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Richard M. Pagni

Richard M. Pagni, Major Professor

Roger C. Minear

Roger A. Minear, Major Professor

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M J Sepaniak

J. W. Elrod

Stephen E. Herbes

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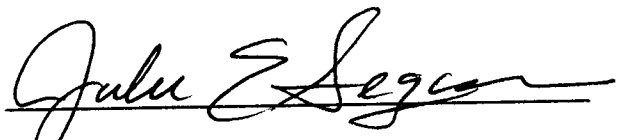
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CHEMICAL CHARACTERIZATION OF SOLUBLE PHOSPHORUS FORMS ALONG A HYDROLOGIC FLOWPATH OF A FORESTED STREAM ECOSYSTEM

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Julie E. Segars
December 1985

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ABSTRACT

The concentration and distribution of soluble phosphorus forms were determined among compartments of a hydrologic pathway in a forested watershed (Walker Branch, Tenn.). Rainfall, throughfall, soil water, groundwater, stream water, and water from two sites in Melton Hill Reservoir downstream of Walker Branch were examined for soluble reactive phosphorus (SRP) and total soluble phosphorus (TSP). Soluble unreactive phosphorus (SUP) was determined from their difference. An increase of TSP from rainfall to throughfall indicated leaching and/or wash-off of phosphorus from the canopy. SRP and SUP decreased markedly as water percolated through the soil, suggesting biological uptake and/or geochemical adsorption of the phosphate groups on soil particles. The temporal pattern of SRP in the upstream site (with a maximum in the summer and a minimum in mid-autumn) and the decrease in SRP and the increase in SUP from upstream to downstream within the stream channel supported previous evidence for biological control of phosphorus dynamics in Walker Branch. Overall, SUP (an estimate of soluble organic phosphorus) constituted a significant fraction of the TSP present in each compartment of the flowpath.

An analytical technique using high-performance liquid chromatography (HPLC) to separate the inositol phosphates (IPs) was developed and used in characterizing organic phosphorus fractions of natural systems. Commercial orthophosphate (PO_4^{3-}), inositol monophosphate (IMP), and inositol hexaphosphate (IHP) were adequately separated from each other on Aminex A-27 resin using a sodium chloride/tetrasodium EDTA gradient elution. The HPLC tech-

nique was also used to separate an enzyme hydrolysate mixture of IPs into five components, to separate IHP from PO_4^{3-} and IMP in an extract of wheat bran, and to examine an alkaline brominated extract of a Walker Branch soil sample, where at least three IP peaks were recognized. Using the HPLC technique, an attempt was made to detect the presence of IPs in a Walker Branch groundwater sample concentrated by membrane ultrafiltration. The concentration process was unsuccessful, possibly due to membrane leakage, so no peaks were detected. The HPLC developmental work indicated that this technique potentially could be used to examine natural waters for the presence of IPs.

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CHAPTER I

INTRODUCTION

Phosphorus has long been recognized as an essential nutrient in aquatic ecosystems and is very often the factor limiting primary and heterotrophic productivity in a given system. The factors affecting phosphorus availability to aquatic producers include inputs and transformations of phosphorus in the atmospheric, terrestrial, and aquatic environments. Although detailed investigations of phosphorus dynamics within individual compartments (e.g., soil, stream water, lake water) of an ecosystem have been published,[1–5] little is known about the transformations and changes in concentration of phosphorus between adjacent compartments or along a continuous hydrologic flowpath. Based on documented changes in the concentration and form of phosphorus within individual compartments, I hypothesize that soluble phosphorus undergoes significant transformation in concentration and biochemical form along a continuous hydrologic pathway in a small watershed and larger aquatic ecosystems downstream and that processes regulating phosphorus within one segment of that hydrologic pathway influence the form and concentration of phosphorus in the adjacent downstream environment. In addition, I predict that seasonal fluctuations in the biogeochemical processes along the flowpath (e.g., rainfall, soil, stream water) will result in changes in phosphorus form and concentration in downstream environments. During this study, I have investigated spatial and temporal phosphorus transformations among the atmospheric, terrestrial, and aquatic environments of a small forested watershed drained by first- and second-order streams (Walker Branch).

The available phosphorus supply in a low-order stream system is dependent upon inputs from outside the watershed (e.g., atmospheric inputs), and from sources within the surrounding watershed (e.g., from weathering, and from recycling). Inputs and biological availability of phosphorus to larger aquatic ecosystems (e.g., reservoirs, lakes) are dependent upon biochemical transformation and loss of phosphorus from low-order stream systems. Therefore, understanding changes in the concentration and form of phosphorus along the hydrologic pathway (precipitation, throughfall, soil water, groundwater, stream, reservoir) is important for determining the availability of phosphorus to aquatic ecosystems.

Orthophosphate (PO_4^{3-}) appears to be the primary species readily available to aquatic organisms. Through cell activity or death and lysis, dissolved organophosphorus (DOP) compounds are released to the aquatic environment. Very little is known about the chemical character, identity, or biological availability of DOP compounds. In order to better understand phosphorus dynamics in the aquatic environment, sources and sinks of biologically available phosphorus need to be identified. Unless the identity and chemical characteristics of DOP compounds are known, their role in the phosphorus cycle cannot be elucidated. As part of my research, an analytical method using high-performance liquid chromatography (HPLC) has been evaluated as a separation tool leading toward the identification of the inositol phosphates (IPs), a specific set of dissolved organophosphorus compounds potentially found in the aquatic environment.

