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INVESTIGATION OF THE CHEMICAL IDENTITY OF SOLUBLE ORGANOPHOSPHORUS COMPOUNDS FOUND IN NATURAL WATERS

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F I N A L R E P O R T

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ABSTRACT

INVESTIGATION OF THE CHEMICAL IDENTITY OF SOLUBLE ORGANOPHOSPHORUS COMPOUNDS FOUND IN NATURAL WATERS

The ability of various laboratory algal cultures to generate substantial quantities of dissolved organic phosphorus (DOP) compounds has been demonstrated. After isolation and concentration of these compounds by low pressure filtration, freeze drying and redissolution, molecular size profiles on Sephadex gels were obtained. Comparison between DOP and inositol phosphate profiles on Sephadex G-25 prompted specific studies for the presence of inositol phosphates in the culture solutions. Alkaline bromination techniques established in soil chemistry research were employed in conjunction with Sephadex gel filtration to generate circumstantial evidence for the presence of inositol monophosphate and the absence of inositol hexaphosphate. The results obtained in conjunction with preliminary volatile derivative studies have provided the base for continued studies on the specific chemical nature of DOP compounds in aqueous systems.

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I. INTRODUCTION

A. Objectives: This project was developed to ultimately establish the chemical identity of the low molecular weight component of naturally occurring dissolved organic phosphorus compounds (DOP). Since these compounds are present at extremely low concentrations in highly complex solutions, specialized techniques must be employed to separate and identify these compounds. As a first step in reducing the complexities of natural systems, investigations were restricted to laboratory systems of pure algal cultures with the following specific objectives:

(a) Evaluate the build-up of DOP in solution for various algal species under differing conditions to provide sufficient DOP for investigation while minimizing the residual soluble orthophosphate concentration.

(b) Utilize molecular size separation techniques to characterize and separate the DOP compounds into size fractions for further study.

(c) Examine the culture solutions for the presence of inositol phosphate compounds which are known to comprise a portion of soil and sediment phosphorus.

(d) Explore derivatization of DOP compounds by silylation to form volatile constituents capable of gas chromatographic separation and subsequent identification.

The above objectives were considered preliminary to the longer term goal of extending techniques and results from this study to use of radiolabeled phosphorus for greater detection sensitivity and use of mass spectrometry for individual compound identification for which funding would be sought as Phase II (which has been assurred, July 1, 1974). The com-

bined results of Phases I and II would ultimately lead to an assault upon the more complex natural aqueous environment DOP system.

B. Background: That DOP forms represent a significant and, at times, the major portion of the total phosphorus in natural waters has been established.[1,2] Of this soluble organophosphorus, 15 to 20 percent is of apparent molecular weight equal to or greater than 50,000.[1-3]

A considerable body of information exists attributing excretion of organic phosphorus compounds to various planktonic sources, live zooplankton,[4,8] live phytoplankton,[9-13] and dead decomposing organisms.[14-17] Certainly, release by cellular death and rupture would be expected and is likely the source of dissolved DNA fragments in algal cultures and natural waters,[1,3] despite recent claims to the contrary.[18] However, the build-up of dissolved organic phosphorus in pure algal cultures prior to the initiation of the death phase, clearly indicates the presence of another release mechanism. It has been proposed that this release may represent metabolic waste disposal across the cell wall.[13] Such a mechanism would be consistent with the high turnover rates reported for soluble organic phosphorus and orthophosphate in aquatic systems.

Knowledge about the specific nature of the DOP compounds is sparse. Jeffery et al.[19] demonstrated the presence of phospholipids in seawater, however, there is at least some reason to suspect pressure damage to marine organisms. Although, Kuenzler[20] found 8 to 11 separate compounds in axenic marine algal cultures based upon thin-layer chromatographic studies, cochromatographing known compounds afforded no identification. These studies and Watt and Hayes[14] activated carbon column characterization of DOP comprise the balance of the information regarding the specific nature of these compounds.

Inositol hexaphosphate and the lower phosphates have been the subject of numerous investigations with respect to their presence and abundance in soil and sediment. The former matrix has a history of over sixty years of investigation while research pertaining to sediment inositol phosphate content has evolved only recently. Table 1 summarizes a portion of the soil research literature.

In 1973, Wildung and Schmidt[31] studied the forms of phosphorus in sediments from Upper Kalamath Lake in Oregon, including inositol hexaphosphate while Weimer[32] measured the quantities of inositol phosphates in selected sediments from two Wisconsin lakes and surrounding watershed soils. Weimer found 12 to 63 percent of the total sediment phosphorus attributable to inositol phosphates. Correspondingly, 32 to 91 percent of the soil phosphorus was comprised of these compounds.

Inositol is the common name for hexahydroxycyclohexane, (1, 2, 3, 4, 5, 6-Cyclohexanehexol). It has a molecular weight of 180.6 and a melting point of 249-250^{o} C. The formula for the compound is $C_6 H_{12} O_6$. There are eight possible isomers of the compound, one of which is racemic. It is possible for phosphate groups to form phosphate ester linkages with the inositol molecule. Since there are six such available sites, a maximum of six phosphate groups can form phosphate ester linkages. There are therefore six possible inositol phosphates, the hexa, penta, tetra, tri, di and mono phosphates each of which theoretically may exist as several isomeric forms. The inositol phosphates have been known by several common names. The hexaphosphate of myo-inositol (myo-IHP) has been called phytic acid and also phytin. Others refer to phytin as the mixed calcium-magnesium salt of IHP. These terms are all loosely used and sometimes include other salts as well as lower inositol phosphate esters (Anderson[33]).

Table 1. Summary of Research on Soil Inositol Phosphates

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, DL-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 hypothesicid presence of polymeric forms or binding to high molecular weight materials.	Moyer and Thomas[30]

For clarity and consistency in this manuscript, the term inositol _____ phosphate with the prefix hexa, penta, tetra, tri, di or mono will be used where applicable. The abbreviations, IHP, IPP, ITetP, ITriP, IDP and IMP will be used for brevity. The term "lower inositol phosphates" will refer collectively to all of the inositol phosphates except the hexaphosphate form.

Wheat bran has been found to be a source of inositol diphosphate (R. J. Anderson[21]) and a source of inositol triphosphate (R. J. Anderson[22]). Inositol hexaphosphate occurs in plant tissue and is reported to be the principal source of phosphate in many grains (Anderson[33]). Caldwell and Black[28] demonstrated that a mixed naturally occurring soil micro-organism community when innoculated into soil samples to which inorganic nutrients had been added, produced IHP. No work uncovered to date has demonstrated that algae are a source of inositol phosphates.

The importance of more fully understanding the natural phosphorus cycle is also related to the analysis of orthophosphate in natural aquatic systems. In recent years, the studies of Kuenzler and Ketchum,[34] Rigler,[35] and Chamberlain and Shapiro[36] have presented evidence that the operationally defined[37,38] orthophosphate analysis may much less closely approximate the conceptual or real levels than has been previously thought. Over estimation by 1 to 2 orders of magnitude may in fact be occurring due to contributions by the DOP fraction.

II. METHODOLOGY

A. Algal cultures: Batch cultures were grown in large volume systems under axenic conditions although bacterial presence was not monitored. A schematic of the culture apparatus is given in Figure 1 and the growth medium composition is presented in Table 2. Culture solution volume

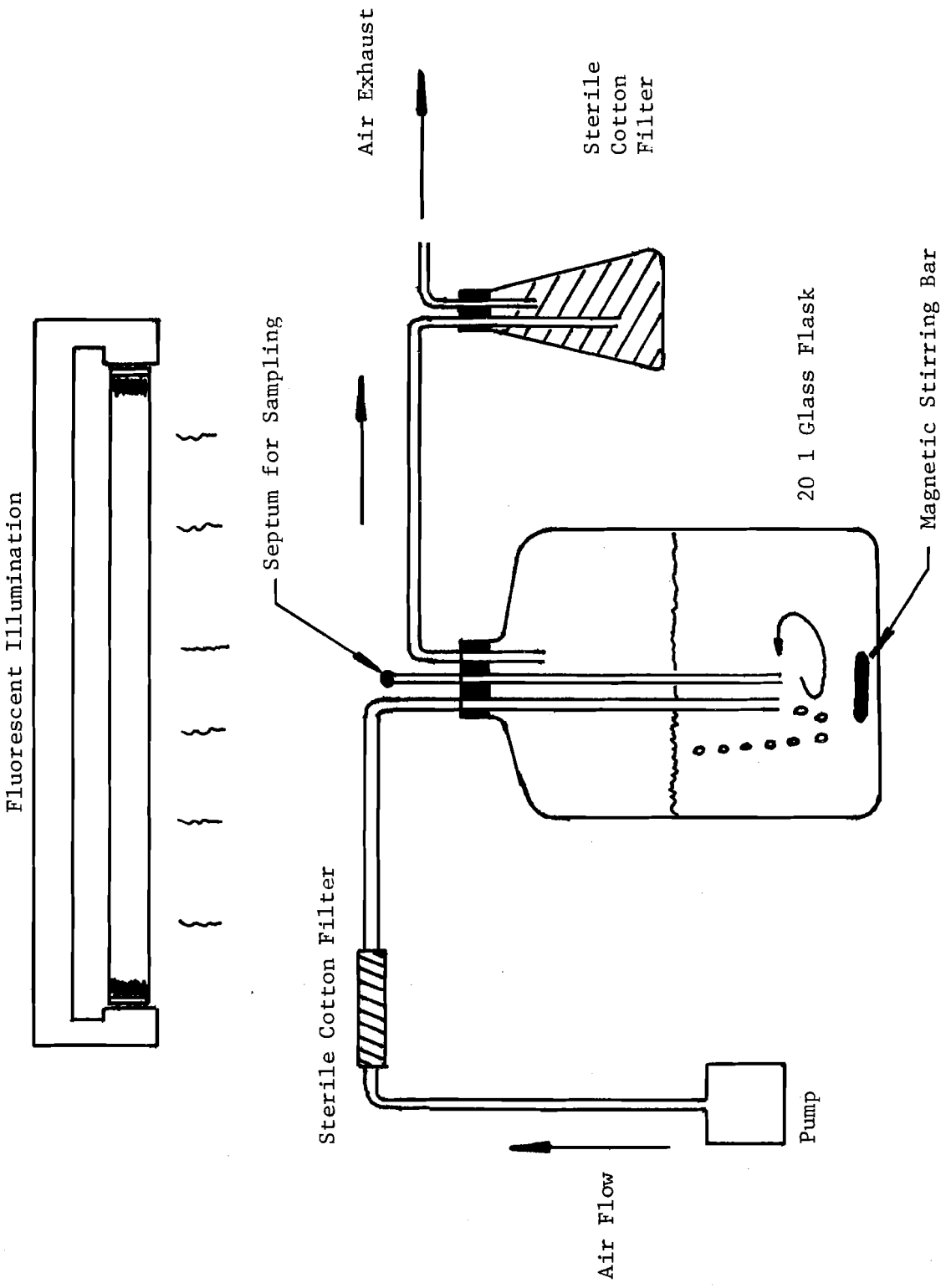

Figure 1. Schematic of Batch Algal Culture Growth Apparatus

Table 2. Composition of Algal Growth Medium.

$NaNO_3$	0.5 mM
$MgSO_4 \cdot 7H_2O$	0.1 mM
NaCl	3.0 mM
$FeSO_4 \cdot 7H_2O$	0.0224 mM
H_3BO_3	0.03 mM
$ZnSO_4 \cdot 7H_2O$	0.01 mM
$MnCl_2 \cdot 9H_2O$	0.01 mM
$Na_2MoO_4 \cdot 2H_2O$	0.001 mM
$CuSO_4 \cdot 5H_2O$	0.002 mM
$Co(NO_3) \cdot 6H_2O$	0.001 mM
NaOH	.07 mM
Na_2 EDTA	.0024 mM
KH_2PO_4	as desired

was initially 10 to 15 liters and all apparatus between the cotton filter systems were sterilized. Trace nutrients capable of precipitation during autoclaving were filter sterilized (0.22 μ membranes) and added to the culture vessel via the septum using sterile technique. Culture inoccula-tion was accomplished in similar fashion.

Incubation was under constant temperature of 20 ± 1° C and fluorescent lighting (conventional, 2 tube, 4 foot units suspended vertically above the culture vessels at a distance of 18 inches).

At periodic intervals, a culture medium sample was withdrawn aseptically via the septum and analyzed for phosphorus forms using the Murphy and Riley[39] ascorbic acid method for orthophosphate and the Menzel and Corwin[40] wet combustion technique for conversion of organic phosphorus to orthophosphate. Phosphorus analysis were preceded by filtration through 0.45 μ membrane filters at low pressure differential.

Culture solutions taken for further investigation were processed and ultimately freeze dried as previously described by Minear.[1,3]

B. Molecular Fractionation: Sephadex gel filtration chromatography was employed for molecular size characterization and separation of DOP compounds.

Gel chromatography is a relatively recent development (within the past 10 years) in liquid phase chromatography. The "gel" which forms the solid phase is a product of the reaction of epichlorohydrin with a carbohydrate polymer known as dextran. Dextran is produced by the bacterium, Leuconostoc mesemteroides while growing on sucrose. The gel produced in this manner is commonly referred to as dextran gel and while its exact chemical structure is not known, it is believed to be of the form shown in Figure 2.

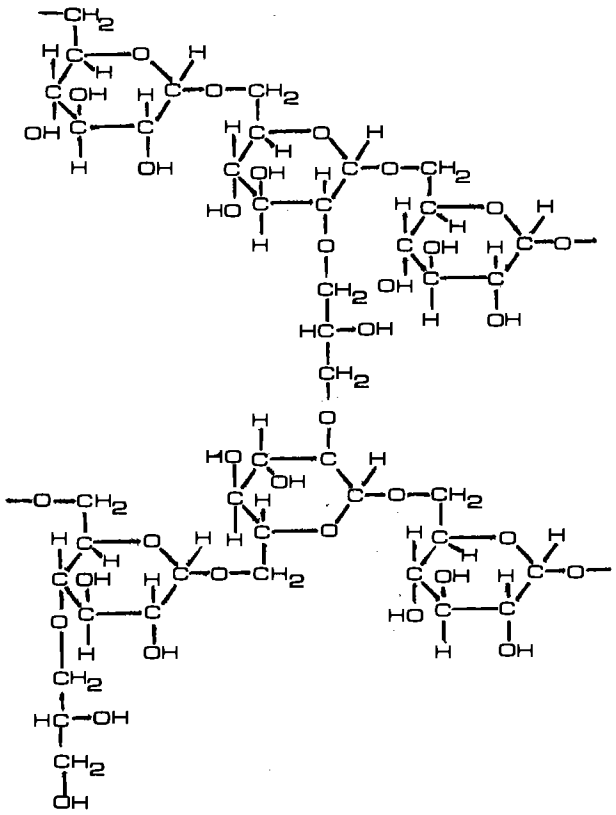

Figure 2. Proposed Structural Element of Dextran Gels. (reproduced from Determann[41])

By controlling the relative amounts of epichlorohydrin and dextran during the reaction, the degree of cross-linking between the dextran chains can be varied. When the dextran gel is put in a suitable solvent, it swells and creates "pores." The degree of swelling of the gel and consequently the size of the pores is a function of the degree of cross-linking between the dextran chains. Thus, gels with various pore sizes can be manufactured. The presence of pores is the basis for the ability of the gel to separate solute molecules on the basis of molecular size. Dextran gels are supplied in the form of beads. They are commercially available under the trade name Sephadex from Pharmacia Fine Chemicals, Uppsala, Sweden.

