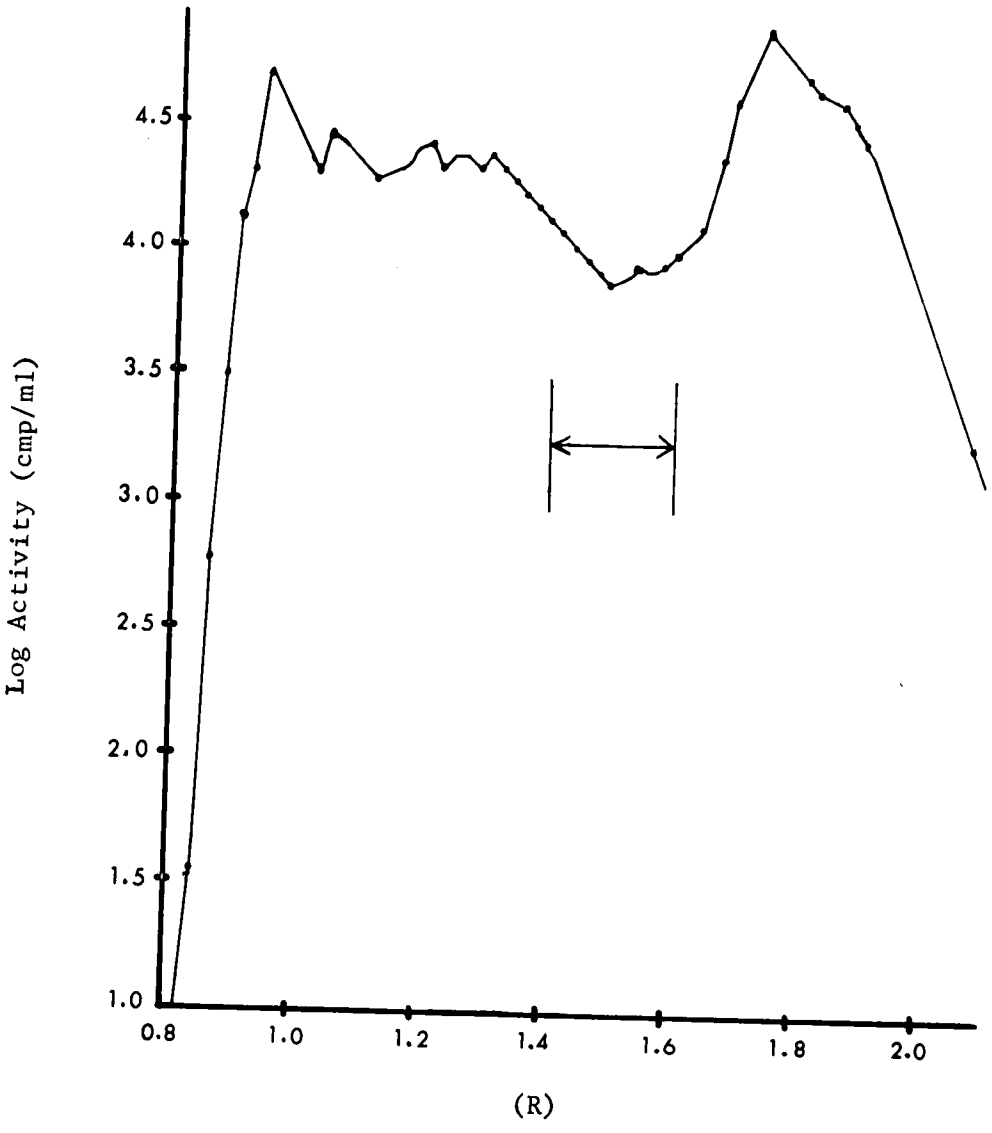


Figure 16. Chromatogram of Whole Culture Sample on Sephadex G-25.