Objectives of Research

In recognizing the scientific community's need to understand our environment, specifically the need to understand nutrient dynamics within aquatic systems, the objectives of my research are

1. to characterize spatially and seasonally the concentration and biochemical form of soluble phosphorus [soluble reactive phosphorus (SRP)/soluble unreactive phosphorus (SUP)] along a hydrologic pathway of a forested watershed (Walker Branch-Melton Hill Reservoir in East Tennessee).

2. to develop and evalute an analytical technique for the separation of the IPs by HPLC to be used in the characterization of DOP compounds in natural waters.

CHAPTER II

LITERATURE REVIEW

Environmental Forms of Phosphorus

Conceptually, phosphorus-containing substances can be divided into different categories based on physical and chemical characteristics. Physically, phosphorus materials are divided into particulate, colloidal, and soluble phosphorus. Distinct separation of the three forms is not easily realizable; however, soluble phosphorus is operationally defined as that which passes through a membrane filter with 0.45-μm pore size.[6] The 0.45-μm membrane filter is used as a convenient and reproducible, although arbitrary, analytical technique for the gross separation of particulate from dissolved forms of phosphorus.

In the environment, phosphorus exists primarily in the pentavalent tetraoxide form. Phosphorus may be chemically present as orthophosphate (PO_4^{3-}), condensed inorganic phosphates, or organophosphorus compounds. Condensed phosphates are very easily hydrolyzed to PO_4^{3-}, so it is generally assumed that in unpolluted, natural waters they are not present.[7–9]

Chemically, soluble phosphorus compounds are operationally fractionated into that which reacts immediately with molybdenum to form a blue complex, SRP, and that which reacts only after oxidation, SUP. The concentration of SRP in a system estimates the concentration of inorganic phosphorus (assumed to be PO_4^{3-}), while SUP estimates DOP concentrations.[10,11] However, discrepancies have been noted. A series of research works seems to indicate that SRP overestimates PO_4^{3-} concentrations. Downes and Paerl[12] found two distinct

fractions of SRP in lake water, a reactive high-molecular-weight fraction and a fraction corresponding to PO_4^{3-}. They suggested that hydrolysis of organic phosphorus to PO_4^{3-} by acid in the analytical reagents was responsible for the reactive high-molecular-weight phosphorus fraction in their samples. White and Payne[13] supported these findings with similar results from a selection of New Zealand lakes and streams. Tarapchak[14] reports evidence for molybdate-enhanced hydrolysis of organic compounds during the measurement of PO_4^{3-}, resulting in its overestimation.

Pettersson[15] proposed an alternate analytical method for the determination of PO_4^{3-} using alkaline phosphatase enzyme inhibition. Hydrolysis of organic phosphorus compounds should not be a problem with this method. In spite of the problems noted with the molybdate analytical method, it is still used widely to gain valuable information regarding soluble phosphorus concentrations and to estimate the various phosphorus forms found in the environment.

Distribution of Soluble Phosphorus Within Hydrologic Compartments

Phosphorus distributions and dynamics within specific hydrologic compartments (e.g., precipitation, soil, stream water, lake water) have been investigated by many researchers. The atmosphere has been recognized as a non-point source of phosphorus to aquatic systems, and atmospheric loading of phosphorus to various systems has been described.[16–19] Although Sober and Bates[16] measured total phosphorus inputs to a lake system in Oklahoma and did not distinguish between soluble and particulate phosphorus, they found a high amount of variability in the temporal pattern of phosphorus inputs. Murphy

and Doskey[19] did distinguish between the forms of phosphorus in their study of precipitation phosphorus inputs to Lake Michigan. They found that precipitation inputs of phosphorus accounted for about 18% of the total phosphorus inputs to the lake. They also found that SUP made up a significant fraction (although often less than 50%) of total soluble phosphorus (TSP) in precipitation.

The literature reviewed appears to be void of discussion on interactions between soluble phosphorus in precipitation and the canopy and its accompanying ecological processes. However, the distribution of soluble phosphorus within a soil system and the processes regulating this distribution have been investigated.[1,20] A combination of biological and geochemical processes appear to control the retention of soluble phosphorus in the Bear Brook, New Hampshire soil system described by Wood and Borman.[1] The sorption and release of phosphorus by soils to the adjacent solutions has been related geochemically to the content of iron and its oxidation-reduction status in soils.[20] The phosphorus-retaining capacity of soils in a watershed can have a substantial impact on the phosphorus levels in the water bodies of that watershed, often regardless of phosphorus inputs to the system.[21]

Cowen and Lee[22] found that leaf litter released significant amounts of SRP and lesser amounts of SUP when leached with distilled water. Preliminary work on Walker Branch leaf litter performed at Oak Ridge National Laboratory by Silver et al.[23] showed an uptake of SRP and a release of SUP by the leaf litter when leached continuously with filtered spring water.

Soluble phosphorus cycling (uptake, release, and transport of phosphorus) within stream systems is a spatially dependent process that can be described as

an imaginary spiral.[24,25] The rate and length of this spiral can be controlled by various stream processes. In Walker Branch, Elwood et al.[26] found that the transformation of soluble phosphorus into particulate phosphorus was predominantly a biologically controlled process. However, in Bear Brook, New Hampshire, Meyer[27] concluded that biotic uptake played a minor role in phosphorus exchange. Elwood[26] discussed why Meyer may have reached this conclusion. To measure phosphorus sorption, Meyer used high concentrations of SRP, which may have exceeded the phosphorus uptake capacity of the microbes. Newbold et al.[28] and Mulholland et al.[29] reported that uptake of phosphorus within Walker Branch was associated with leaf decomposition. Both Meyer (in Bear Brook) and Rigler [30] (studying Dartmoor catchments in Great Britain) concluded that phosphorus enters streams in the soluble form and accumulates on the stream bed during periods of base flow but is transported out primarily in the particulate form during periods of high flow. This transformation from soluble to particulate can have significant implications to downstream systems. Meyer and Likens[2] found annual stream flow to be a major factor affecting phosphorus transport through the Bear Brook stream system.