The separation of solute molecules or the basis of molecular size by gel chromatography can be visualized by a comparatively simple model. Figure 3 shows a schematic representation of separation of two different sized molecules by means of gel chromatography. The small black dots represent the smaller (in actual molecular size) molecules while the large black dots represent the larger molecules. The large white dots represent the swollen gel beads. The mixture of the larger and smaller molecules is applied at the top of the column and an eluent reservoir (not shown) continually applied an eluent to the top of the column. In (A) the solute mixture begins its passage through the column. Since the gel beads have pores of various sizes, the solute molecules can diffuse into the beads providing the largest pore size is larger than the biggest molecule. If, however, some solute molecules are larger than the largest pores, they <u>cannot</u> diffuse into the gel bead and are said to be <u>excluded</u> from the gel. To simplify the example in Figure 3, it will be assumed that this is the case for the large black dots. In (B), the eluent has

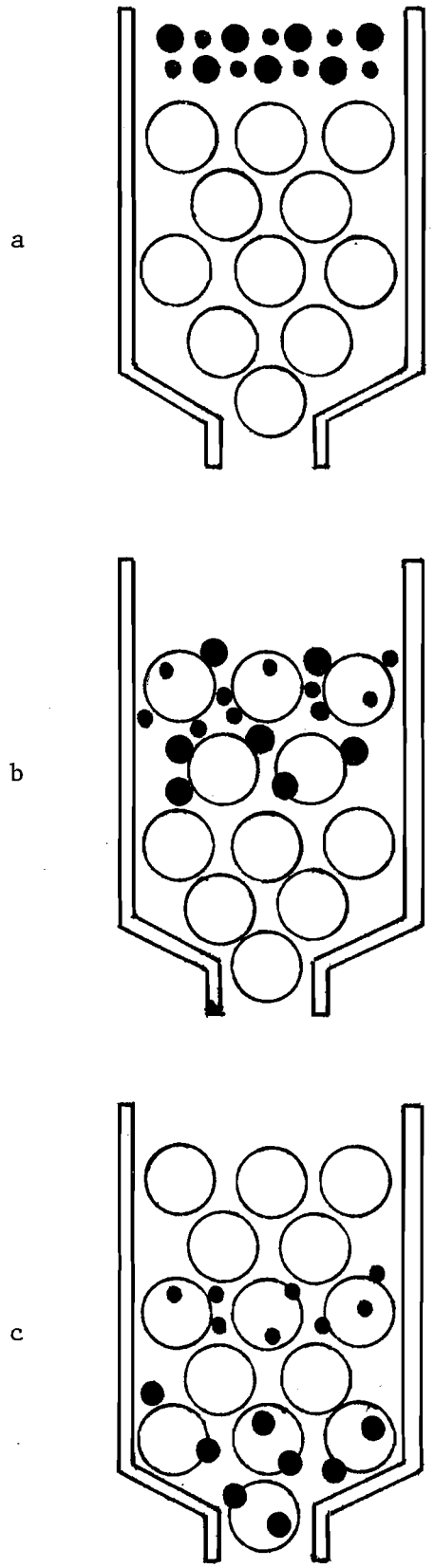

Figure 3. Schematic Representation of Gel Chromatography in Three Phases

transported the large molecules further down the column but the small molecules now must diffuse out of the gel beads before they can continue down the column. As the process repeats itself, the larger molecules become further separated from the smaller ones. In (C), there is a complete separation of the smaller from the larger molecules. The larger molecules will therefore be eluted from the column ahead of the smaller molecules.

A simple way to view the separation of molecules is to observe the volume of solvent which the molecules can occupy. The largest molecules cannot penetrate the pores of the gel and therefore cannot diffuse into them. The maximum volume they can occupy is that which surrounds the gel. This volume is called the Exclusion Volume or V_o. The smaller molecules can diffuse into the pores, and depending on relative sizes of the smallest pores and the smallest molecule (or ion), can diffuse into a maximum volume equal to the volume around the gel beads plus the solvent volume inside the gel beads. This is called the Inner Volume or sometimes the Inclusion Volume (V_i). The remaining volume is the volume of the gel matrix itself, (V_m). This can be seen mathematically as

$$V_t = V_e + V_o + V_m \qquad (1)$$

As a consequence of diffusion being the mechanism for separation of solute molecules, it follows, therefore, that solute molecules will be eluted in order of decreasing molecular size provided that there is no interaction between the solute molecules and the gel matrix. Certain conditions are necessary to ensure this lack of interaction among which is maintenance of adequate ionic strength in the eluting solution (Determann[41], Minear[3]). The volume at which a specie is eluted is simply called the Elution Volume, (V_e). The V_e of a specie may be equal to V_o

or V_i or maybe somewhere in between. Table 3 lists the elution parameters which are most commonly used in gel chromatography studies.

An important distinction must be made between the molcular size and molecular weight of a substance. While an increasing molecular weight, especially of complex biological substances, usually means an increase in molecular weight, this is an oversimplification. Table 4 lists the various forms of Sephadex gels available and the fractionation range for dextrans. These fractionation ranges for dextrans can only be taken as approximate fractionation ranges for other molecular species. Association with water molecules, molecular geometry, solvent-solute interaction and lastly the possible interaction of the solute specie with the gel matrix may not result in the same fractionation ranges for other molecular species. To assign a molecular weight to an unknown substance on the basis of its elution volume from a gel, is not a valid procedure.

Gel studies were conducted in upward flow columns using in-line sample injection by three way valve. Elution was accomplished with 0.05 N NH_4HCO_3 to maintain basic conditions and adequate ionic strength. Individual fractions were collected for analysis by an automatic fraction collector.

C. Inositol Phosphate: Preliminary work with thin-layer chromatography on cellulose layers was abandoned due to inadequate detection sensitivity for inositol hexaphosphate either by phosphate detection or residual inositol after hydrolysis. Consequently, no presentation of this methodology is included.

Instead, alkaline bromination procedures employed in soil chemistry investigations were combined with gel chromatography to assay the presence of inositol phosphates. This consisted of obtaining G-25 profiles before and after alkaline bromination while other organic compounds, including the organphosphate, would be destroyed.

Table 3. Parameters for the Description of the Elution Behavior in Gel Chromatography

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	$K_d = (V_e - V_o)/V_t$ V_i (volume inside of the gel grains); dependent on the amount of dry gel and its solvent regain upon swelling (S_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).
K_{av}	$K_{av} = \frac{V_e - V_o}{V_t - V_0}$	independent of the geometry and packing of density of the column; all columns are easily measured; greatest accuracy for large V_e (small molecules).

Table 4. Properties of Commercial Dextran Gels, Sephadex*)

Type	Particle size**) (dry; in μ)	Water regain (ml/g)	Gel Bed (ml/g)	Approximate Separation Range Peptides and glob. proteins	Dextran fractions
G-10	40-120	1.0±0.1	2-3	up to 700	up to 700
G-15	40-120	1.5±0.1	2.5-3.5	up to 1,500	up to 1,500
G-25, coarse	100-300				
G-25, medium	50-150	2.5±0.2	4-6	1,000- 5,000	100- 5,000
G-25, fine	20-80				
G-50, coarse	100-300				
G-50, medium	50-150	5.0±0.3	9-11	1,000- 30,000	500- 10,000
G-50, fine	20-80				
G-75	40-120	7.5±0.5	12-15	3,000- 70,000	1,000- 50,000
G-100	40-120	10.0±1.0	15-20	4,000-150,000	1,000-100,000
G-150	40-120	15.0±1.5	20-30	5,000-400,000	1,000-150,000
G-200	40-120	20.0±2.0	30-40	5,000-800,000	1,000-200,000

*) According to statements by the manufacturer: Pharmacia Fine Chemicals, Uppsala (Sweden).
**) All types are also available in the particle size >>Superfine<< (10-40 μ).

The general alkaline bromination procedure consisted of the following:

1. To a particular volume of sample (5.7 - 98 ml) were added 10 ml of 5 N NaOH and 10 ml of saturated bromine water (excess bromine plus distilled water allowed to equilibrate for 24 hours).

2. Heated to boiling for one hour with periodic addition of distilled water as necessary.

3. Neutralization to pH 6.5 - 6.8 with 6 N HCl.

4. Filtration to remove precipitation formed upon neutralization.

When variations in the above procedure were used, they are indicated in the results section.

Of four specific chemical tests for inositol and inositol phosphates (Feigl,[42] Fischler and Kurten,[43] Nagui and Kimura[44]) the following procedure from Feigl was used:

"On a spot plate, a drop of aqueous test solution is taken to dryness with a drop of concentrated nitric acid. A drop of 5 percent calcium chloride and a drop of concentrated ammonia are added to the residue. After evaporation, the spot plate is kept at 180°. According to the quantity of inositol present, a brown red to light red residue is obtained at once or after 4-10 minutes at the most. Limit of identification 2.5 µgm inositol."

D. Trimethylsilyl Derivitization: The formation of trimethylsilyl ethers and esters has been used extensively to increase the volatility of polar hydroxy and acidic compounds for gas chromatographic analysis. A review of these procedures and a bibliography of specific applications has been assembled by Pierce.[45] Specific applications to phosphate compounds have been described by Hancock[46] for adenosine derivations, various

riboncleotides by Hashizume and Sasaki,[47] sugar phosphates by Sweeley et al.,[48] Horning et al.,[49] Eisenberg and Bolden,[50] and Hashizume and Sasaki.[51] Aqueous orthophosphate conversion to the TMS derivative has been reported by Wiese and Hanson[52] and Butts and Rainey[53] converted orthophosphate and several other oxygen anionic species to volatile TMS derivations for simultaneous analysis by gas-liquid chromatography.

Derivitization studies were conducted by addition of solvent (Dimethylformamide or pyridine TMS grade, Pierce Chemical Company) to the solid sample followed by addition of Hexamethyldisilane (HMDS) and trimethylchlorosilane (TMCS) according to Sweeley et al.[48] or Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) according to Butts and Rainey[53] in serum capped vials. Known compounds and culture solids were either used directly or were converted to the ammonium salts with a cation exchange resin on the ammonium cycle followed by lyophilization to remove water.

Chromatography was conducted on a Varian 1740 gas chromatograph using all glass systems containing various liquid pluses. Flame ionization detection was used generally although phosphorus specific detection with the alkali flame ionization detector was attempted.

III. ALGAL PRODUCTION OF DOP

Four algal species (Chlamydomonas reinardtii, Chlorella pyreniodosa, Anabaena flos-aqua, and Navicula pelliculosa) were grown in pure culture under various conditions of initial orthophosphate concentration and with or without CO_2 supplementation. Variation in initial orthophosphate concentration was examined in order to obtain a balance between the organic phosphorus levels achieved and the residual orthophosphate concentration remaining in solution. At high initial orthophosphate concen-

tration, sufficient in organic phosphorus remains in solution such that the DOP material is not reutilized for continued algal growth.[1] However, under these conditions, organic phosphorus evaluation becomes difficult from any analytical standpoint because of the analysis methodology. When the initial orthophosphate level is too low, complete uptake of orthophosphate is experienced, the DOP fraction seems to be reutilized as a phosphorus source, and low DOP levels result.

Supplementation of the air stream with pure CO_2 (2-4 percent by volume) resulted in rapid algal growth in some cases, substantiating previous work[1,3] but was abandoned because of CO_2 flow control problems encountered. This resulted in erratic growth behavior and inhibition of cultures in some cases. Instead, slower growth rates were accepted utilizing only atmospheric air.

Preliminary studies with roughly 6 mg/l of orthophosphate demonstrated that erratic results for organic phosphorus levels were obtained which were likely attributable to analytical imprecision i.e., subtraction of two large numbers (total P - ortho P = DOP). The balance of the cultures were grown with initial orthophosphate levels of 3-4 mg/l.

Of the cultures studied, Navicula proved to grow so slowly and exhibited much less orthophosphate uptake than the other three cultures. Thus, while appearing to produce DOP, the data were judged unreliable and further work was conducted with the remaining three algae. Representative results for Chlorella pyreniodosa and Anabaena flos-squa are presented in Figure 4-8. Results for Chylamydomonas reinhardtii duplicated previous findings by Minear[1,3] with the only difference being slower growth rates in the absence of CO_2 supplementation.

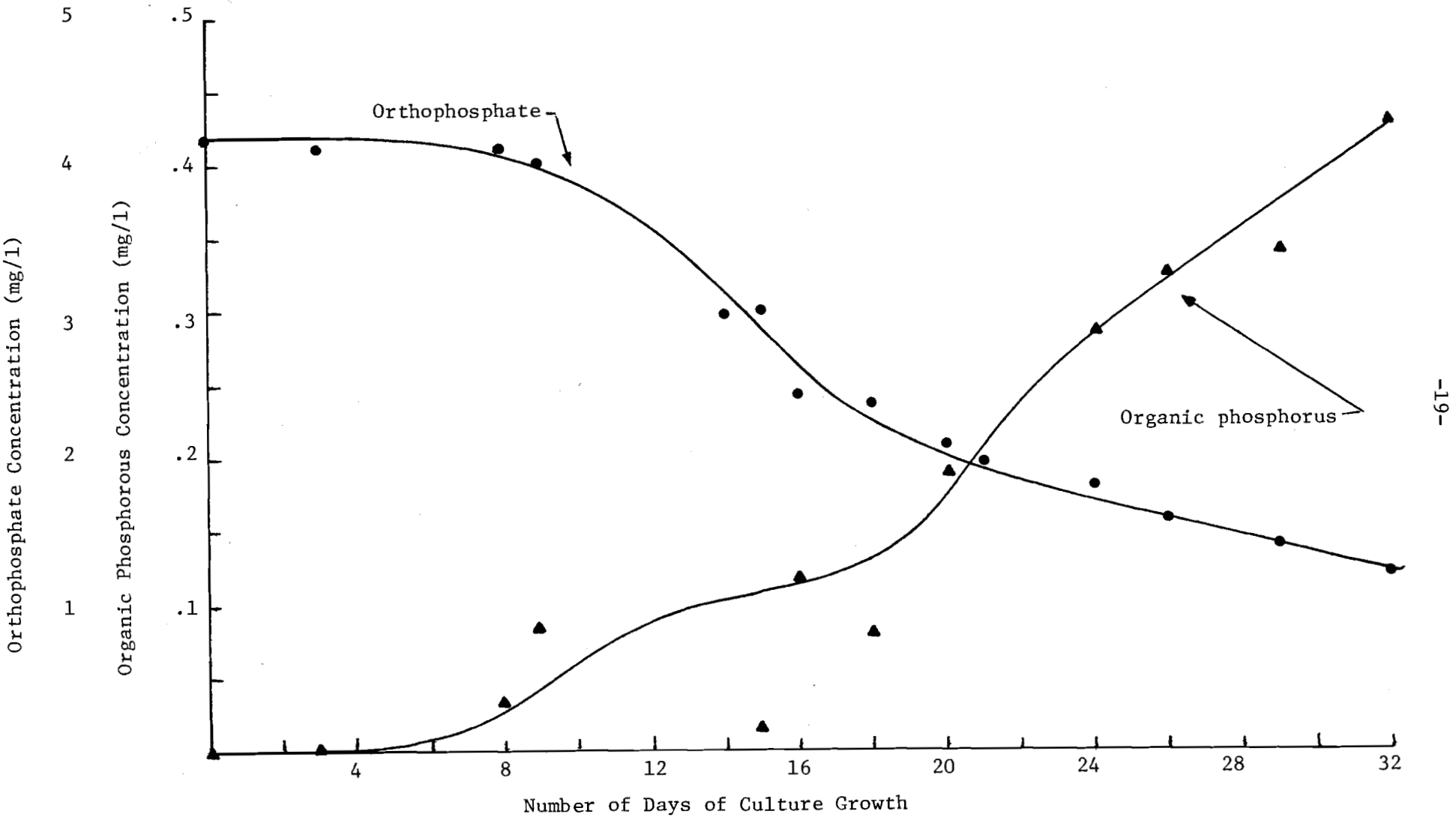

Figure 4. Orthophosphate Uptake and DOP Production by Chlorella Culture No. 1

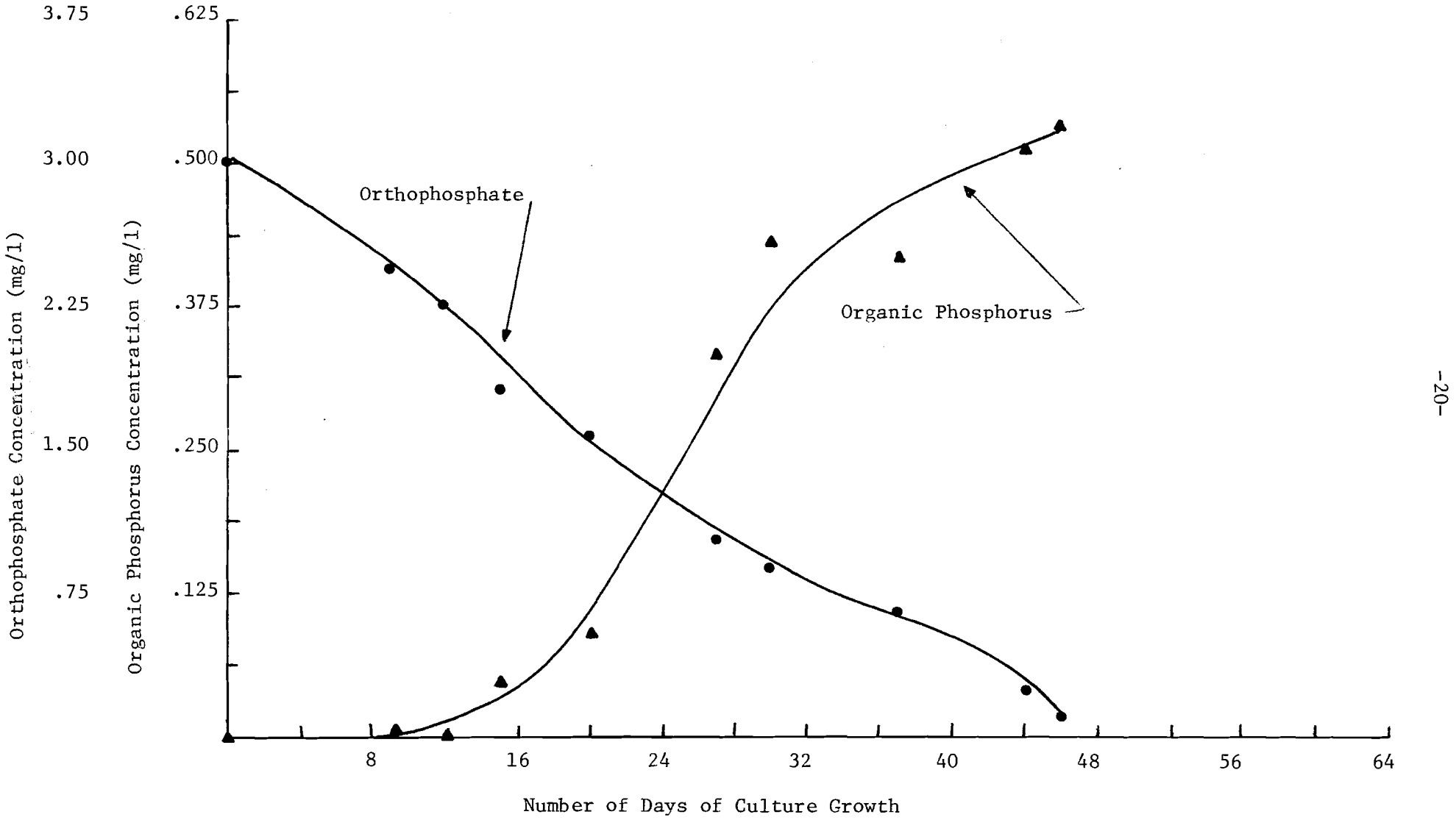

Figure 5. Orthophosphate Uptake and DOP Production by Chlorella Culture No. 2.