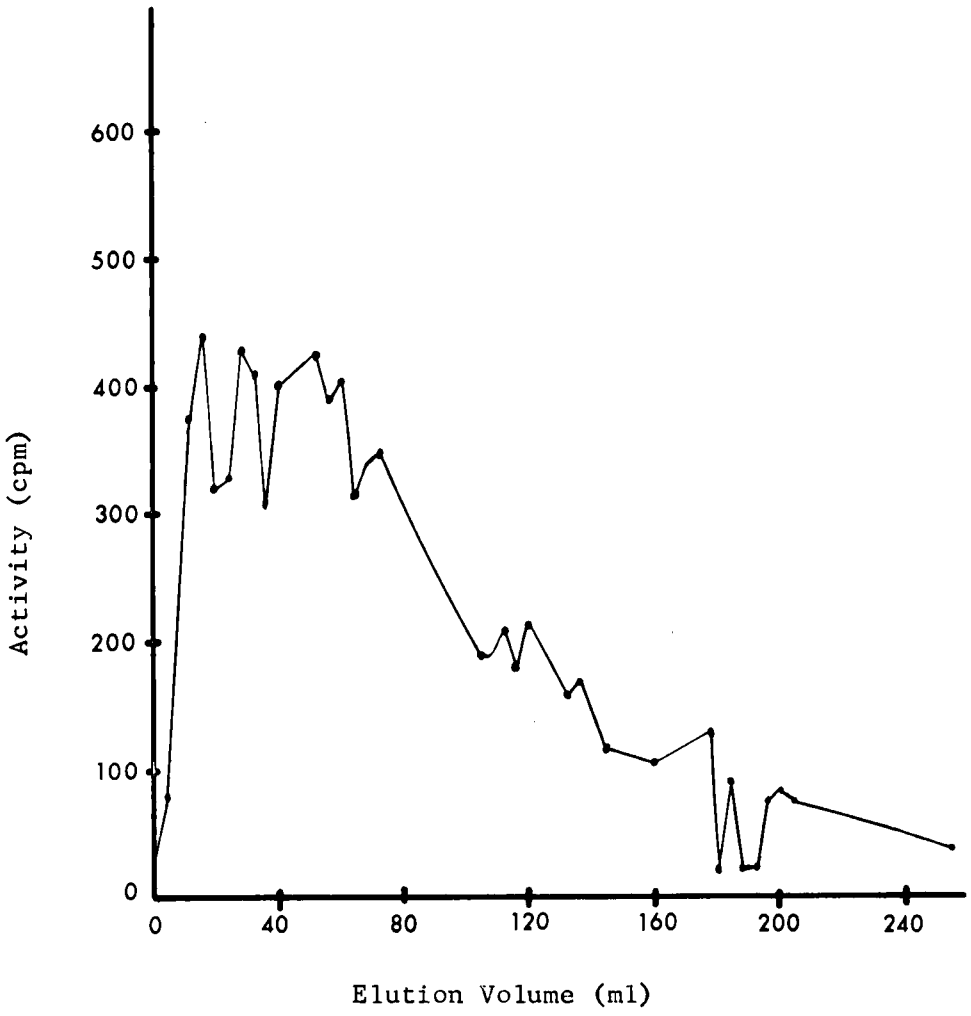


Figure 17. Chromatogram of Sephadex Fraction R = 1.5 from Whole Culture Sample on Aminex A-27.

Table 12. Summary of Alkaline Bromination Recovery for the Whole Culture Sample

Sample	Initial DOP mg	Initial PO_4 mg	Initial % DOP	Final DOP mg	Final PO_4 mg	Final % DOP	% Total P Recovery	% Breakdown to PO_4
Whole Culture	0.97	0.42	69.8	0.77	0.55	58.3	95.0	41.7