Soluble phosphorus distribution and dynamics in several lake impoundments and reservoirs have been described extensively by many researchers.[3,31,32] Seasonal patterns of soluble phosphorus concentrations have been related to biological activity and to morphometric and other features of a lake.[33]

Even though research has been done on the patterns of phosphorus concentrations and the associated mechanisms of phosphorus control within individual compartments—focusing primarily on transformation between soluble and parti-

culate fractions—very little is known about the exchange and transformations of soluble phosphorus between adjacent compartments in a hydrologic pathway.

Characterization of Dissolved Organophosphorus Compounds

Dissolved organophosphorus compounds can make up a significant fraction of TSP in stream water;[34] however, little is known about their chemical character, identity, and bioavailability. One method of characterizing DOP is by determining molecular weight profiles using Sephadex gel liquid chromatography. Minear[35] and Peters[36] identified three types of total dissolved phosphorus: PO_4^{3-}, low-molecular-weight phosphorus [(LMWP), mol. wt ~250], and a high-molecular-weight phosphorus [(HMWP), mol. wt > 10^6]. Peters[36] showed that at least a portion of the DOP is available to lake biota. Stevens and Stewart[37] concentrated, fractionated, and characterized DOP compounds first into acid and alkali soluble fractions then into high-, medium-, and low-molecular-weight fractions. They used algal bioassays to test the availability of some fractions. Francko and Heath[3] used ion-exchange chromatography and gel filtration to classify complex phosphorus compounds in lake water. They observed the release of orthophosphate from the various compound classes after treatment with ultraviolet radiation or alkaline phosphatase to indicate the degree of bioavailability of each class.

In further characterization of DOP, Koenings and Hooper[38] described the apparent presence of hydrolysis products of ribose nucleic acids and free nucleotides in a Michigan bog. Their tentative identifications were based on the comparison of the elution positions of the DOP materials using ion-exchange

chromatograpy and their absorbance spectra to elution positions and absorbance spectra of known compounds reported in the literature. Minear[35] substantiated the presence of DNA fragments in algal exudates by a combination of chemical and biochemical assays. Francko and Wetzel[39] identified cyclic adenosine 3′-,5′-monophosphate in lake water using enzyme bioassay techniques. Other than these, no conclusive identifications of single DOP compounds have been made.

In attempts to identify specific components of the DOP fraction in fresh waters, a particular group of compounds, the inositol phosphates (IPs), have been emphasized.[40–44] The IPs are a homologous series of compounds with one to six PO_4^{3-} groups substituted by ester linkages onto hexahydroxycyclohexane, or inositol. The IPs have been studied extensively as constituents of soil organic phosphorus.[45–50] See Dalal[51] for an excellent review of soil organic phosphorus research. IPs have been observed in lake sediments[52,53] and inferred in lake water through gel filtration studies[41] and through enzymatic techniques.[40] In addition, Weimer,[54] Minear and Walanski,[42] McEntyre,[43] and Hixson[44] established that algae were a potential source of the less substituted IP esters in the aquatic environment. These results suggest that the IPs are possible components of stream DOP.

The Inositol Phosphates

Inositol is the common name for 1,2,3,4,5,6-hexahydroxycyclohexane ($C_6H_{12}O_6$). It has a molecular weight of 180.16. Inositol is widely distributed in plants and animals and is a growth factor for microorganisms. There are nine possible stereoisomers of inositol. Seven are optically inactive, or meso; the

other two are a racemic mixture. Figure 1 shows the chemical structure of the nine stereoisomers of inositol and their common names. The most prevalent natural form of inositol is cis-1,2,3,5-trans-4,6-hexahydroxycyclohexane, commonly known as myo-inositol.

A phosphate group may form an ester bond with any of the six hydroxyl groups of inositol, resulting in six types of IPs based on the number of phosphate groups substituted onto the ring. the mono, di, tri, tetra, penta, and hexaphosphates of inositol. Each stereoisomer of inositol can form several structurally different IPs of each type, depending on the location of the substituted phosphate group (i.e., myo-inositol can form four different monophosphates). However, only the myo-inositol phosphate compounds have been identified in plants.[55] Phosphate esters of the DL-, scyllo-, and myo- isomers have been identified in soils, and are believed to be formed by bacterial synthesis.[56] The phosphate esters of the myo-inositol isomer are the compounds most often described in soil, food, and environmental chemistry literature. Figure 2 shows the molecular structure of myo-inositol hexaphosphate.

Most analytical procedures used to separate the IPs have separated the compounds by the number of phosphate groups attached (i.e., the mono, di, tri, tetra, penta, hexaphosphates) and have not distinguished the various structural isomers of each type of the homologous series. The separation technique presented in this thesis has separated the IPs in this same manner.