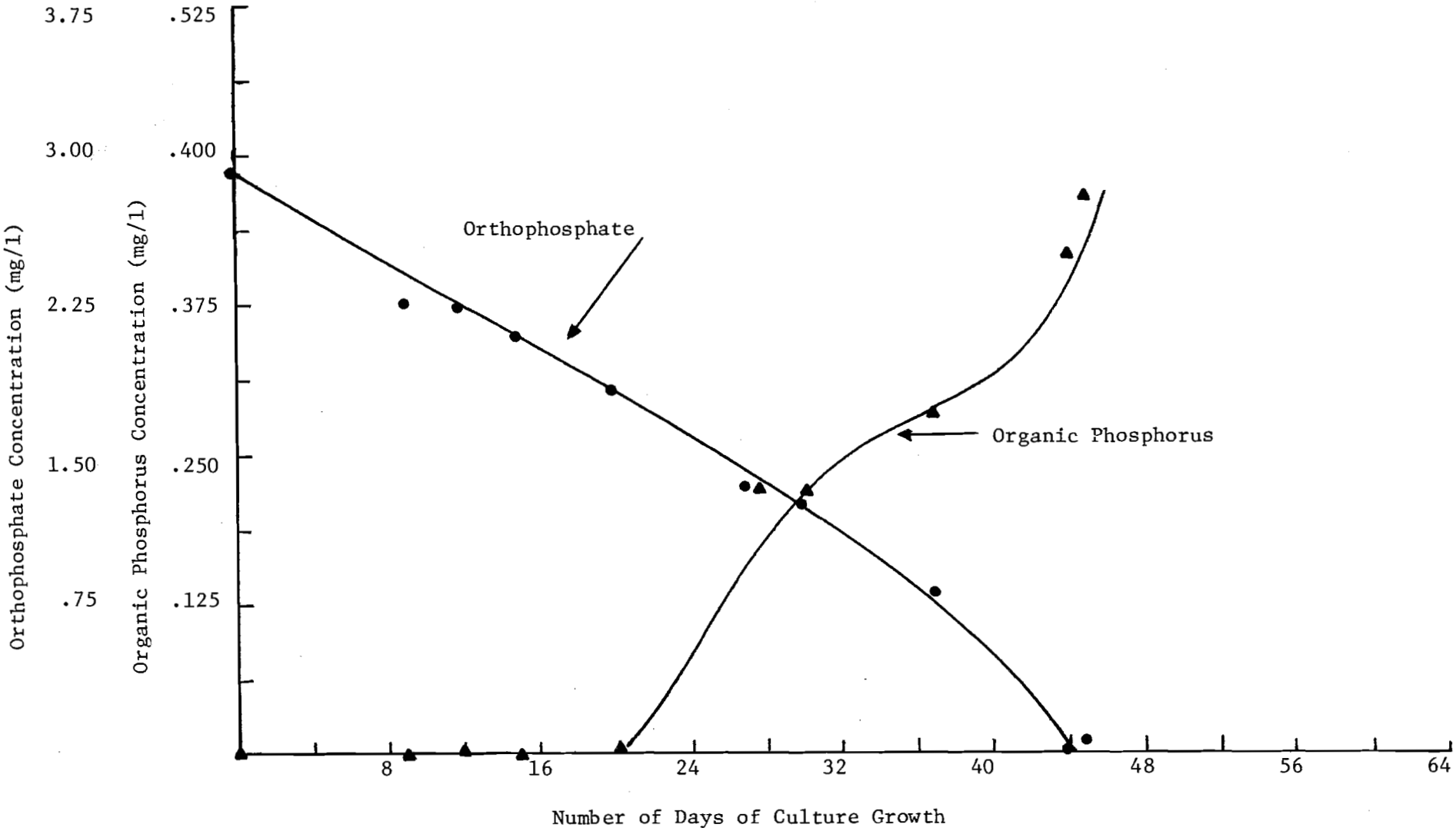

Figure 6. Orthophosphate Uptake and DOP Production by Chlorella Culture No. 3.

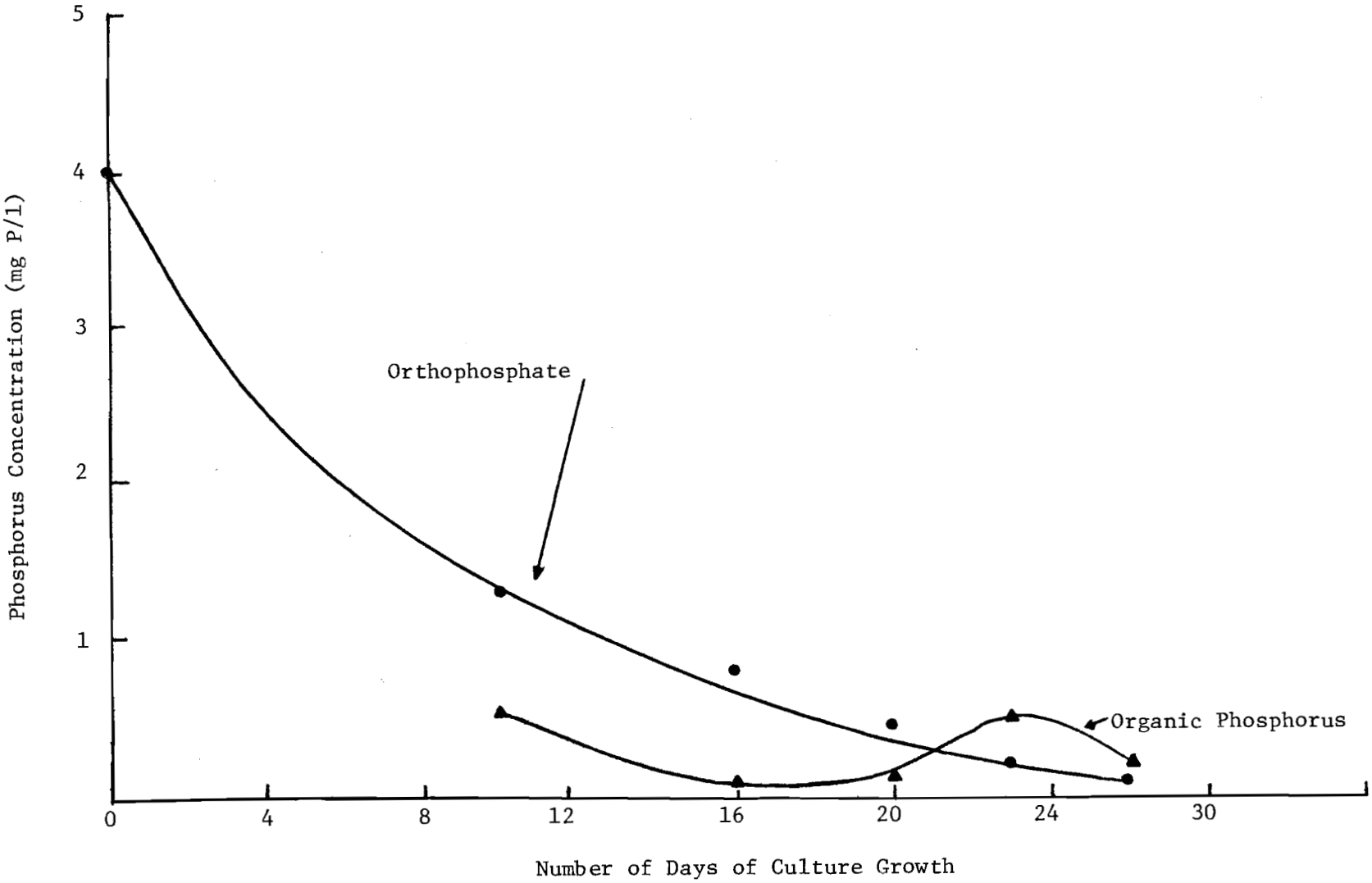

Figure 7. Orthophosphate Uptake and DOP Production by Chlorella Culture No. 4

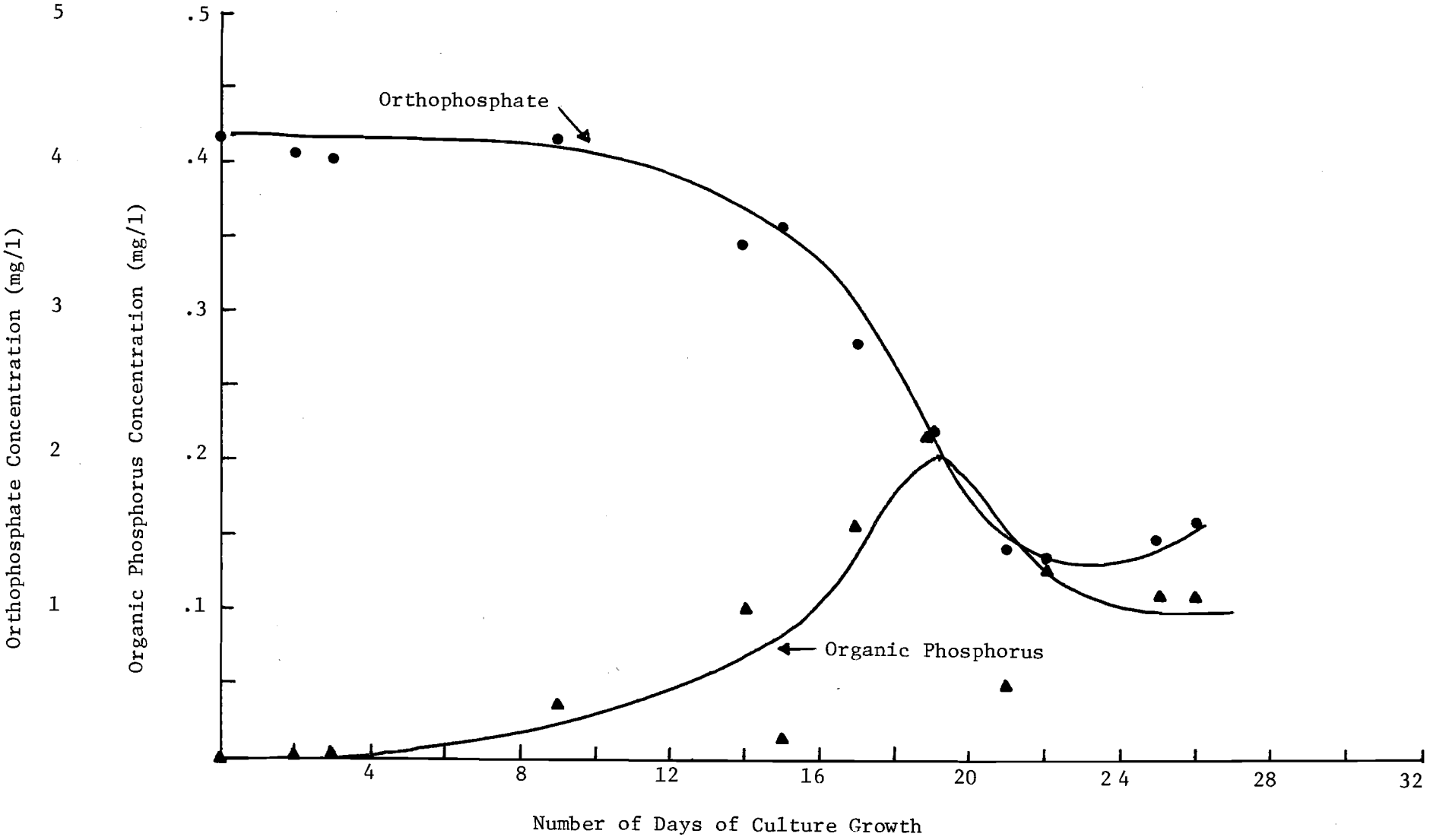

Figure 8. Orthophosphate Uptake and DOP Production by Anabaena Culture No. 1.

In all cases, the build-up of DOP required one to two weeks and thus is likely attributable primarily to release by cells that have died. The point of these investigations was not to define a mechanism for release but to establish conditions for obtaining DOP concentrations sufficient for further examination. From the data, it is apparent that DOP concentration of 0.1 to roughly 0.5 mg/l can be readily achieved and with proper selection of initial orthophosphate levels can be made to represent the major proportion of the total soluble phosphate.

A summary of culture media processed for further study is presented in Tables 5 and 6. These samples were utilized for further investigation in the Sephadex fractionation and inositol phosphate studies to follow. Data on the percent recovery of orthophosphate and organic phosphate from reconstituted solids is presented in Table 7.

IV. SEPHADEX GEL FILTRATION

Sephadex G-75, G-25, G-15 and G-10 gels were examined with respect to their separation capabilities with known compounds and DOP compounds. The latter materials were reconstituted in concentrated solution by dissolution of the freeze dried culture isolates. Physical dimensions of the G-75, G-25 and G-10 columns are presented in Table 8.

A. G-75 Results: Figure 9 provides definition of the void volume as determined by Blue Dextran 2000 (a dyed dextran of known molecular weight greater than 2×10^6) and the inclusion volume, which corresponds to the elution position of orthophosphate. It can be seen that inositol hexaphosphate is resolved from the orthophosphate peak.

DOP from the Chlamydomonas culture No. 1 was found to duplicate results previously reported by Minear[1,3] and are not presented. Figures

Table 5. Final Algal Culture Media Conditions and Recovery Data for Solids.

Culture	Number of Days of Growth	Final Ortho P Concentration mg/l	Final Organic P Concentration mg/l	% Organic P	Total Vol. of Media Collected	Weight of solids recovered
Anabaena #1	26	1.6018	0.1093	6.8%	8000 ml	1.726 gm
Chlorella #1	34	1.1857	0.4303	26.6%	8000 ml	1.707 gm
Chlorella #2	46	0.0964	0.5302	84.6%	8500 ml	1.597 gm
Chlorella #3	45	0.0750	0.4659	86.1%	8700 ml	1.750 gm
Chlorella #4	28	.085	.200	70.2%	6000 ml	0.97 gm
Chlamydomonas #1	41	.100	.068	40.5%	6000 ml	1.114 gm

Table 6. Algal Culture Subsample Inventory.

Culture	Fraction	Weight of Solids Recovered	Volume of Media in the Fraction
Anabaena #1	A	578 mg	3000 ml
	B	730 mg	3000 ml
	C	418 mg	2000 ml
Chlorella #1	A	641 mg	3000 ml
	B	676 mg	3000 ml
	C	390 mg	2000 ml
Chlorella #2	A	437 mg	3000 ml
	B	460 mg	3000 ml
	C	700 mg	2500 ml
Chlorella #3	A	639 mg	3000 ml
	B	567 mg	3000 ml
	C	544 mg	2700 ml
Chlorella #4	A	467 mg	3000 ml
	B	504 mg	3000 ml
Chlamydomonas #1	A	534 mg	3000 ml
	B	580 mg	3000 ml

Table 7. Summary of Phosphorus Concentrations in Reconstituted Algal Culture Solids Solutions.*

Algal Culture	Ortho P, µg			Organic P, µg		
	Theoretical*	Recovered	%	Theoretical*	Recovered	%
Anabaena #1A/B	9611	1710	17.8	656	171	20.3
Anabaena #1C	3203	2340	72.7	219	128	58.5
Chlorella #1B	3555	1186	33.0	1290	760	59.0
Chlorella #1C	2372	670	28.2	860	448	52.2
Chlorella #2C	269	60	22.3	1485	924	62.2
Chlorella #3A	225	71	31.5	1398	710	50.8
Chlorella #4A/B	510	48	9.4	1200	519	43.2

*Theoretical value based upon 100% recovery of material from original solution volume.

Table 8. Sephadex Column Dimensions

Gel	Column Diameter, cm	Gel Bed Height, cm	Eluent	Flow Configuration
G-10	1.5	77	0.05 N NH_4HCO_3	Downward
G-25 (medium)	2.6	78	0.05 N NH_4HCO_3	Upward*
G-75	5.0	87	0.05 N NH_4HCO_3	Upward*

*Special upward flow adaptors utilized.