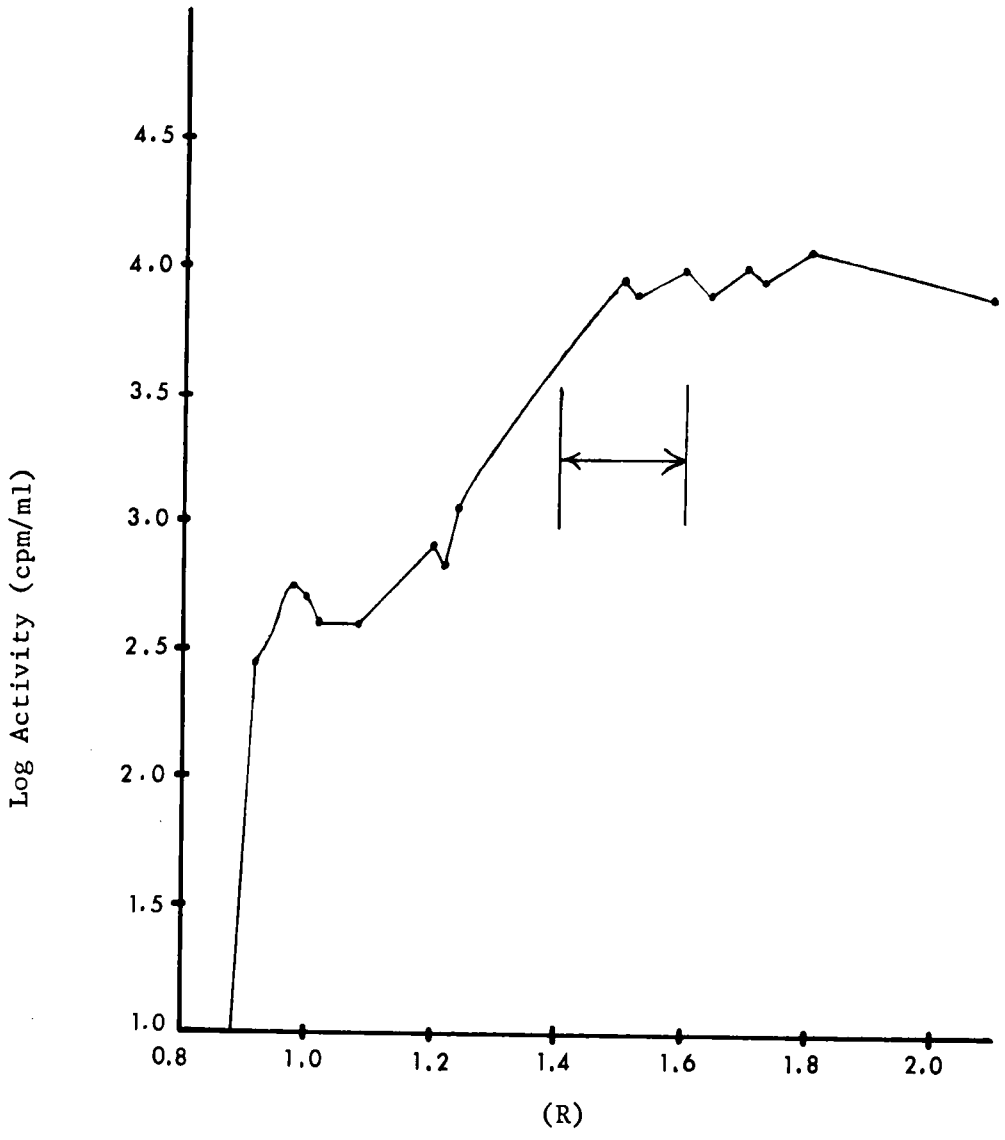


Figure 18. Chromatogram of Brominated Whole Culture Sample on Sephadex G-25.

extraneous organic material. This chromatogram also showed what could be a double peak in the areas of IMP and orthophosphate.

The fractions representing IMP (R = 1.4-1.6) were concentrated by vacuum evaporation followed by HPLC on Aminex A-27. Inspection of this chromatogram, shown in Figure 19, revealed that peaks in the orthophosphate region are more pronounced and free of tailing. Two additional distinct peaks occurred at 108 ml and 192 ml. Neither of these peaks matched standard IMP and IHP samples chromatographed before and after the culture studies. Results from the ABWC chromatogram confirmed that a clean-up procedure such as bromination followed by Sephadex chromatography enhanced the resolution of the peaks obtained by HPLC.

Alkaline Bromination of Sephadex Whole Culture Fractions R = 1.40-1.60. The whole culture Sephadex fractions from R = 1.40 to 1.60 (excluding one fraction R = 1.50) were subjected to alkaline bromination followed by a sample concentration step prior to being rechromatographed on Sephadex G-25. Inspection of this chromatogram, shown in Figure 20, showed that the majority of the extraneous organic matter was destroyed. Three aliquots were taken from this sample for HPLC work and are summarized in Table 13. Aliquot #1 represented the rechromatographed fractions R = 1.35 to 1.50. This sample was concentrated by vacuum evaporation prior to being chromatographed on the HPLC. The resulting chromatogram, shown in Figure 21, revealed that the peaks are sharper and more pronounced in the orthophosphate region. Additional peaks out of the orthophosphate region are positioned at 108 ml and 120 ml.