The IPs have been known by various names in the literature. The hexaphosphate of myo-inositol has been called phytate, phytic acid, and phytin. For clarity and consistency in this manuscript, I use the term "inositol phosphate" with the prefix hexa, penta, tetra, tri, di, or mono. For brevity the respective

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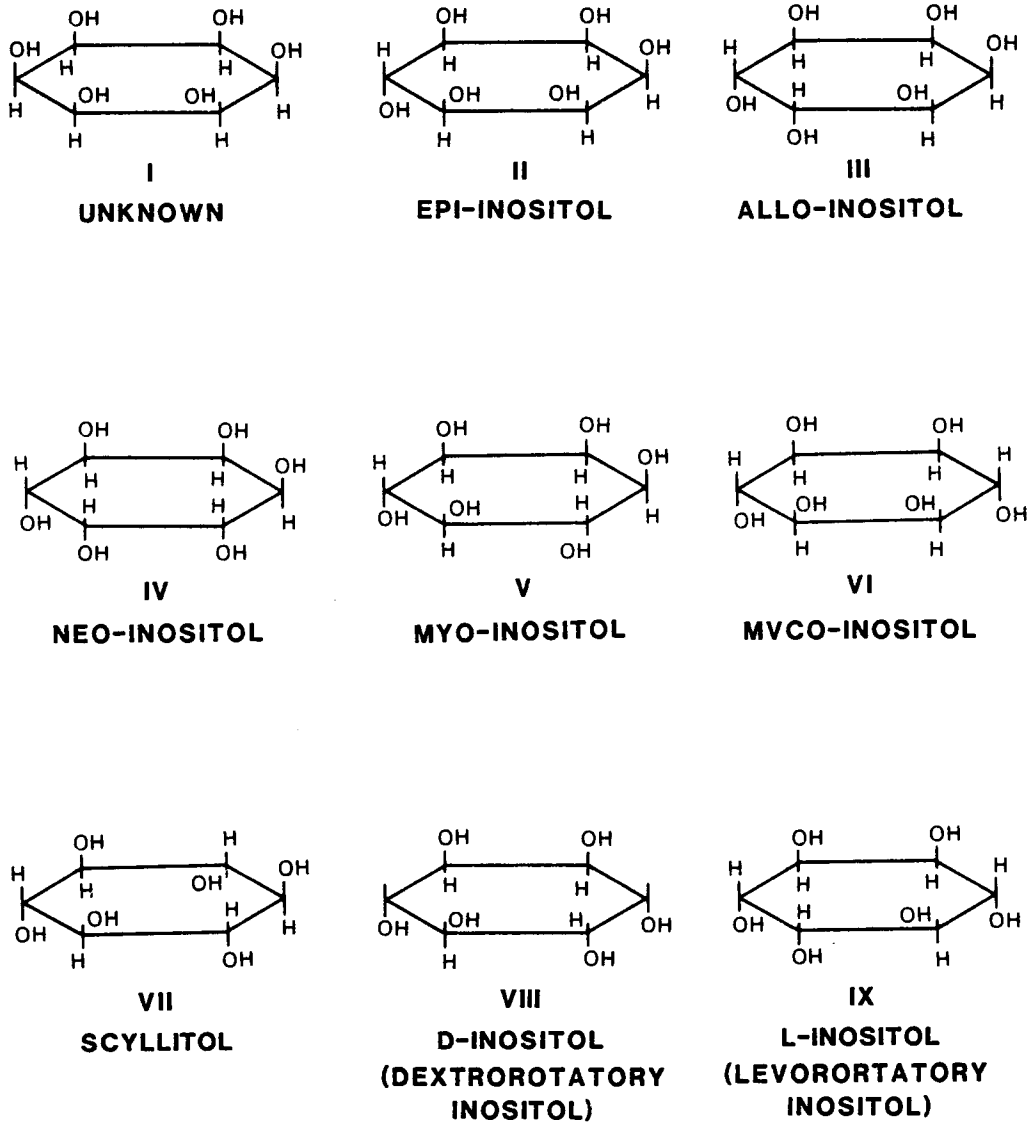


Figure 1. The chemical structure of the isomers of inositol. Source: The Vitamins: Chemistry, physiology, pathology. Eds. W. H. Sebrell, R. S. Harris. New York Academic Press. 1954. p. 324.