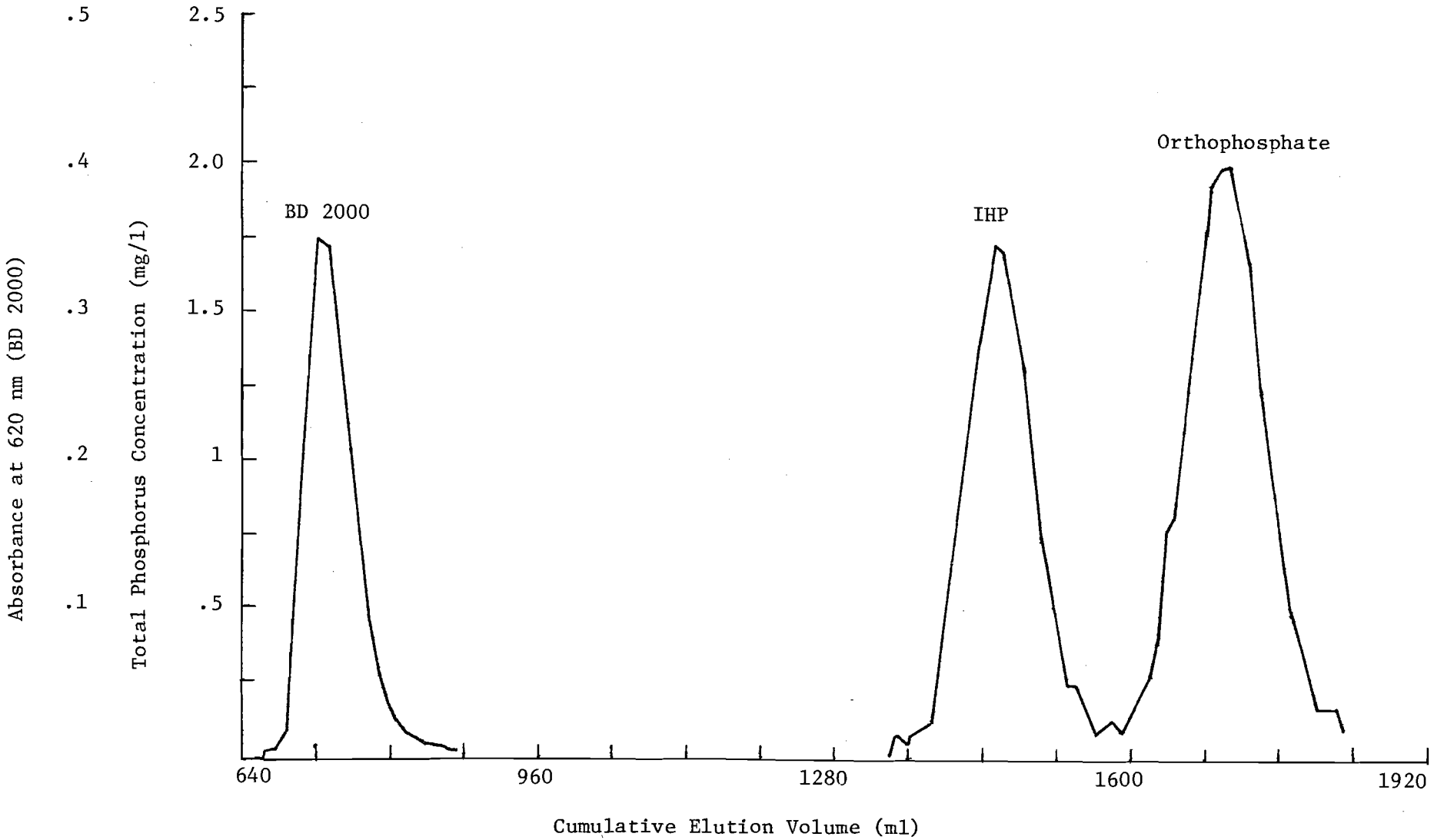

Figure 9. G-25 Chromatogram of Blue Dextran 2000, IHP and Orthophosphate

10 and 11 show the behavior of Chlorella No. 1B and 4A/B DOP on Sephadex G-75. The bulk of the DOP is obviously relatively low molecular weight material although a small amount of DOP is excluded from the column and some material is present which corresponds to the intermediate molecular weight range.

The low molecular weight peaks from the Chlamydomonas and Chlorella systems were collected, freeze dried and redissolved for further investigation on other gels.

B. G-10 Results: The void volume determination for the Sephadex G-10 column shown in Figure 12 and the elution profile for the G-75 low molecular weight fraction of the Chlorella No. 4A/B DOP shown in Figure 13 indicate that no separation of orthophosphate from DOP was achieved on the G-10 gel. The primary reason for this lack of separation was due to essentially complete exclusion of the orthophosphate and the DOP from the G-10 gel. Very similar results were experienced with the G-15 gel for the G-75 low molecular weight fraction collected from the Chlamydomonas No. 1 system.

C. G-25 Results: Figure 14 demonstrates the separation characteristics of the Sephadex G-25 gel with respect to known phosphorus compounds. This particular gel was deemed the most desirable for separation and characterization purposes since the high and intermediate molecular weight materials (between IHP and the inclusion peak of the G-75 profile) would be excluded from the gel but the bulk of the culture DOP would be expected to elute between the position of the IHP and orthophosphate. This observation can be made by examining the relative elution of the DOP peak and IHP on the G-75 column.