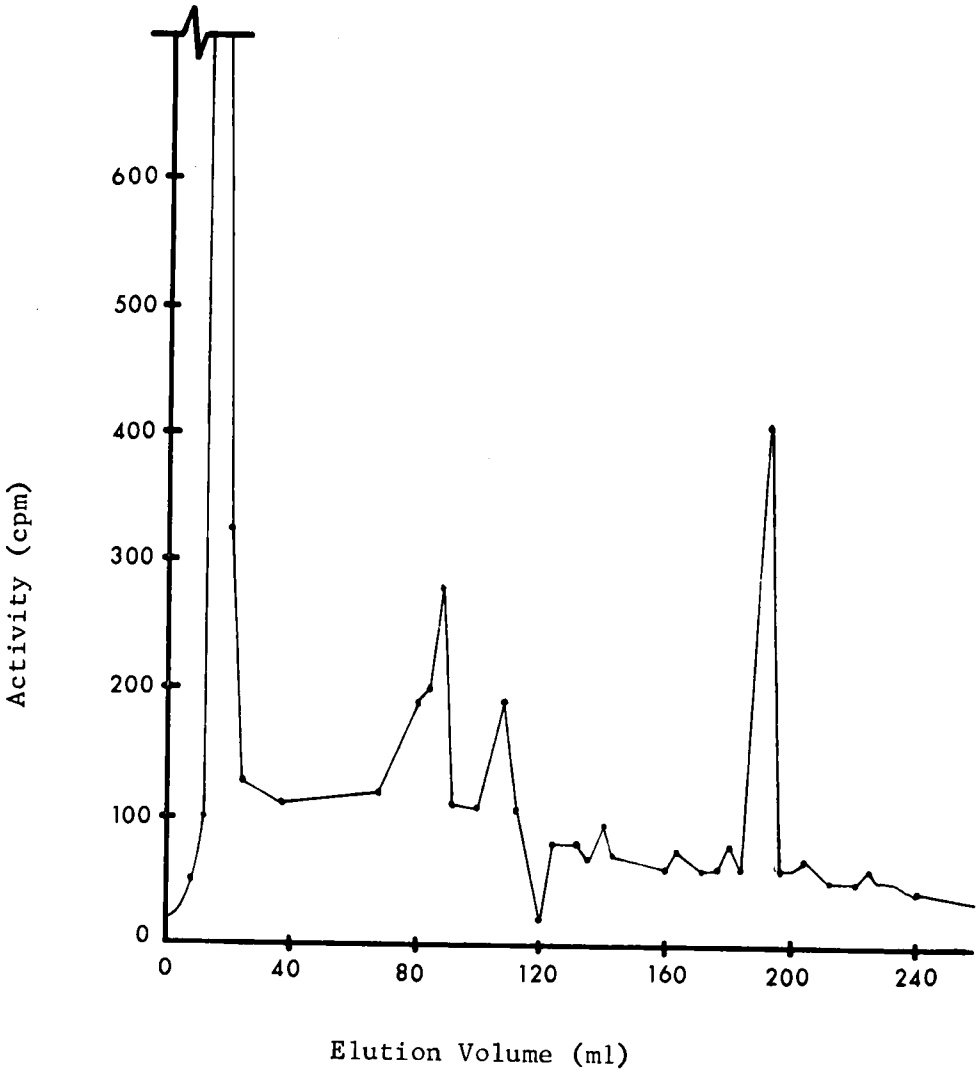


Figure 19. Chromatogram of Sephadex Fraction R = 1.4 - 1.6 from ABWC Sample on Aminex A-27.