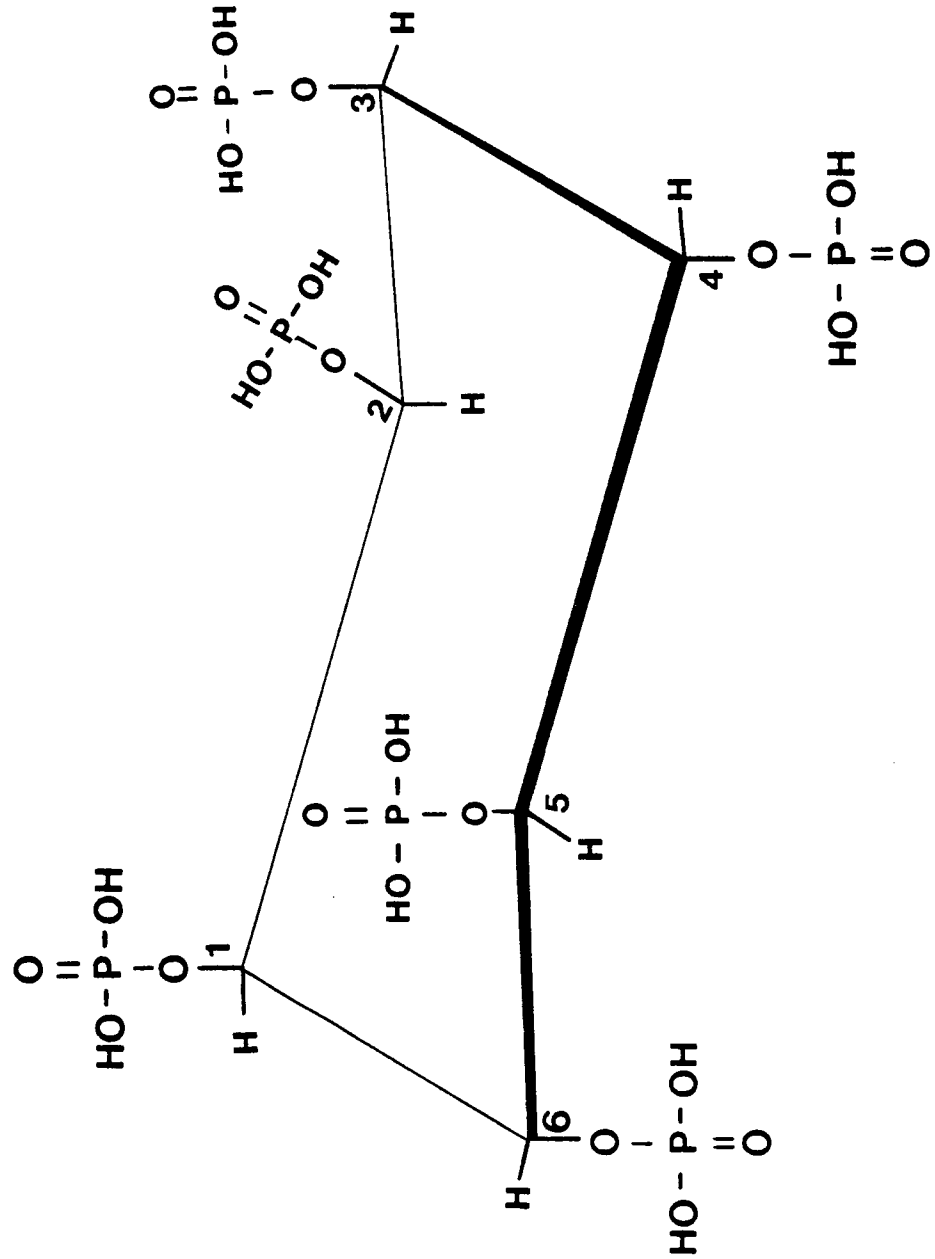


Figure 2. The molecular structure of myo-inositol hexaphosphate.

abbreviations IHP, IPP, ITetP, ITriP, IDP, and IMP are also used. "Lower inositol phosphates" refers collectively to all of the IPs except the hexaphosphate form. The term "intermediate inositol phosphates" is used for all of the IPs except the hexa and mono forms.

Separation And Identification of Inositol Phosphates

Because IPs were first identified as constituents comprising 1–58% of soil organic phosphorus,[57] analytical procedures for extracting, separating, and identifying the homologous series (mono- through hexaphosphate) have been developed and periodically modified in attempts to better understand storage and release of soil nutrients. Soil chemists have used thin-layer chromatography (TLC),[49] paper chromatography,[47] and combinations of paper electrophoresis, paper-partition chromatography and ion-exchange chromatography[45] to isolate the IPs from other organic constituents of soils.

Initial isolation of the IPs usually involved precipitation of the humic acid from a sodium hydroxide soil extract by addition of acid, hypobromite oxidation of all other organic matter except the IPs, followed by precipitation of the IPs as their ferric salts.[45] Because the IPs have been shown to be stable to alkaline bromination, it has been used as an isolation technique by many researchers interested in the IPs.[41,42,44,50,52,58] After isolation, the IP extract can be analyzed by a variety of chromatographic techniques.

Cosgrove[44] reportedly resolved the penta- and hexaphosphates of inositol and identified their respective stereoisomers (myo-, DL-, and scyllo-) in soils. He used De-Acidite anion-exchange chromatography for the initial separations

and confirmed the presence of inositol isomers by paper-partition chromatography. Hong and Yamane[49] separated the six types of phosphate derivatives of inositol (mono- to hexaphosphate) produced from a hydrolysate mixture of Na-IHP using TLC developed with methanol:water:concentrated NH_4OH:acetic acid with volume ratios of 50:30:15:5. They tested the procedure on fulvic and humic acid extractions and found distinct IHP spots in both and fainter spots corresponding to the intermediates in the fulvic acid sample.

In extracts of lake sediments, the IP homologous series has been separated by ion-exchange chromatography using an HCl linear gradient.[52] Gel filtration chromatography has also been used to separate IHP, IMP, inorganic phosphorus, and DNA standards prior to use on lake waters.[41] Minear and Walanski[42] obtained circumstantial evidence for the presence of IMP in algal culture solutions by employing alkaline bromination in conjunction with Sephadex gel filtration. McEntyre[43] followed with supporting evidence for the presence of IMP and the absence of IHP in algal cultures using TLC separations of algal culture samples and IP standards.

Food and cereal chemists have also been interested in the determination of IPs, particularly IHP, in foods. IHP, or phytate as it is called by cereal chemists, is found in all plant seeds, with highest concentrations in mature legumes and cereal grains.[55] Research has focused on the nutritional interactions of IHP in food systems. IHP complexes certain minerals like calcium and zinc, potentially decreasing their bioavailability. An excellent review deals with IHP nutritional implications.[59]

The two most common methods of quantifying IHP in food samples (after an initial extraction with either trichloroacetic acid, hydrochloric acid, or sul-

furic acid) are precipitation with ferric iron and anion-exchange chromatography.[55] In the ferric salt precipitation method, the iron content of the salt is determined colorimetrically and the IHP concentration is calculated from this value, assuming a constant 4 Fe:6 P molecular ratio in the precipitate.[60] Haug and Lantzsch[61] used a faster modification of the ferric precipitation method. However, the specificity of precipitation is uncertain,[62] and the method does not give quantitative results at low concentrations.[63]

Cosgrove[64] and Ellis and Morris[63] used anion-exchange chromatography to isolate IHP from various food samples. Stepwise elution with HCl and NaCl, respectively, resulted in a single peak of IHP, while all other phosphorus compounds were eluted previously under lower ionic conditions. Igaue et al.,[65] on the other hand, used linear gradient elution of 0.0 to 0.6 M HCl on a Dowex (Cl^- form) anion-exchange column to resolve the six phosphorylated derivatives of inositol (mono- through hexa-) extracted from maturing rice plant cells.