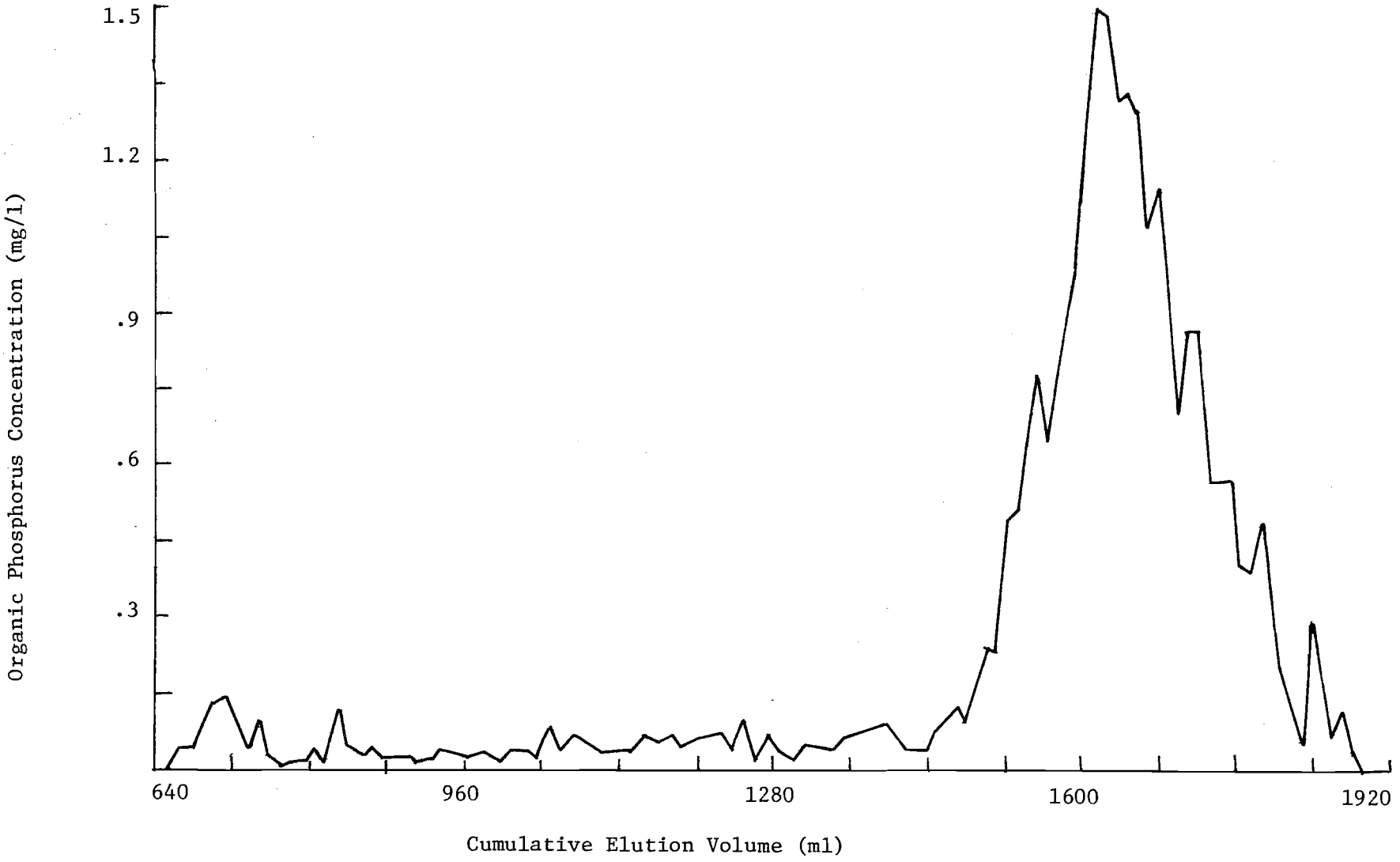

Figure 10. G-75 Chromatogram of Chlorella No. 1, Fraction B, Media Solids

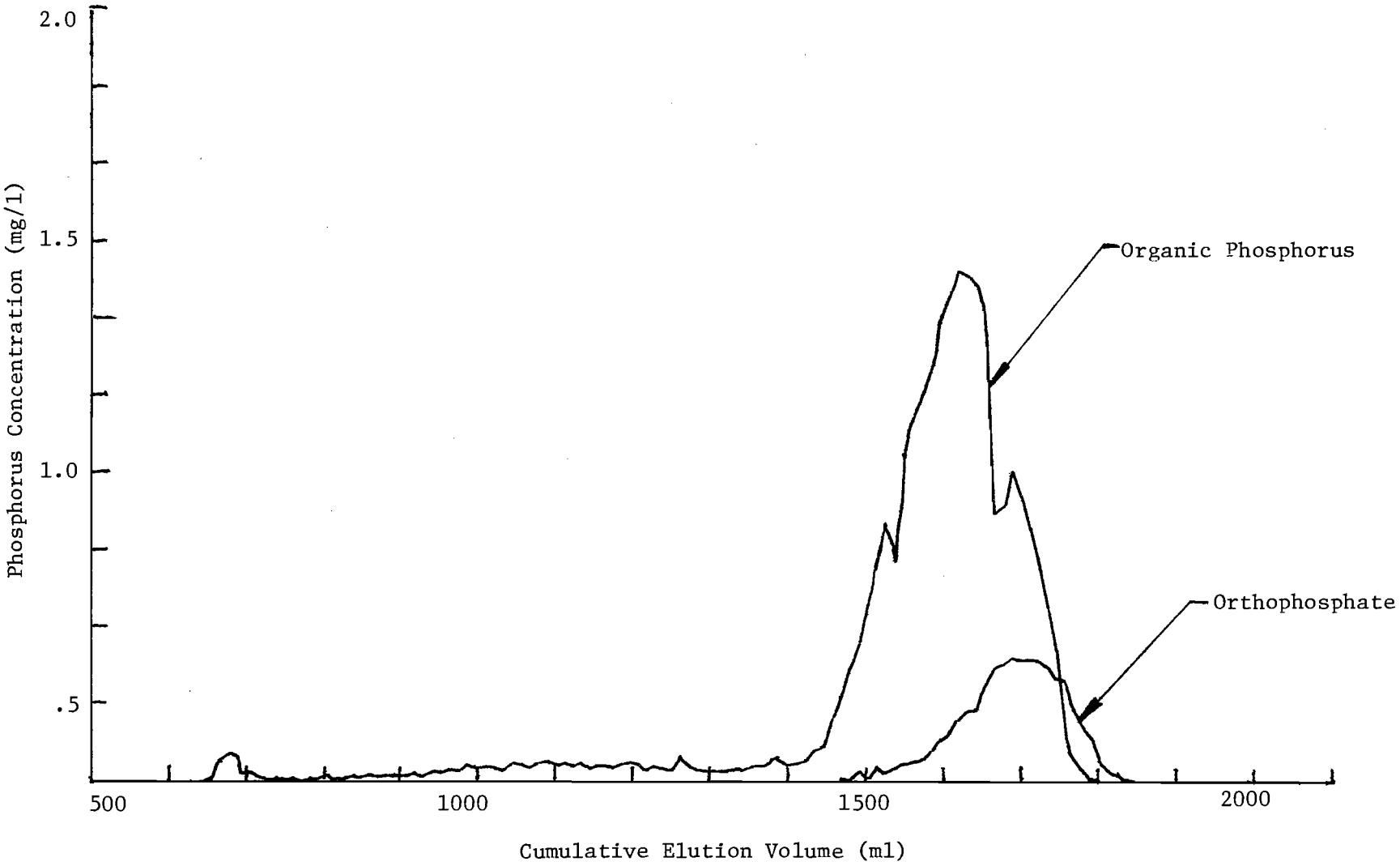

Figure 11. G-75 Chromatogram of Chlorella No. 4, Fractions A and B, Media Solids

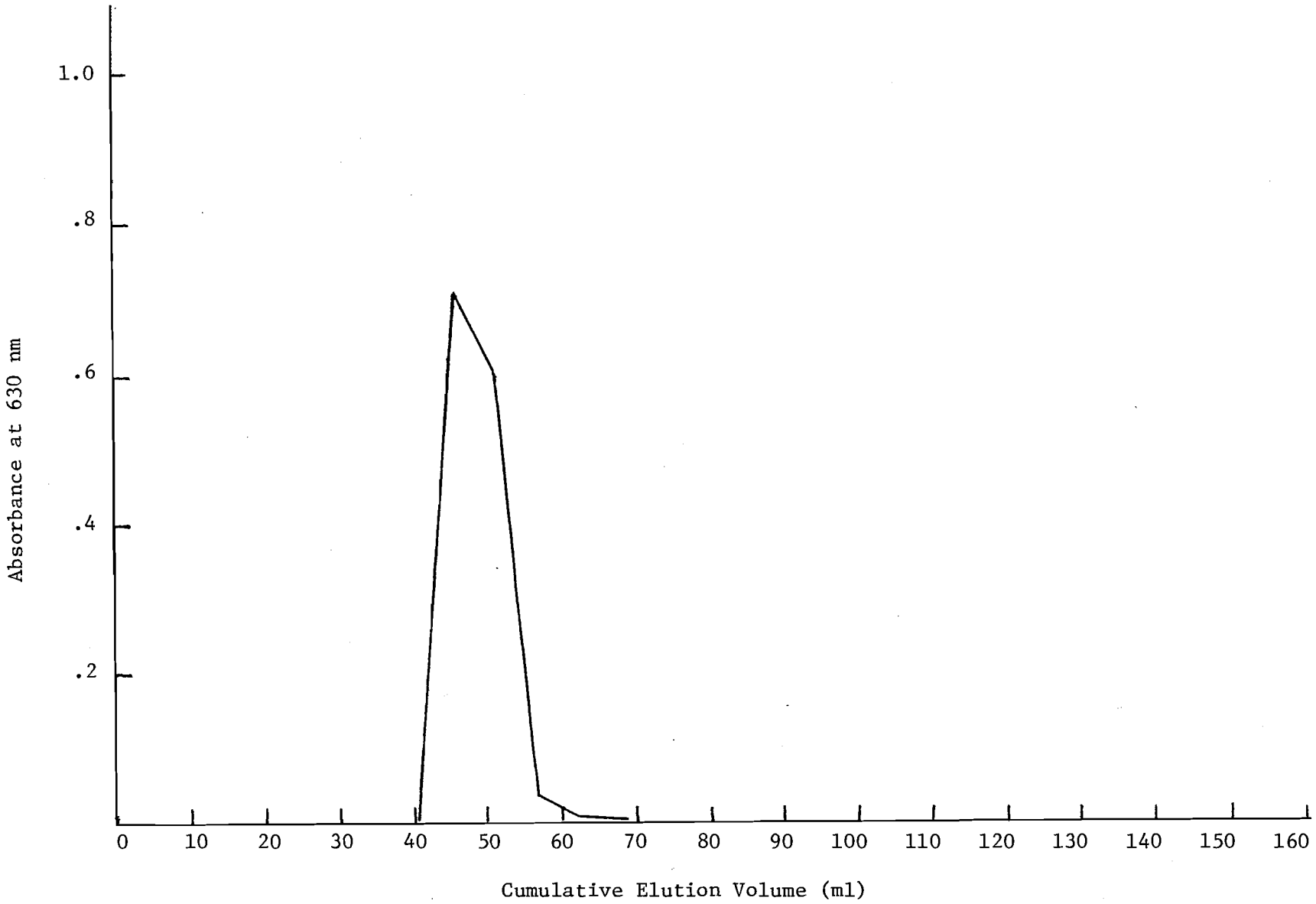

Figure 12. G-10 Void Volume Definition with Blue Dextran 2000

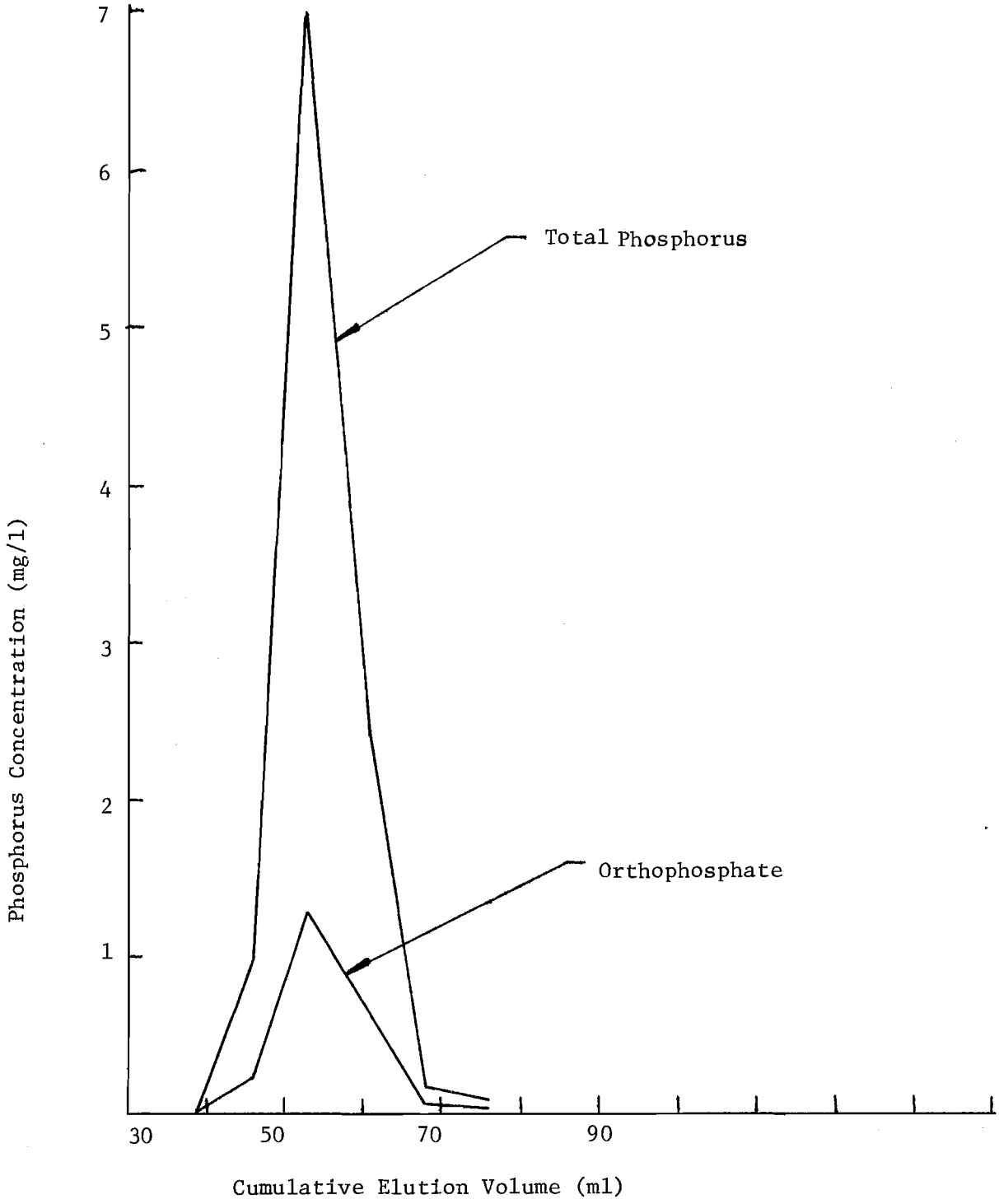

Figure 13. G-10 Chromatogram of G-75 Low Molecular Weight Fraction of Chlorella No. 4, Fractions A and B, Media Solids

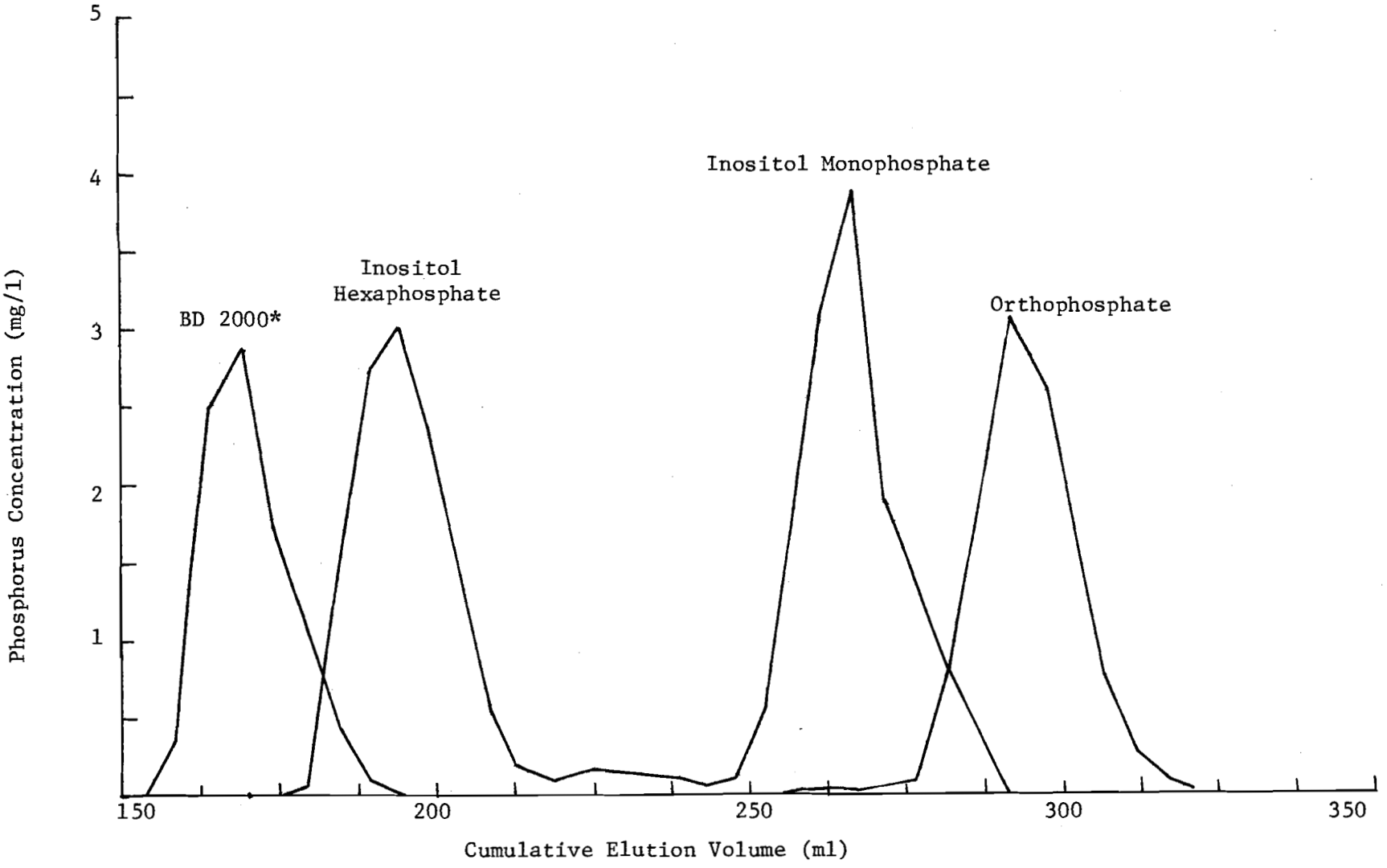

Figure 14. G-25 Chromatogram of Blue Dextran 2000, IHP, IMP, and Orthophosphate

*(Determined by absorbance at 620 nm. Ordinate does not apply)

Intermediate Inositol Phosphates

Also of interest are the elution volumes of the intermediate inositol phosphates; i.e., IDP, ITriP, ITetP, and IPP. Because these compounds were not found to be commercially available and work with enzymes to breakdown IHP was considered beyond the scope of this investigation, some other estimate of their elution volumes was sought.

Determann[41] reports that the elution volumes of substances on Sephadex are a function of the log of the molecular weight and is linear with respect to the parameter K_{av} where:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

and:

V_e = elution volume of substance

V_o = exclusion volume of column

V_t = total volume of column

A plot of the log of molecular weight vs K_{av} is usually linear. Consequently, if one assumes the inositol phosphates follow this phenomenon, it is possible to predict the elution volumes of the intermediate inositol phosphates when the V_e of the IHP and IMP are known. By calculating the K_{av}, these two points can be plotted. A straight line is then drawn between them and the K_{av} for the intermediate inositol phosphates can be found. From the K_{av}, the elution volumes can be calculated, because the molecular weight of the individual inositol phosphates is known. Table 9 gives the results of this calculation and plot.

Table 9. Measured and Calculated Elution Volumes, and K_{av} for the Inositol Phosphates.

Compound	Molecular Weight	Log MW	K_{av}	Elution Volume ml
IHP	660	2.819	.1023	190
IPP	580	2.763	.1550	201
ITetP	500	2.699	.2100	213
ITriP	420	2.623	.2750	227
IDip	340	2.531	.3500	243
IMP	260	2.412	.4512	265

Since intermediate inositol phosphates were unavailable, there was no way to test the hypothesis that a plot of log MW vs K_{av} would be linear. However, some estimate of the elution volumes is needed and when reference is made to a peak possibly being an intermediate inositol phosphate it should be kept in perspective.

Actual culture DOP molecular size distributions on Sephadex G-25 are presented in Figures 15, 16 and 17 for two separate Chlorella and one Anabaena culture respectively. Figures 16 and 17 demonstrate the importance of maintaining low orthophosphate concentrations in the cultures as the organic response truncates in Figure 15, quite likely due to loss of analytical sensitivity.

An important feature of these profiles is that significant quantities of DOP are absent in the elution region of inositol hexaphosphate. Equally significant is that appreciable DOP is present at elution positioning corresponding to IMP and IDP. These observations led to ensuing investigations to evaluate the presence of lower inositol phosphates in the culture media.