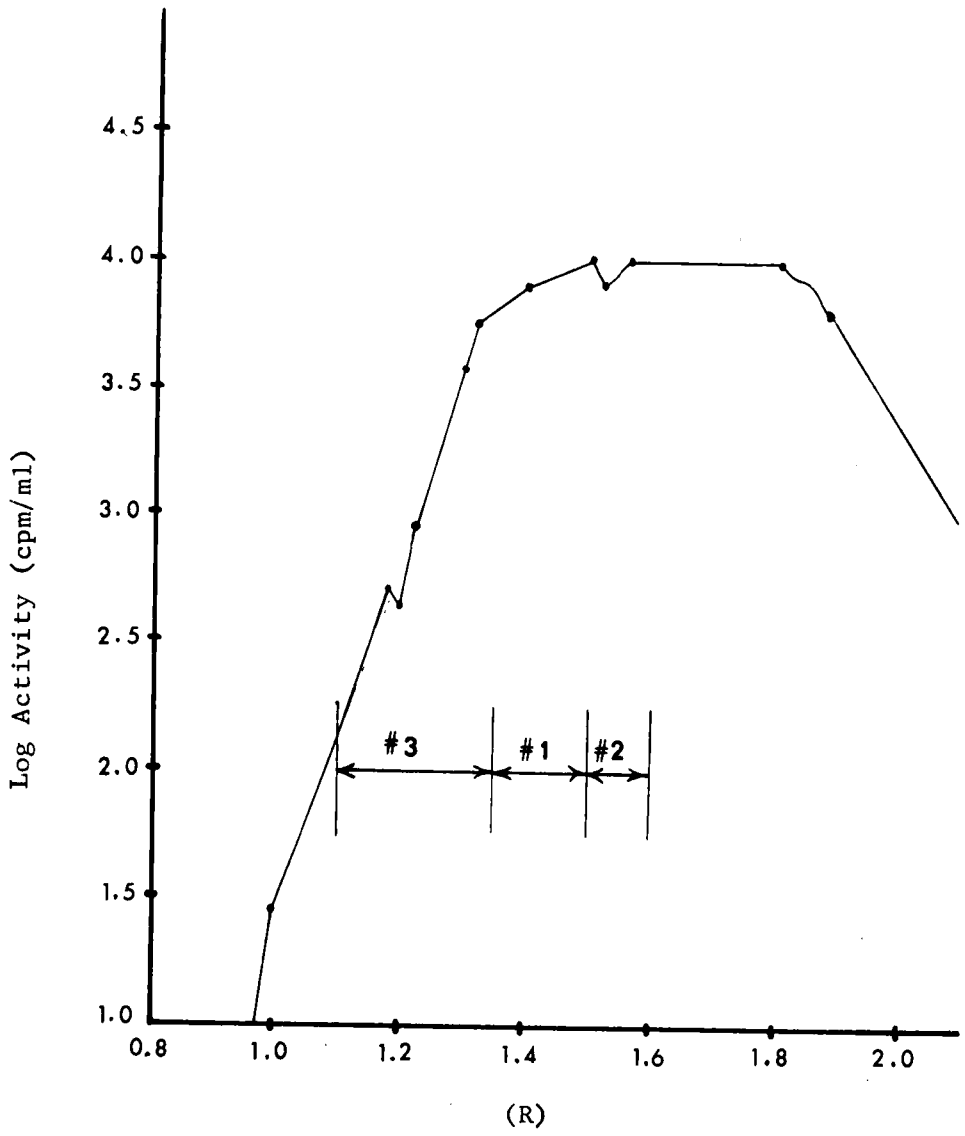


Figure 20. Chromatogram of the Rechromatographed Fractions R = 1.4 - 1.6 from the Whole Culture Sephadex Fractions.

Table 13. Rechromatography of Whole Culture Brominated Fraction

Sample	R Values
Aliquot #1	1.35 - 1.50
Aliquot #2	1.50 - 1.60
Aliquot #3	1.10 - 1.35