An alternative method of plant IHP determination has been proposed using P-31 fourier transform nuclear magnetic resonance spectrometry (^{31}P-FT-NMR).[62] The ^{31}P-FT-NMR technique appears to be specific for IHP, and lower IP peaks may also be distinguished.

Because the ferric precipitation method has been reported as unreliable,[63] column chromatography is too time consuming, and NMR instrumentation is more costly, several analytical methods for IHP in foods have been developed using HPLC.

Tangendjaja et al.[66] first used a reverse phase C_{18} column with a sodium acetate mobile phase and a refractive index detector to separate IHP from inositol. However, only inositol was detected, and IHP eluted with the solvent front

in the void volume. Camire and Clydesdale[67] attempted to get better resolution of IHP from the acid solvent by precipitating the ferric salt prior to injection onto a reversed phase column, but again they recognized the problem of little or no retention of IHP on the C_{18} column. Graf and Dintzis[68] used anion-exchange chromatography as a purification step prior to reversed phase C_{18} chromatography. Again IHP eluted within the void volume. With a similar procedure, Knuckles et al.[69] adjusted the pH of the solvent to pH 6 to prevent dissolution of the column packing for more reproducible results.

Only Lee and Abendroth[70] have developed an HPLC method in which IHP elutes significantly later than the dead volume. They succeeded in doing this by using a tetrabutylammonium-formate ion pair on an octadecyl (C_{18}) column. Food samples still required anion-exchange pre-cleanup.

In all the analytical methods for IHP in foods, analysis of lower IPs was ignored or only alluded to.[68] However, in the study of DOP released from algal cells grown in cultures, an anion-exchange HPLC method to separate and identify the homologous series of IPs was proposed by Hixson.[44] Hixson separated commercially available IHP and IMP from each other on Aminex A-27 resin with ammonium bicarbonate at pH 8.8 as the eluent. However, he had problems with the standard IPs hydrolyzing to possible IP intermediates and PO_4^{3-} while on the column. He speculated that this was due to an alkaline hydrolysis process. However, Brazell et al.[71] used the same anion-exchange resin, Aminex A-27, to separate successfully (without hydrolysis) less stable polyphosphates using as eluent NaCl solutions containing 5 mM Na_4EDTA to maintain a pH of 10.

Hixson used a predicted linear relationship between the log of the elution position and the log of the number of units in the homologous series to calculate the elution positions of the commercially unavailable IP intermediates (standard IHP and IMP elution positions established the slope of the line). Use of this linear relationship is based on similar work done with separation of homologous series of compounds by gel filtration.[72,41] The on-column hydrolysis allowed him to compare calculated values of intermediate IP-retention parameters with retention parameters of the hydrolysis-produced peaks. Therefore, Hixson demonstrated the possibility of using ion-exchange HPLC to separate the IPs. However, the on-column hydrolysis prevented use of the technique on real DOP samples. The separation required long analysis times (as long as twenty hours to elute IHP). Column efficiency was also poor, with the PO_4^{3-} peak exhibiting considerable tailing. These problems indicated the necessity for further modifications of the analytical method to separate the IP homologous series.

CHAPTER III

EXPERIMENTAL PROCEDURES

Field Studies

Study Site

The field studies were conducted in a 38.4-hectare experimental watershed on the Oak Ridge National Environmental Research Park located in the Ridge-and-Valley province of eastern Tennessee. A detailed description of the watershed was reported by Curlin and Nelson.[73] The focus of the flowpath research was the West Fork of Walker Branch. The stream is fed by springs and seeps that arise in dolomitic limestone. Pieces of chert and weathered limestone are abundant in the upper layers of the watershed soils and are also the dominant materials on the stream bottom. The West Fork of Walker Branch joins the shorter and more seasonally sporadic East Fork after flowing approximately 500 meters from the West Fork's source. Walker Branch then empties into Melton Hill Reservoir 1.1 kilometers below the confluence of the two forks. Figure 3 is a map of the watershed showing the watershed boundaries and the stream channels. Melton Hill Reservoir drains an area of 865.8 hectares, so Walker Branch accounts for only 4.4% of the total reservoir watershed.

Sampling Scheme

General. Discrete water samples were collected monthly at several locations in Walker Branch watershed to represent a possible hydrologic pathway. Because samples were collected at point locations in the watershed, it cannot be