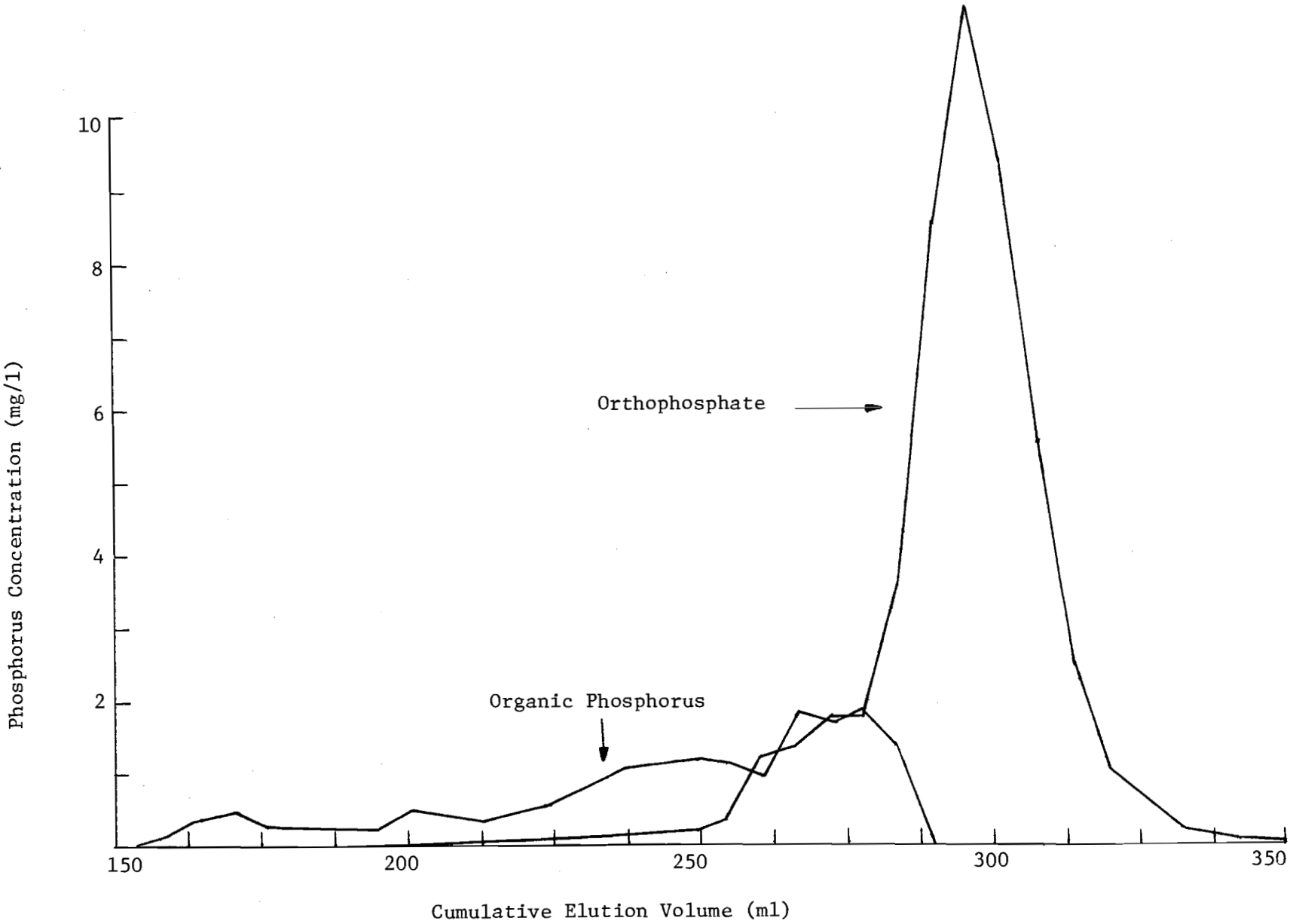

Figure 15. G-25 Chromatogram of Chlorella No. 1, Fraction C, Media Solids

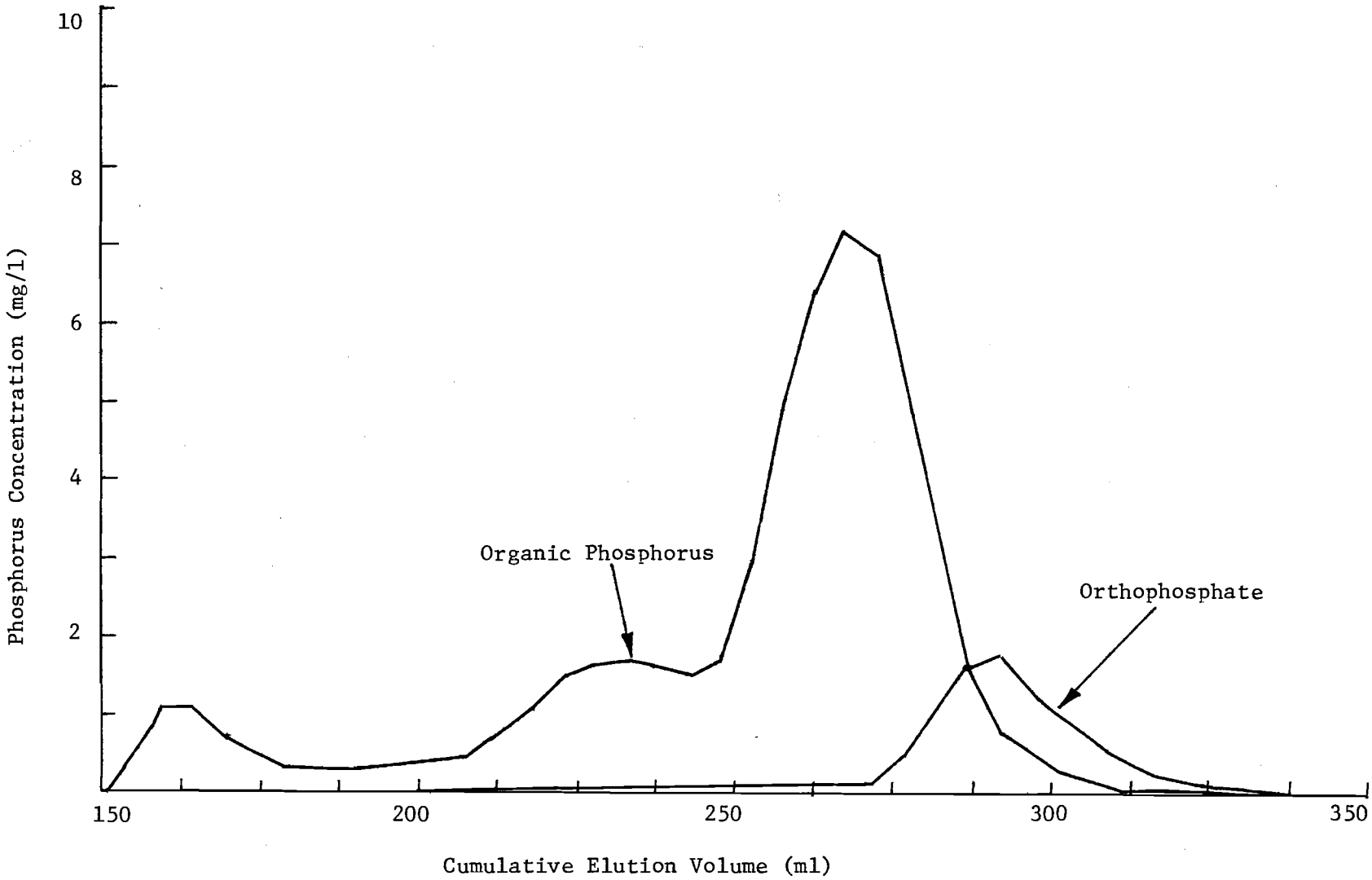

Figure 16. G-25 Chromatogram of Chlorella No. 3, Fraction A, Media Solids

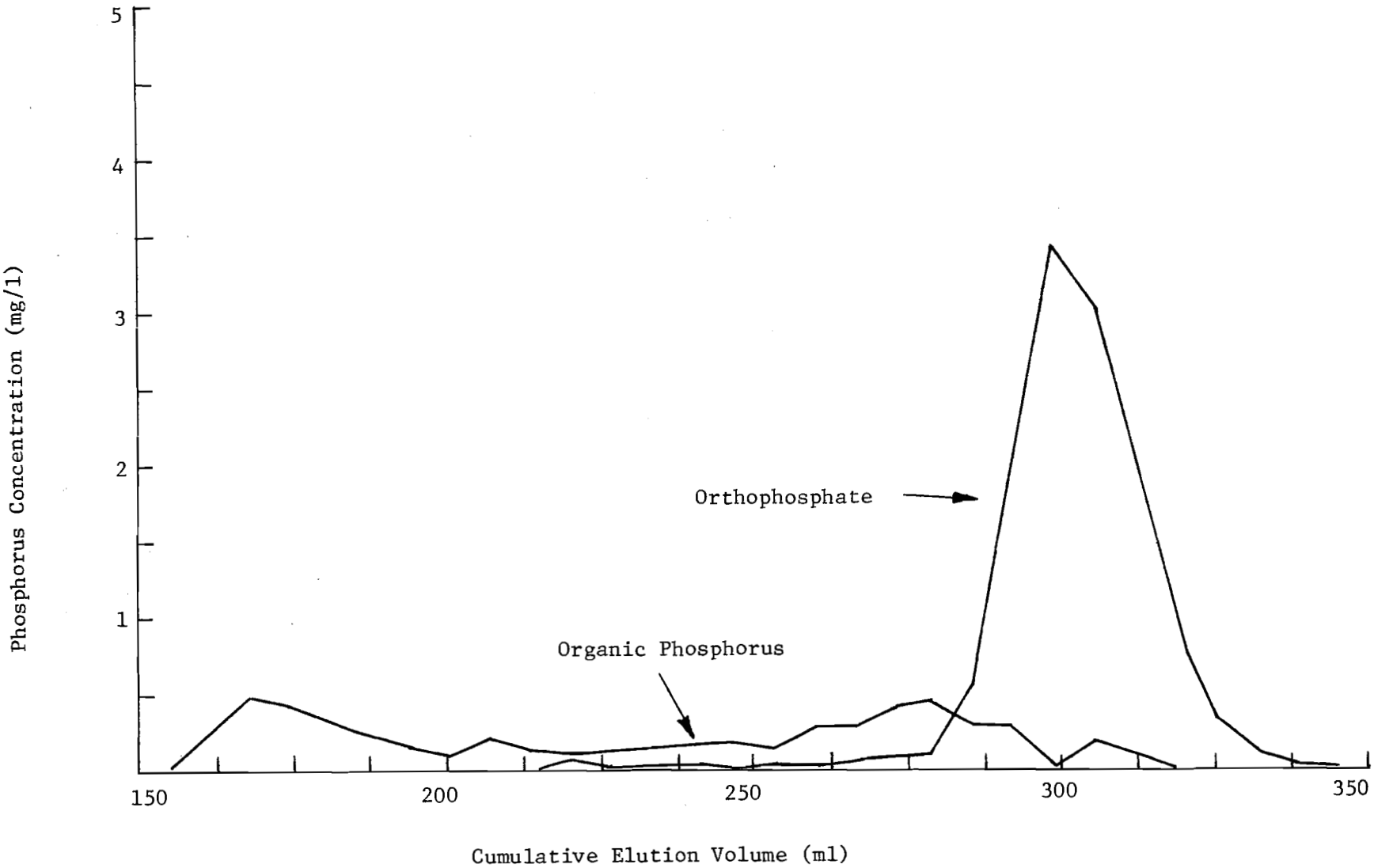

Figure 17. G-25 Chromatogram of Anabaena No.1, Fraction C, Media Solids

D. Conclusions: The results obtained indicate that the G-25 gel affords the best separation of the culture DOP exclusive of the highest molecular weight material. Since the latter was present in relatively low abundance, the G-25 gel also operates to selectively purify the low molecular weight fraction of high and intermediate molecular weight constituents, those containing DOP and other organic materials. Such purification is illustrated in Figure 18 which is a G-25 profile taken by using sample absorbance at 400 nm. In this case, yellowish colored organics in general would be monitored and serve as a rough index of the organic content since a visible spectrum of the bulk solution prior to chromatographing yielded maximum absorbance at 400 nm.

V. BROMINATION STUDIES

A. Alkaline Bromination of IHP and IMP: Previous investigators have shown that inositol phosphates were resistant to the severe oxidizing conditions of alkaline bromination. In order to use this technique to destroy other organic phosphorus compounds and recover inositol phosphates from the algal culture solids, known inositol phosphates were subjected to the procedure to evaluate (1) recovery, (2) conversion to other compounds and (3) changes in G-25 elution behavior. The G-25 results obtained for IHP and IMP (both obtained from Sigma Chemical Company) are shown in Figures 19 and 20. Table 10 summarizes reaction conditions and recovery data.

Low total phosphorus recovery for the IHP solution is accounted for by the formation of a precipitate upon neutralization. For the IMP solution, the precipitate was redissolved in base, analyzed, and a composite value reported. The significant aspects of these results and the chromatograms are:

(1) IHP survives alkaline bromination although not quantitative

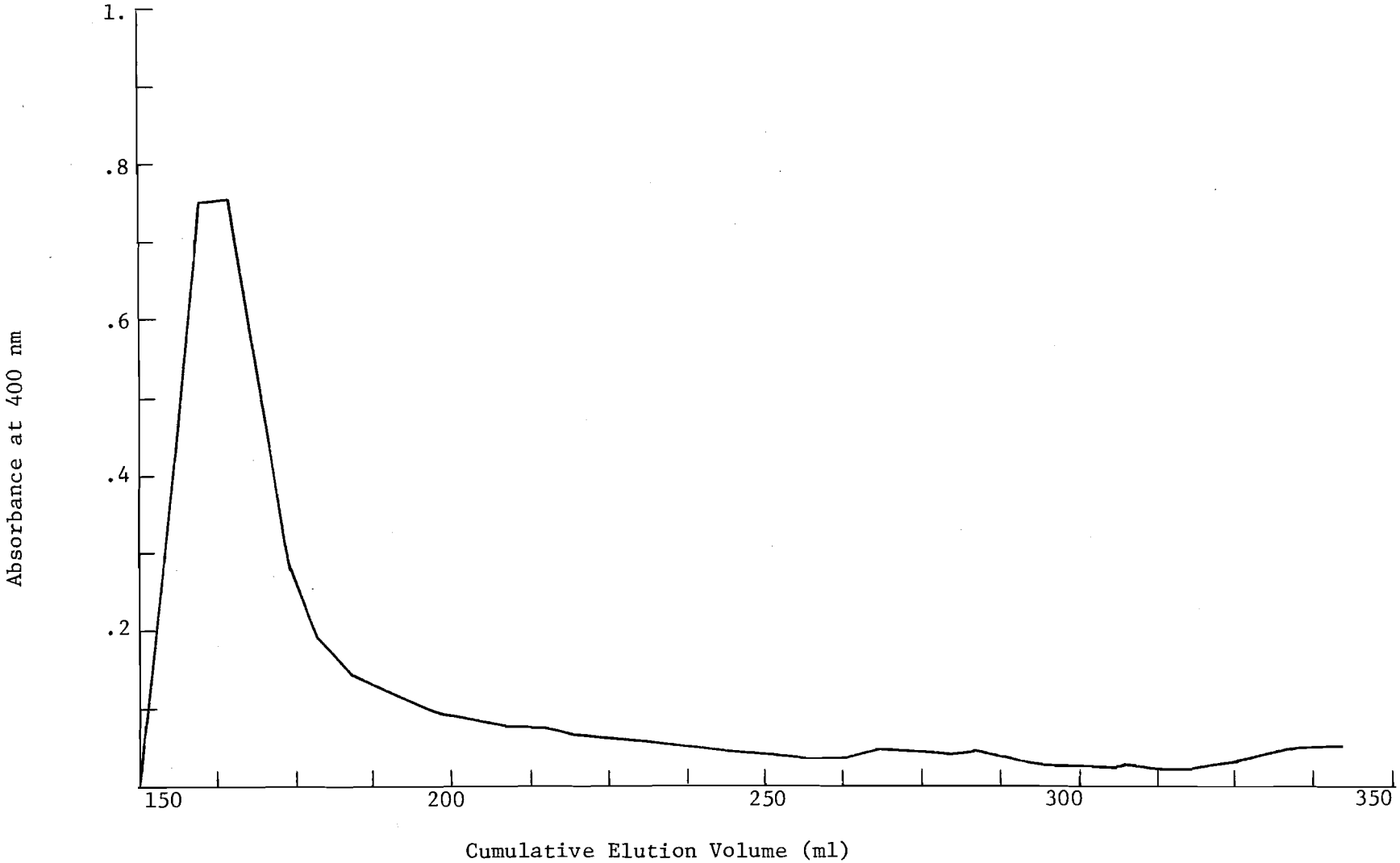

Figure 18. G-25 Chromatogram of Chlorella No. 3, Fraction C, Media Solids Monitoring Absorbance at 400 nm

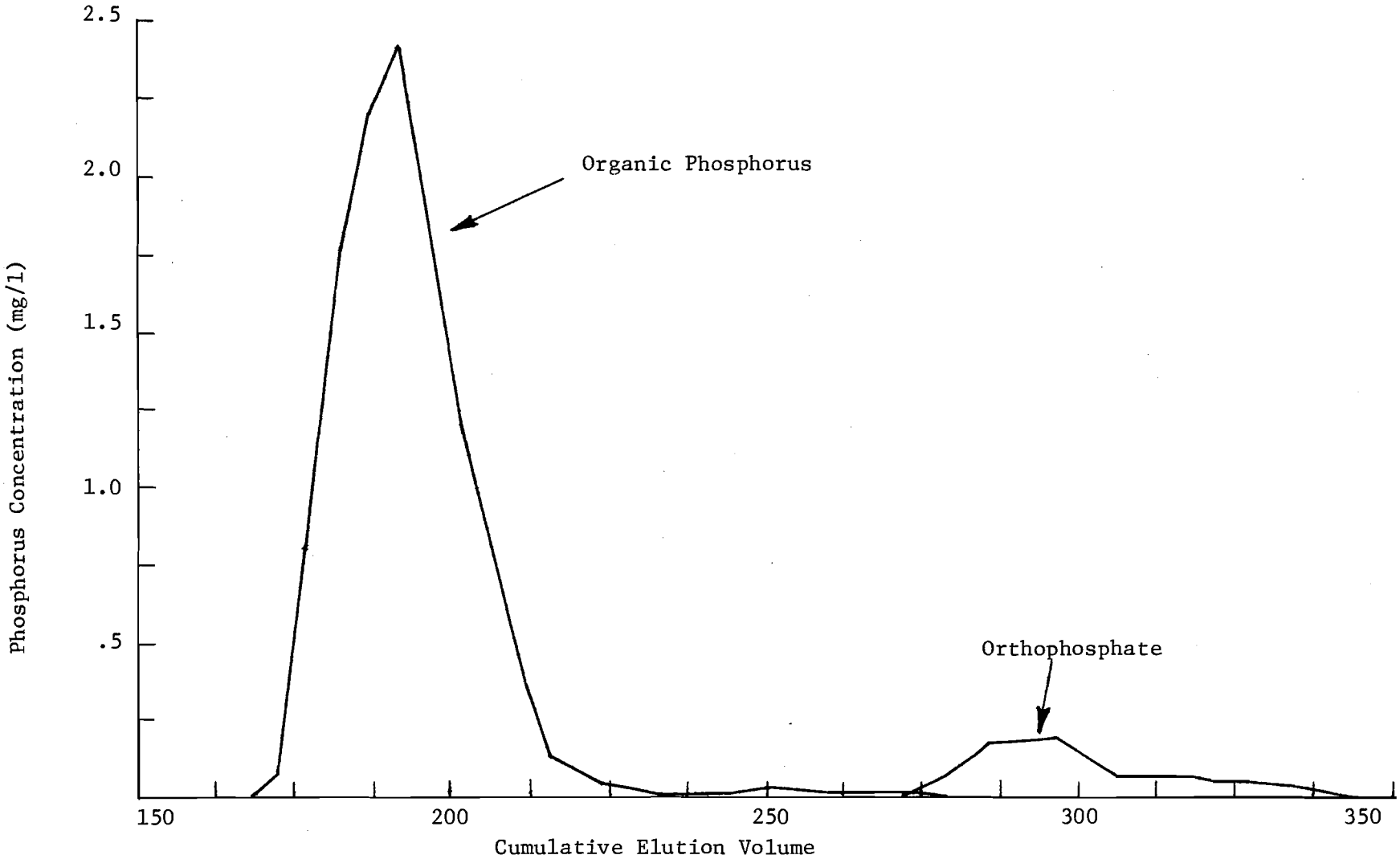

Figure 19. G-25 Chromatogram of Inositol Hexaphosphate after Alkaline Bromination

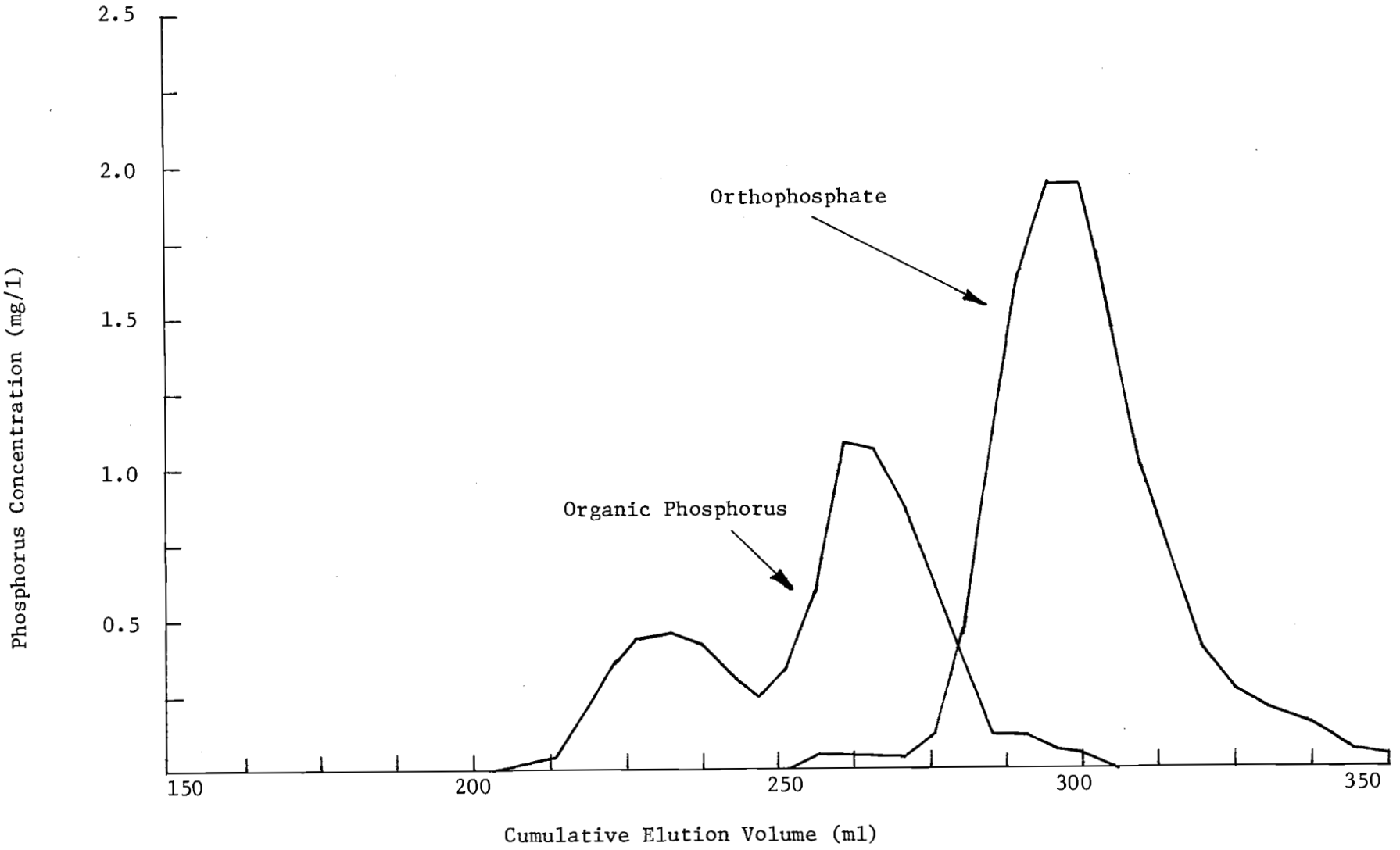

Figure 20. G-25 Chromatogram of Inositol Monophosphate after Alkaline Bromination

Table 10. Summary of Alkaline Bromination Conditions and Results for IHP and IMP.