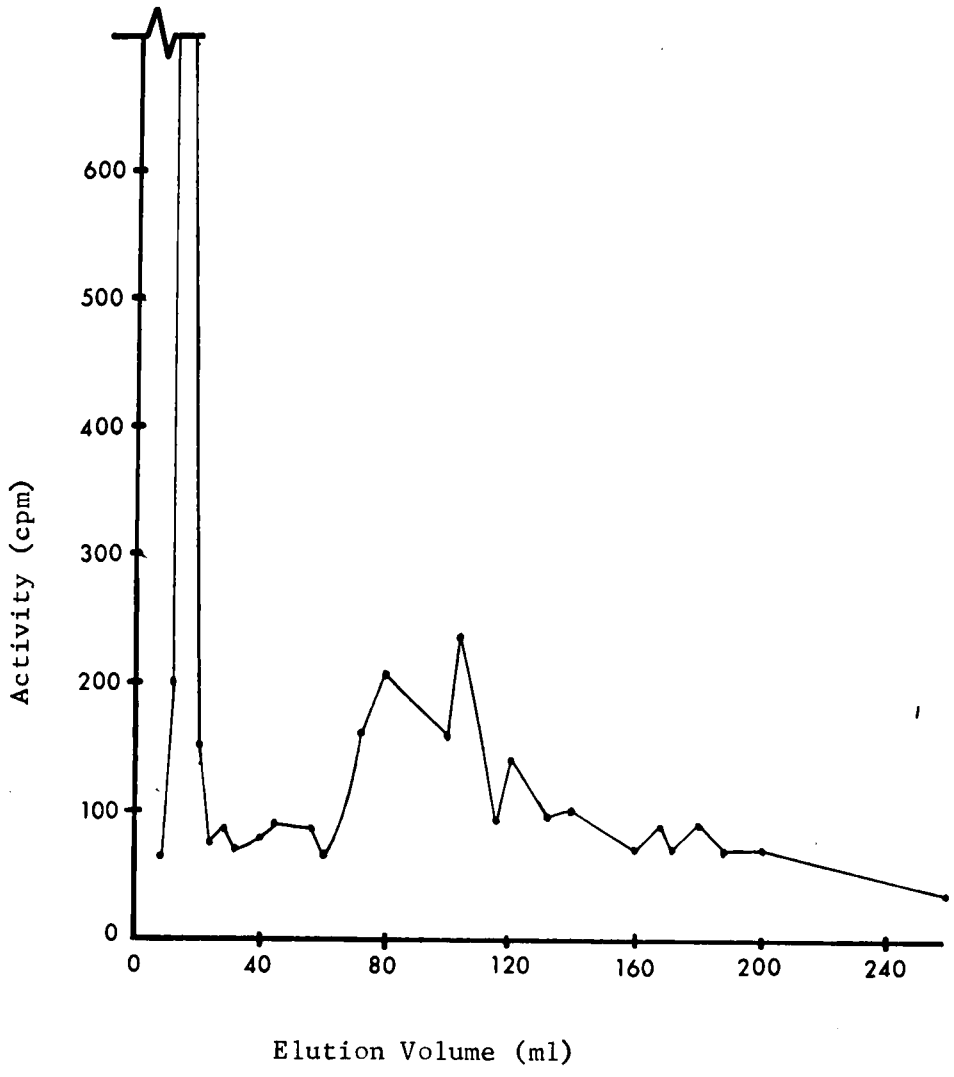


Figure 21. Chromatogram of Aliquot No. 1 on Aminex A-27.

The matching of the peak at 108 ml with a peak at the same position from the brominated whole culture could indicate a breakdown compound or could be one of the four isomers of IMP. The small peak at 120 ml could possibly be IMP or an experimental artifact.

Aliquots #2 and #3 represented the rechromatographed fractions R = 1.50 to 1.60 and 1.10 to 1.35, respectively. These regions would represent the area between IMP and orthophosphate and the area between IHP and IMP. After each sample was concentrated, the samples were individually chromatographed on the HPLC. The resulting chromatograms are shown in Figures 22 and 23. Both chromatograms reveal two peaks which are in the orthophosphate region. No additional information concerning peaks out of the orthophosphate region were obtained from these aliquots.