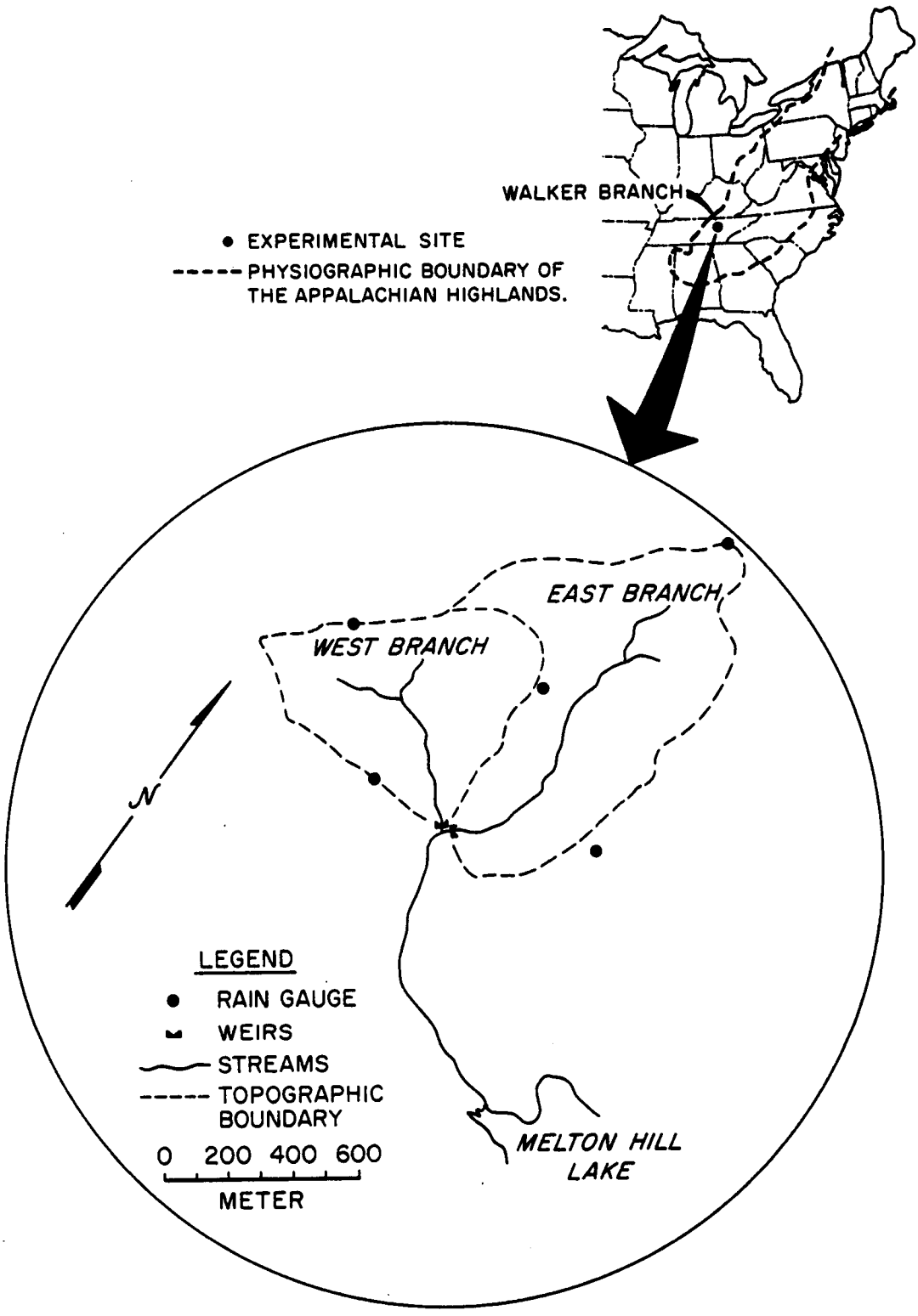


Figure 3. Map of Walker Branch Watershed.

assumed (except for the stream sites) that these points actually make up a continuous hydrologic pathway, (i.e., direct flow from one sample location to another). The sites sampled, however, do represent general compartments of a hydrologic flowpath.

Sampling began in June of 1984 (rain samples were collected in May 1984) and continued through May 1985. Sampling usually took place in early morning while air temperatures were low. All water samples were collected in polyethylene bottles prewashed in 25% HCl and rinsed throroughly with deionized water. Samples were either filtered upon collection in the field or returned to the laboratory and filtered as soon as possible (usually within thirty minutes, see page 25 for details on filtration). Samples were put on ice immediately after collection for transport to the laboratory. Upon arrival at the laboratory, the samples were filtered (if not previously filtered) and analyzed for SRP (see page 25 for details of phosphorus analysis). During any delay of more than 30 minutes between collection and analysis, samples were refrigerated at 4 °C.

Sample Locations. An automatic rainfall sampler was located at the site labeled Ra, as shown in Fig. 4. Acid-washed bottles and funnels were placed on the wet deposition side of the sampler at some time (usually not more than two weeks) prior to a rain event, and a cover automatically sealed air and moisture out of the containers until a rain event had begun. The bottles were collected as soon as possible after the end of the rain event (generally the next day if the event was at night). Aliquots (250 ml) were brought into the laboratory for analysis. At least one precipitation event was collected each month (except April 1985 when the samplers malfunctioned). Rainfall samples were designated with the sample prefix "Ra."