Compound	Solution Volume	Initial DOP	Initial PO_4	Initial % DOP	Final DOP	Final PO_4	Final % DOP	% Total P Recovered	ml 5 N NaOH	ml Sat. Br_2-H_2O	Boiling Time
1. IHP	10 ml	1232 µg	0.42 µg	99.9	409 µg	41.8 µg	90.7	36.6	10	10	60
2. IMP	5.7 ml	650 µg	0 µg	100	246 µg	402 µg	38	99.7	10	10	60
3. IMP	10 ml	1150 µg	0 µg	100	0 µg	---	0	0	10	20*	60

*An additional 10 ml of bromine water added after 40 minutes of boiling.

and does not yield intermediate inositol phosphates upon degradation.

(2) IMP survives alkaline bromination although excess bromine added during the reaction (No. 3, Table 10) can result in complete destruction of the IMP.

(3) Possible production of a higher phosphate might occur from bromination of IMP (Figure 20) although the presence of higher phosphates was not evaluated prior to bromination. The commercial IMP was designated as only 90 percent pure, consequently the extra peak may reflect on impurity.

(4) The respective elution positions on G-25 of IHP and IMP after alkaline bromination were coincident with the unbrominated solutions (Figure 14).

The peak at V_e = 23 ml in Figure 20 corresponds roughly to ITriP based on the values of Table 9 but its nature and origin remained uncertain.

B. Orthophosphate Peak Tailing: Observation of Figures 19 and 20 reveals strong tailing which could imply binding in solution with residual, low molecular weight material which in turn is adsorbed by the gel matrix (Determann[41]). Alternatively, the bromination procedure was considered to be responsible for this behavior and was so investigated.

Figure 21 demonstrates that the presence of bromine in an orthophosphate solution was not responsible for the tailing. However, the reaction conditions and subsequent sample treatment result in a final solution of high ionic strength. To simulate this situation, orthophosphate in 0.8 N NaCl was chromatographed and exhibited pronounced tailing (Figure 21). Because the higher molecular weight constituents quickly separate from the inorganic species on the column, the DOP

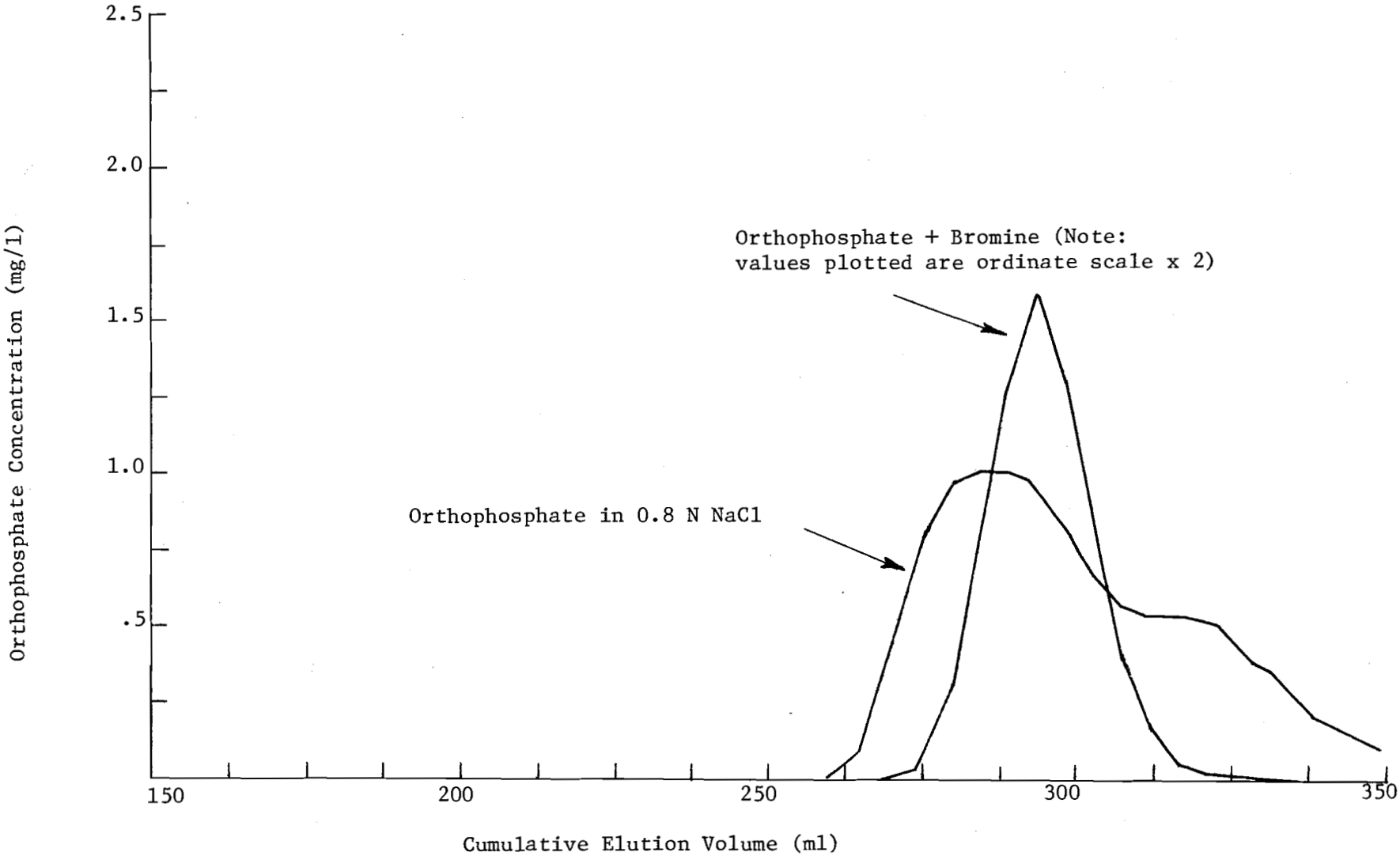

Figure 21. G-25 Chromatogram of Orthophosphate plus Bromine Water and Orthophosphate plus 0.8 N NaCl

peaks are of expected symmetry and should experience no effects from the high ionic strength.

C. Bromination of Algal Culture Solutions: To define the possible presence of the lower inositol phosphates in the DOP of algal cultures, alkaline bromination to destroy other organics and the non-inositol phosphates was undertaken. Rechromatographing the brominated solutions on G-25 would then provide a mechanism for assigning tentative identity to the oxidation resistant phosphorus compounds. Table 11 identifies the culture subsamples subjected to alkaline bromination and summarizes the reaction conditions, recoveries obtained, and indicates the figures corresponding to the respective G-25 chromatograms after alkaline bromination.

(1) Figure 22. Of note in the chromatogram, is the absence of any high molecular weight organic phosphorus. Bromination of the sample has completely destroyed this higher molecular weight material. There is no peak at the elution volume of IHP. Nor is there a peak at the calculated elution volumes of IPP, ITriP, or ITetP. There is, however, a distinct peak at $V_e = 250$ ml. This peak is close to the calculated elution volume of IDP which is 243 ml. The V_e of 243 ml is the calculated value based on the assumption that the inositol phosphates are eluted as a linear function of the log of their molecular weight with respect to the parameter K_{av} (Table 9). It is possible, therefore, that this peak may be IDP.

The next peak is eluted at $V_e = 265$ ml. This is the elution volume of IMP. Conceivably the material eluted at this point is IMP. The peak begins to dip and is then followed by a "shoulder." This is

Table 11. Summary of Alkaline Bromination Conditions and Results for Algal Culture

Sample	Solution Volume ml	Initial DOP µg	Initial PO_4 µg	Initial % DOP	Final DOP µg	Final PO_4 µg	Final % DOP	% Total P Recovered	ml 5 N NaOH	ml sat. Br_2-H_2O	Boiling Time	G-25 Chromatogram
Chlorella #1-C	10	179	268	40.0	30	275	9.8	68.2	10	15	90[a]	Fig. 22
Chlorella #2-C	98	906	59	93.9	587	185	76.0	80.0	10	10	60	Fig. 23
Chlorella #2-C Rechromatographed[b]	22	--	--	--	56	36	60.9	--	10	10	60	Fig. 24
Anabaena #1-C	10	51	96	34.7	7.5	110	6.4	79.5	10	10	60	Footnote C
Anabaena #2-A&B	48	128	164	43.8	121	147	45.1	91.8	10	10	60[d]	Fig. 25

a. Ten ml bromine water added initially and boiled 60 minutes, 5 ml addition bromine water plus 30 minutes boiling.

b. Fractions from V_e = 248 to V_e = 296 pooled, rebrominated and rechromatographed.

c. G-25 chromatogram inconclusive because total phosphorus in collected fractions below the reliable limit for the analysis procedure using 1 cm light path.

d. Boiling was reduced below other bromination procedures due to faulty hot plate. Reduced oxidation a likelihood.

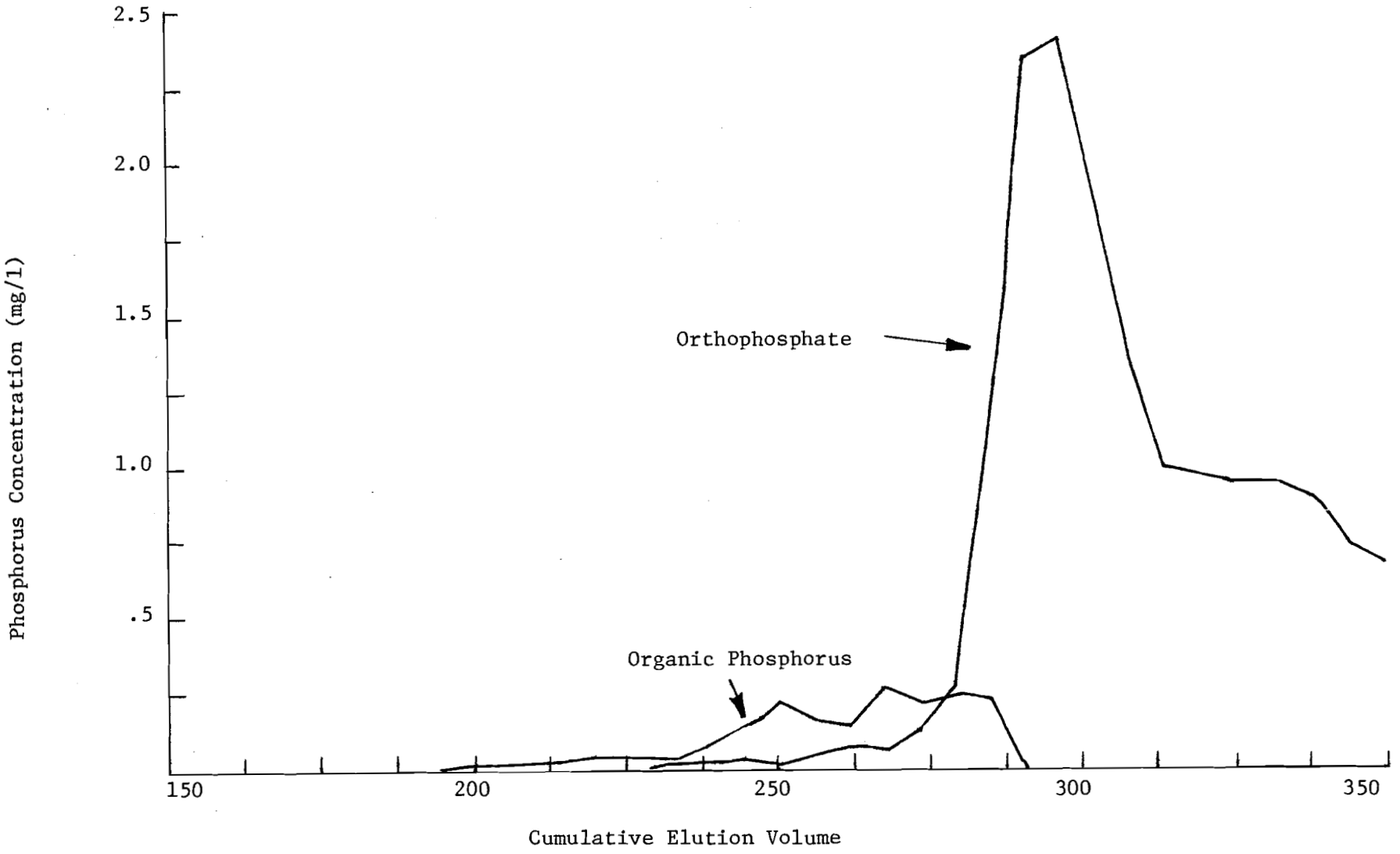

Figure 22. G-25 Chromatogram of Brominated Chlorella No. 1, Fraction C, Media Solids

believed to be an analysis artifact created by the extremely high orthophosphorus concentration of the sample relative to the organic P concentration. If the DOP eluted in these two peaks is IMP and IDP, it would represent 16.8 percent of the original DOP of the sample if there is 100 percent recovery. Since neither 100 percent recovery of IHP nor of IMP was achieved with standard solutions, the 16.8 percent figure may then be viewed as a lower limit. In other words, the amount of IMP and IDP may be greater than 16.8 percent of the DOP recovered from the culture solids.

(2) Figure 23. The chromatogram shows that all of the higher molecular weight DOP has been destroyed. There is no peak at the elution volume of IHP; i.e., V_e = 190 ml and none at the calculated elution volume of IPP; i.e., V_e = 201 ml. This indicates the absence of these compounds in quantities sufficient to detect their presence either because they were not in the culture media or they were not recovered in sufficient quantities after bromination.

At approximately V_e = 207 ml, an organic phosphorus peak begins to develop which has its maximum at V_e = 240 ml. The major DOP peak occurs at a V_e of 268 ml. Organic phosphorus fragments can be seen from V_e = 300 ml to 350 ml. The tailing of the orthophosphorous peak, i.e., the "salt effect," is also evident.

The possibility of non-inositol DOP fragments making up a part on even the bulk of this material exists. Certainly some fragments are present as evidenced in the elution volumes greater than V_e = 300 ml.

In order to verify the presence of inositol phosphates in the elution volumes under the organic phosphorus peaks, the eluted fractions from

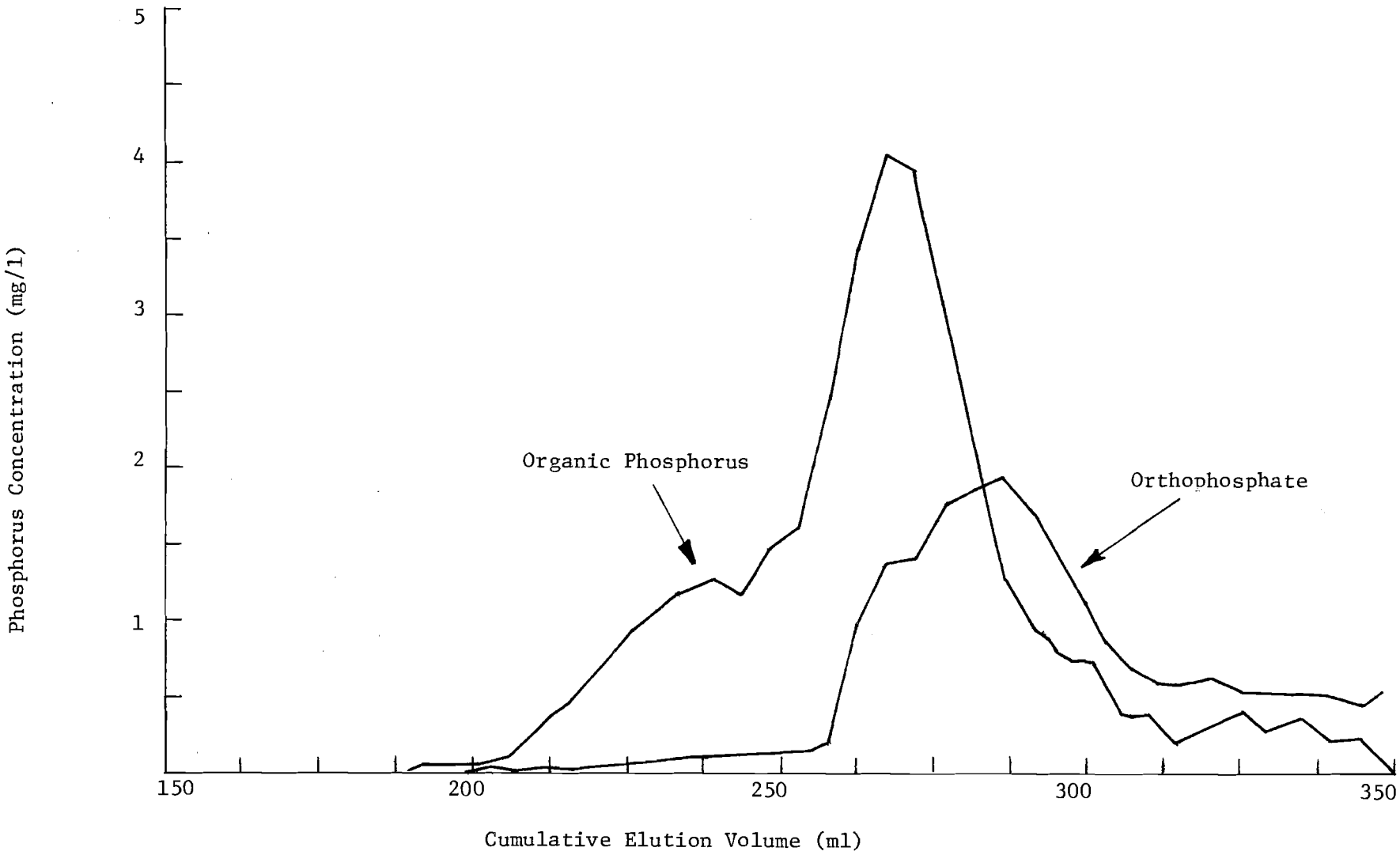

Figure 23. G-25 Chromatogram of Brominated Chlorella No. 2, Fraction C, Media Solids

V_e = 248.2 ml to 296.2 ml were pooled. This amounted to 28 ml of pooled samples. Of this, 6 ml was removed for performing the Fiegl[42] color specific test for inositol (see page).