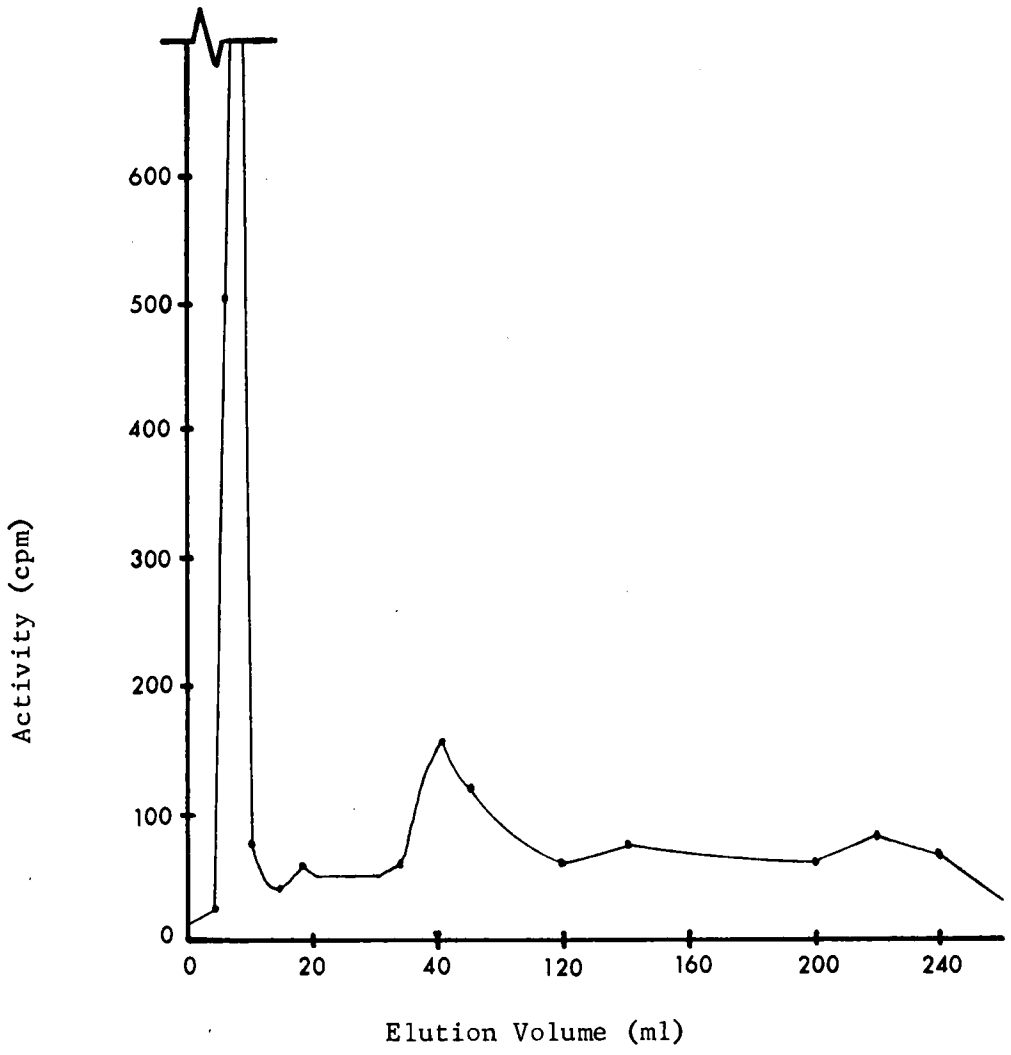


Figure 22. Chromatogram of Aliquot No. 2 on Aminex A-27.