(3) Figure 24. The remaining 22 ml of pooled sample was then subjected to alkaline bromination a second time in the same manner as the original Chlorella No. 2 C.

Inspection of the chromatogram reveals the presence of two distinct peaks. One peak at V_e = 265 ml and another at V_e = 244 ml. The former peak appears at the V_e of IMP and the latter peak at the calculated V_e of IDP (Table 9).

(4) Figure 25. Inspection of the chromatogram reveals several things. The most important observation is that the alkaline bromination treatment did not destroy all of the non-inositol phosphates. DOP can be seen in the high molecular weight exclusion volume, V_e = 168 ml. Obviously excluded DOP survived the oxidative conditions of the treatment. Also noticeable are a series of a very irregular peaks after V_e = 288 ml. These irregular peaks had not been observed in previous chromatograms and their irregularity does not suggest a single component. A large peak at V_e = 269 ml does imply the likely presence of a single component. The peak is sharp, symmetrical and elutes prior to the inclusion volume. A comparison of the Anabaena phosphorous profile before alkaline bromination, (Figure 17) reveals that a much higher percentage of the DOP is below the exclusion limit of the G-25 gel.

Results for the Anabaena culture are less conclusive than those for the Chlorella cultures. The major problem seemed to be incomplete oxidation of the sample likely due to reduced heating conditions during

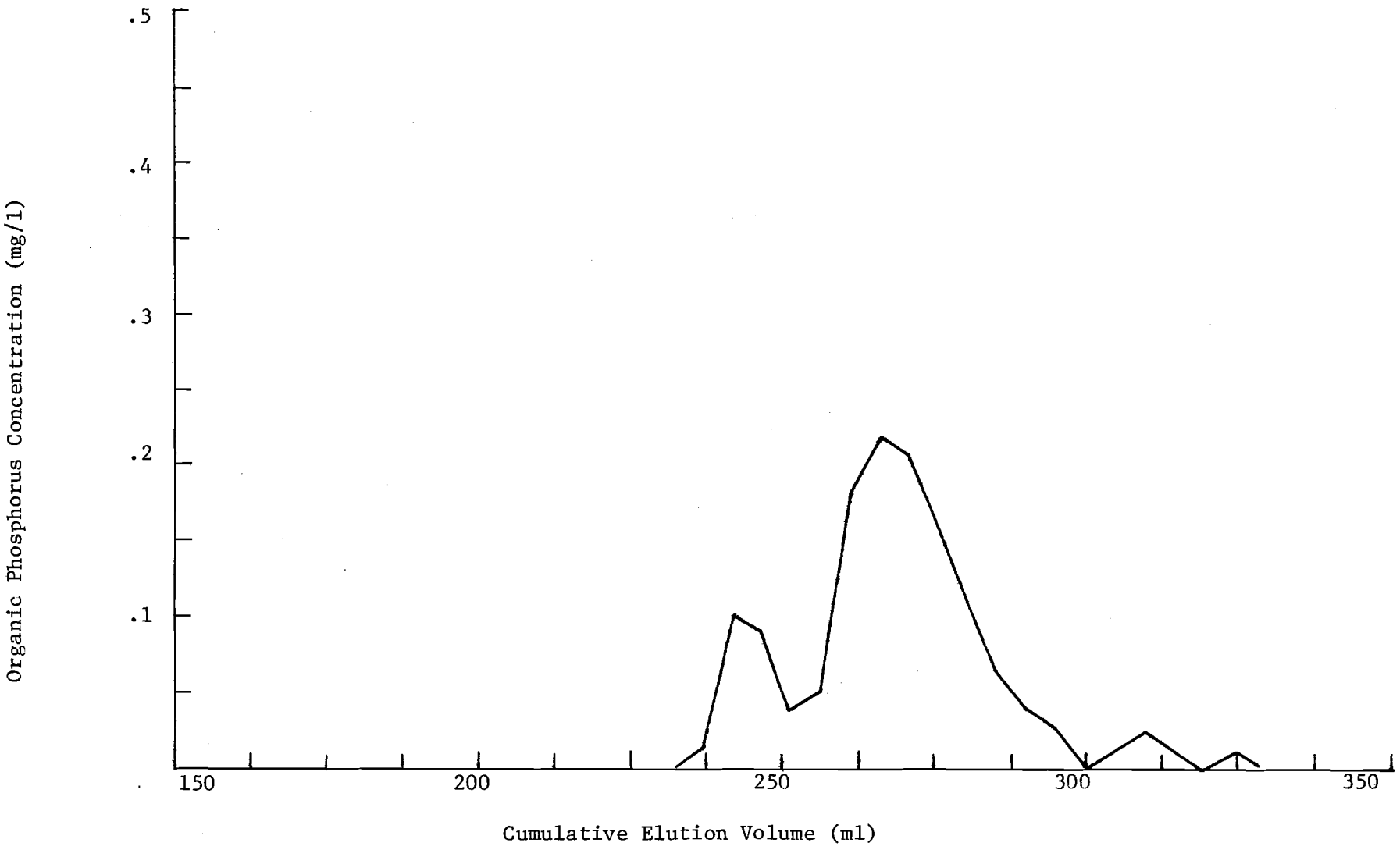

Figure 24. G-25 Chromatogram of Low Molecular Weight Rebrominated Chlorella No. 2, Fraction C, Media Solids

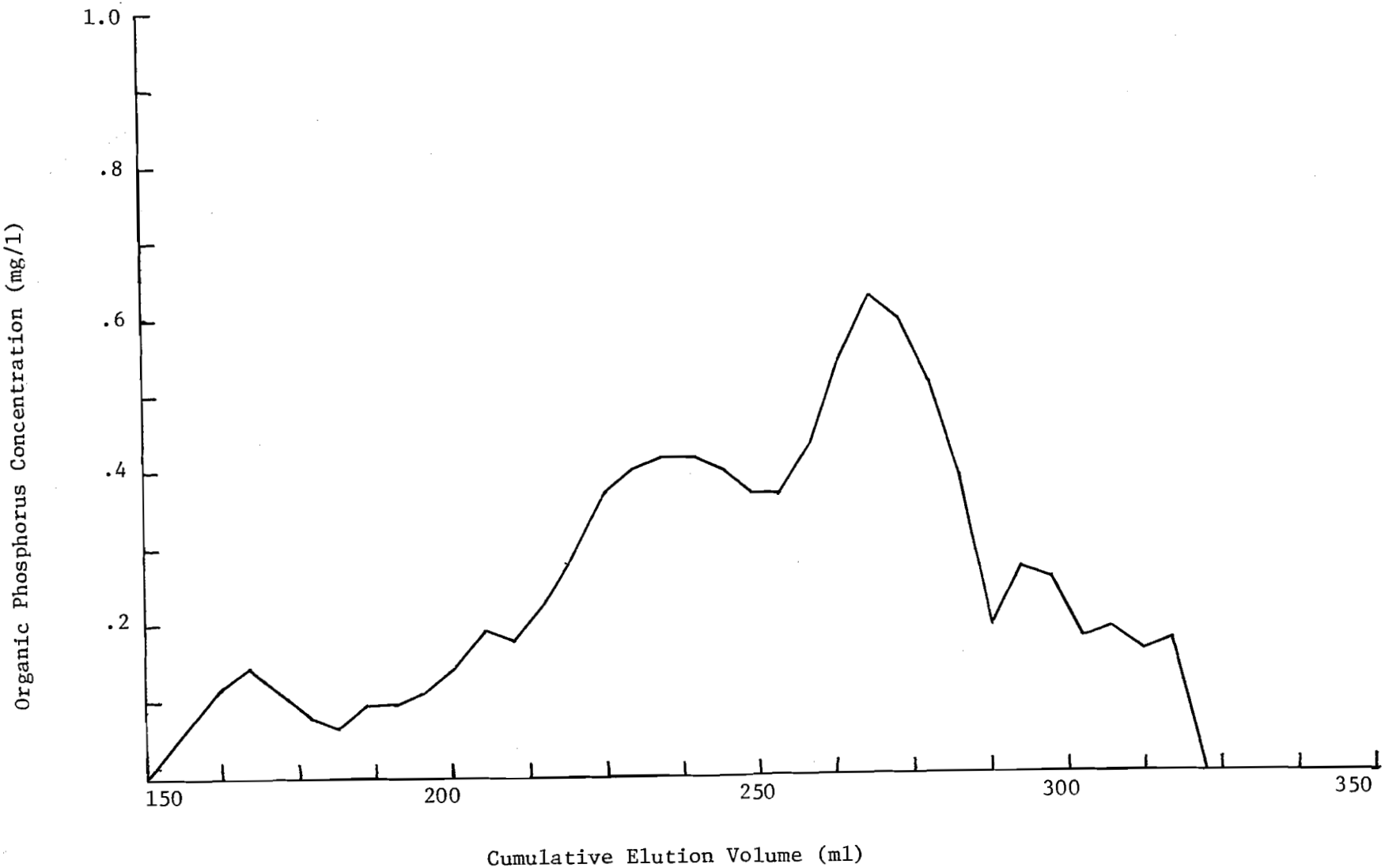

Figure 25. G-25 Chromatogram of Brominated Anabaena No. 1, Fractions A and B, Media Solids

oxidation (Footnote d, Table 11). Limited sample and low overall DOP prevented a rebromination as was achieved with the Chlorella No. 2 C sample. While the presence or absence of inositol phosphates is uncertain, the large peak at V_e = 267 ml does not rule out the possible presence of IMP.

D. Spot Tests: The Fiegl[42] spot test for inositol was applied to the IMP samples subjected to alkaline bromination (No. 2 and 3, Table 10). The first gave a positive response (light brown) while the second yielded a negative result as would be expected. In addition, the fractions from the G-25 chromatogram of brominated IMP (V_e = 210 to 290, Figure 20) were collected, combined and subjected to the spot test procedure. Positive results were obtained.

A portion of the composite fractions recovered from the G-25 chromatogram of the brominated Chlorella solids (V_e = 248 to 296, Figure 22) were treated in a similar manner and yielded a positive response. Although, certainly not conclusive without additional confirmation, these results do strengthen the possible presence of IMP and perhaps other inositol phosphates in algal culture DOP.

E. Conclusions: The results of the bromination studies coupled with Sephadex separations and the inositol spot test on known compounds and the algal culture solids provides strong circumstantial evidence for the presence of IMP and possibly other lower inositol phosphates in the overall algal culture DOP. In spite of the presence of IHP in soil and lake sediment organic phosphorus, the results of these studies show that the algae studied are not a source of IHP. Before a definitive statement could be made relative to natural water DOP, a great deal more work is necessary with a much wider range of algal species. Specific and conclusive identification of algal IMP obviously remains to be accomplished.

VI. SILYLATION STUDIES

Attempts to form trimethylsilyl derivatives of known phosphorus compounds are summarized in Table 12. Both HMDS-TCMS and BSTFA procedures were used in either dimethyl formamide (DMF) or pyridine solvent. Although gas chromatographic peaks were obtained, little information of value resulted from these studies because of instrumental problems and the central focus of the research activities in other areas. The basic research plan was to assign specific identity to phosphorus derivatives by use of the phosphorus specific alkali flame detector. Attempts to achieve proper function of this detector were not successful in the time available. Consequently, the peaks for known compounds and those obtained upon silylation of the Chlorella No. 4, Fraction A/B, sample recovered from the Sephadex G-10 gel (chromatogram of Figure 13) cannot be attributed to phosphorus containing derivatives.

Even though these studies were essentially non-productive with respect to meaningful information, they are mentioned for the sake of completeness. Furthermore, some basic information was obtained regarding useful columns and temperatures for separation procedures which will be of value in continuing studies to be initiated in the summer of 1974. (See Significance of Research section). Of the columns investigated (OV-17, DC-200, QF-1, SE 30 and SE 52) only the SE 52 yielded separations at a temperature of 220 to 240^{o} C. All compounds investigated including the G-10 sample had several peaks in common. The first objective of continuing studies will be to assign an identity to these peaks. At this point, it must be assumed that these common peaks represent reaction by-products of the silylation reactants.

Table 12. Retention Time Data for Trimethylsilylation Products on All Glass Gas Chromatographic Columns (FID Detector)

Column 1. Five percent QF-1 on Varaport 30 (100/120)
Operating conditions: Column temperature - 140° C
Injection temperature - 190° C
Detector temperature - 200° C

Compound	Derivatization	Peak Retention Time (relative to injection)	
$(NH_4)_2HPO_4$	BSTFA in DMF	6.3 min.	6.72 min.
$(NH_4)H_2PO_4$	BSTFA in DMF	6.2 min.	7.3 min.
Control	BSTFA in DMF	6.3 min.	---

Column 2. Five percent SE 52 on Varaport 30 (100/120)
Operating conditions: Column temperature - 240° C
Injection temperature - 280° C
Detector temperature - 280° C

Compound	Derivatization	Peak Retention Times (relative to solvent front)		
NH_4 Adenosine 5'-phosphate	HMDS-TCMS in DMF (2 hr.)	6.00 min.	8.63 min.	10.31 min.
NH_4 Adenosine 5'-phosphate	HMDS-TCMS in DMF (18 hr.)	5.63 min.	8.44 min.	10.13 min.
NH_4 Adenosine 5'-phosphate	BSTFA in DMF (2 hr.)	5.63 min.	9.75 min.	12.38 min.
NH_4 Adenosine 5'-phosphate	BSTFA in DMF (18 hr.)	5.63 min.	---	12.38 min.
Inositol Hexaphosphate (NH_4)	HMDS-TCMS in DMF (15 min.)	5.81 min.	---	---
Inositol Hexaphosphate (NH_4)	HMDS-TCMS in DMF (2 hr.)	5.81 min.	9.75 min.	---
NH_4-β Glycerophosphate	HMDS-TCMS in DMF	5.44 min.	8.44 min.	9.94 min.
G-10 isolate	BSTFA in DMF (2 hr.)	5.72 min.	8.06 min.	9.94 min.
G-10 isolate	BSTFA in DMF (24 hr.)	5.63 min.	---	9.94 min.

VII. SIGNIFICANCE OF RESEARCH

The primary significance of this project has been the tentative identification of inositol monophosphate and possibly inositol diphosphate in algal culture DOP. Secondly, inositol hexaphosphate, a primary constituent of soil and lake sediment organic phosphorus, was not found to be a major constituent of the algal culture DOP although its presence could not be conclusively ruled out. These results now provide a specific compound upon which further research can focus with the objective of first conclusive identification and quantitation in algal culture systems and ultimately in natural waters.

The DOP production characteristics of several algal cultures have been established. These results can be applied directly to further investigations to be initiated July 1, 1974, under the Phase II project approved for funding by The University of Tennessee, Water Resources Research Center. Similarly, the investigations with Sephadex gel filtration have established that the G-25 gel affords the most useable separation characteristics for fractionating and desalting the DOP compounds.

The need for more a sensitive phosphorus determination, even with the concentration procedures employed, has been documented and has prompted the Phase II experimental design to center about the use of radio-labeled phosphorus 32. Sufficient background information was generated in the completed research to make this feasible.

A positive step toward better definition of the natural phosphorus cycle has been accomplished by providing a small amount of additional knowledge about the DOP compartment in this cycle. Avenues for further

research have been established by the completed project and these results have aided in the formulation of a proposal submitted and approved as mentioned above.

As a first step in communication of these results, a paper has been submitted to the 37th National Meeting of the American Society of Limnology and Oceanography. A master's thesis entitled, "Inositol Phosphates in Algal Cultures" by Mr. Kenneth A. Walanski, has been generated by this project and journal publication of the research is anticipated.

Additional educational benefits have been derived from this project through partial support of two other graduate students and the practical research experience gained through their participation.

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