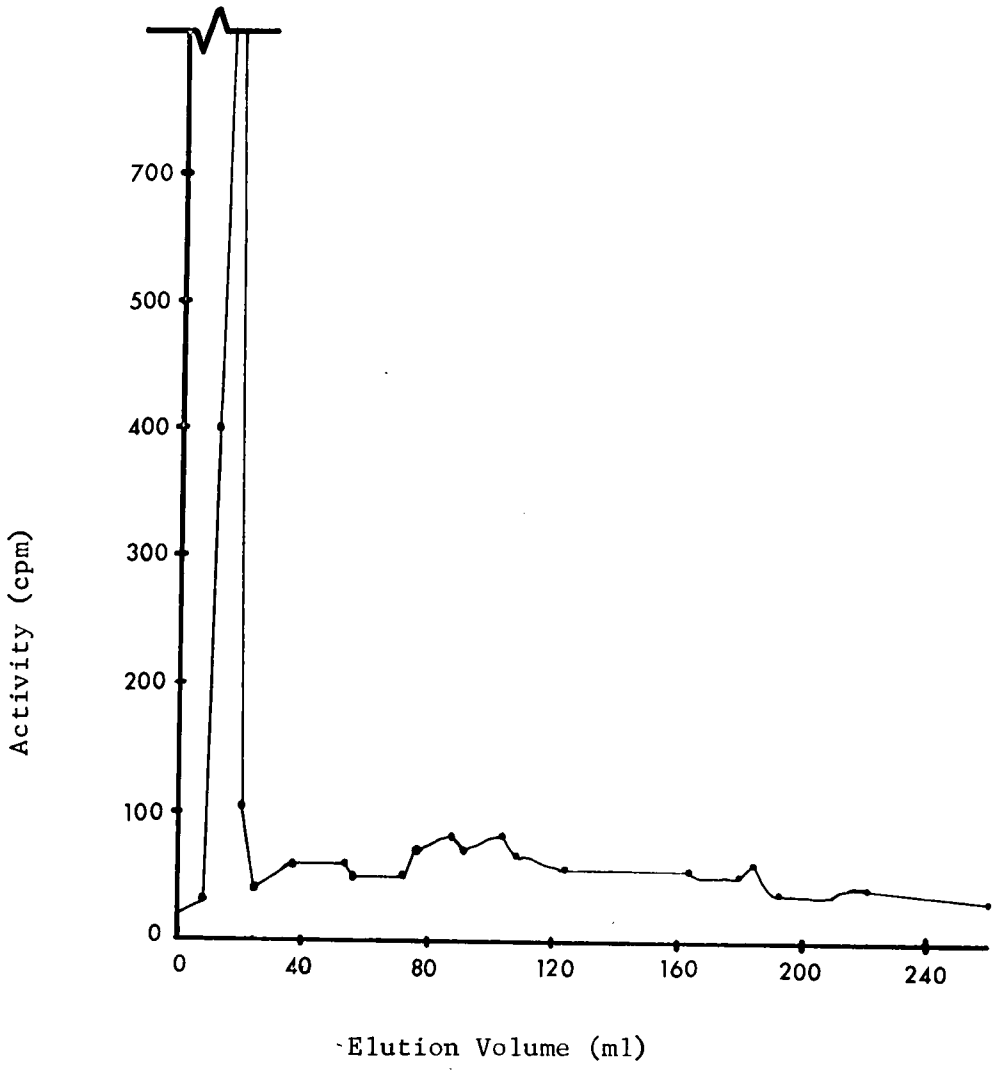


Figure 23. Chromatogram of Aliquot No. 3 on Aminex A-27.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The primary implication of this research investigation has shown that high performance liquid chromatography can be utilized as a chromatographic means of separating the inositol phosphates. The breakdown of the inositol phosphate standards, while on the HPLC column with NH_4HCO_3 at pH 8.8, can be speculated to be due to an alkaline hydrolysis process. The actual hydrolysis mechanism was not identified by this study since it was beyond the scope of this investigation. Intermediate phosphates' elution positions were obtained from a log-log plot of unit number in a homologus series versus elution position. Identification of the breakdown components from IHP was based upon comparison of the calculated elution values from log-log plot with the observed elution values from the IHP chromatogram. The agreement between the calculated elution values and the observed elution values indicate that IHP does breakdown while on the column to the intermediate inositol phosphates and to orthophosphate.

Culture studies utilizing Sephadex G-25 and alkaline bromination presented evidence that not all non-inositol phosphate organic compounds, released by the algal culture, are completely oxidized by the first bromination. Results of the incomplete oxidization are

evident in the chromatograms of the whole culture brominated samples. Bromination followed by rechromatography on Sephadex G-25 of the IMP fraction results in a chromatogram which contains less non-inositol phosphate compounds. Chromatograms of the culture samples from the HPLC study were less conclusive. The HPLC chromatogram of the whole culture sample shows the need for sample pretreatment or clean-up procedure. This chromatogram is inconclusive due to the tailing of the orthophosphate. Cleaner and sharper peaks are presented in the HPLC chromatograms of culture samples which underwent bromination. Only the orthophosphate peaks from the culture study were matched with elution position of standards. Nevertheless, the culture results do not preclude the release of any inositol phosphate due to the breakdown of the compounds to orthophosphate on the HPLC column. Several major peaks present in HPLC study of the culture are not inositol phosphates. These peaks were not identified, but are speculated to be isomers of the inositol phosphates since they appear in three of the chromatograms. In the chromatograms of the Sephadex fraction, R = 1.5, from the whole culture sample and of aliquot #1, the peak at elution volume, equal to 120 ml, could possibly be IMP.

Recommendations for Further Research

Refinements in the HPLC separation procedure are necessary for consistency of results and to ensure that no breakdown products occur while on the liquid chromatograph's resin. Further analysis could be completed on the preserved samples separated during this

study. These analyses should include gas chromatography and/or mass spectrometry for additional identification information. Evaluation and refinements of the alkaline bromination procedure are necessary to ensure complete oxidization of extraneous organic material present in the algal culture. Evaluation of standard inositol phosphates on HPLC using NH_4HCO_3 at pH 7.0 as the eluent are necessary to determine if breakdown of the IHP and IMP standards could occur. Other solvent systems should be investigated, including the use of derivatizing the samples to form a volatile compound followed by an organic solvent system HPLC and compatible resin.

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VITA

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