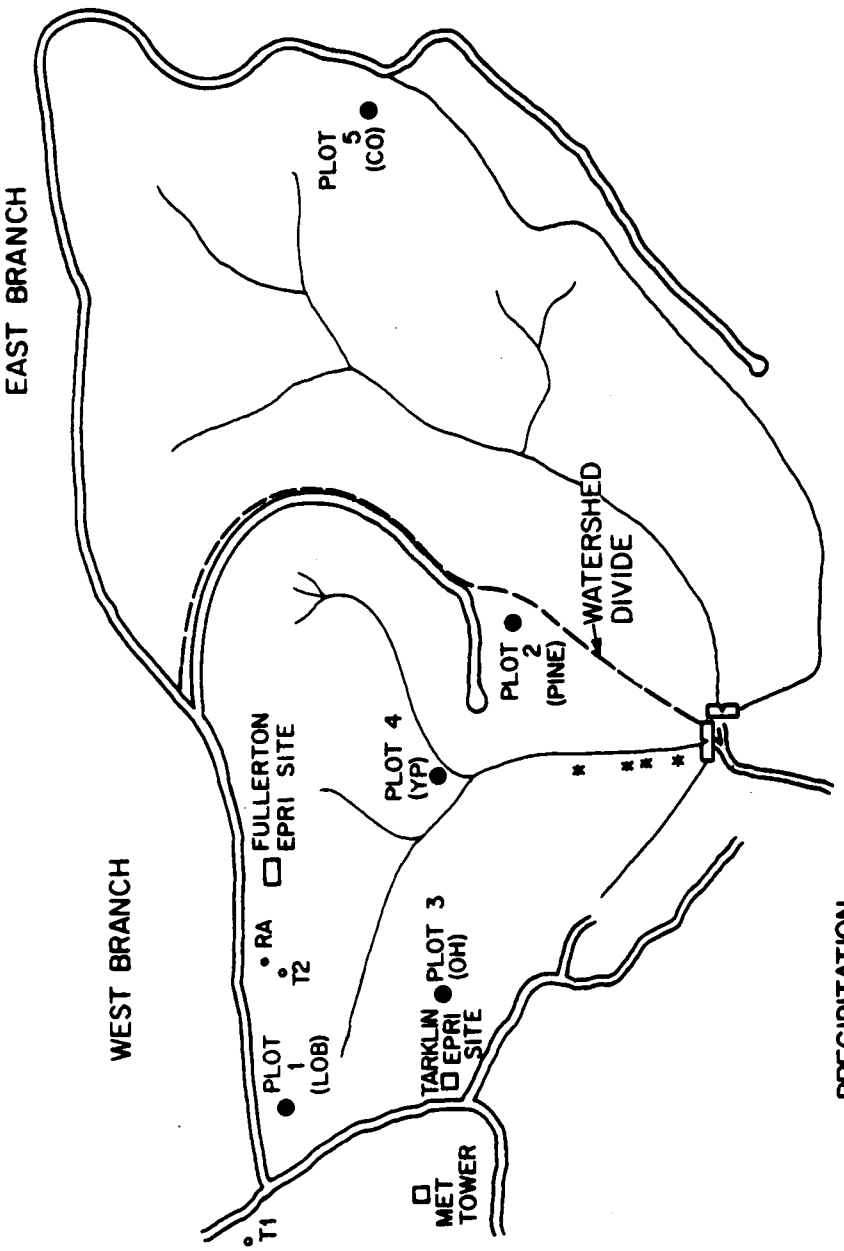


Figure 4. Map of Walker Branch Watershed showing terrestrial sampling locations.

Similar automatic samplers were placed under two canopy (tree cover) types for throughfall collections. (1) chestnut oak canopy—located at the northwest corner of the watershed, designated sample prefix T1, (2) tulip poplar canopy—located in the forest near the rain sampler, designated sample prefix T2 (see Fig. 4). These canopy types were chosen because they are the two dominant forest cover types in the watershed. Throughfall sampling procedures were identical to rainfall collection procedures. In most cases rainfall and throughfall samples were collected for the same precipitation event.

Soil solution samples were collected monthly from soil lysimeters placed in the B-Horizon (~1 meter depth) at locations shown in Fig. 4. These lysimeters were installed as part of another research project on the Walker Branch Watershed. The soil solution sites were located upslope from the stream, above the water table, so at any time (except immediately after a rain event) the soils were dry. Water in the upslope soil system originated as precipitation and percolated directly into the soils with little runoff. Soil solution samples are designated by SW with a number corresponding to each location. In December 1984, detailed soil solution sampling occurred at the EPRI sites labeled Tarklin and Fullerton (see Fig. 4), which included lysimeters located just below the litter, in the A-Horizon (depth of 8 cm for both sites), at the A/B boundary (17 cm at Tarklin site, 38 cm at Fullerton site), and B-Horizon (50 cm at Tarklin, 80 cm at Fullerton) soil layers.

Riparian zone groundwater samples were collected by sinking four polyvinyl chloride wells (9 cm diameter) at different sites longitudinally along the stream bank until the water table was reached. The wells ranged from 0.75 to 1.2 meters deep and 1 to 2 meters from the stream bank. See Fig. 4 for well loca-

tions. To sample the wells, a length of Tygon tubing was inserted into each well and the entire contents of the well pumped out using a Nalgene Mityvac handpump. The wells were then allowed to refill with fresh water over a period of 20 to 30 minutes, and a 250-ml sample was removed from each well with the hand pump. These samples were prefiltered prior to membrane filtration to prevent clogging of the membranes (see page 25 for filtration details).

A sample was collected from a perennial spring located midway along the West Fork of Walker Branch. Spring flow is monitored by a Parshall flume with a stage height recorder. Presumably, phosphorus in the spring water reflects groundwater in the rock unit from which the spring emerges.

Stream water samples were taken at four locations. (1) upstream—located ~100 meters above the spring, (2) midstream—located 2 meters below the spring, (3) downstream—located ~50 meters above the weir, and (4) below the confluence—located midway between the confluence of the East and West Forks and Melton Hill Reservoir. See Fig. 5 for stream and spring site locations.

To complete the sampling scheme and for reference phosphorus concentrations, Melton Hill Reservoir was sampled in two locations. The cove into which Walker Branch flows was sampled at a location approximately 50 meters from the road, 3 meters off shore, and 0.5 meters below the surface (see Fig. 5). The main channel was sampled beneath the Solway Bridge using a weighted Nalgene bucket on the end of a 30 meter rope lowered from the bridge. Attempts were made to collect the reservoir sample in the main channel from beneath the surface of the reservoir. All stream, spring, and lake water sample bottles were rinsed with the sample water, emptied, then a sample collected.

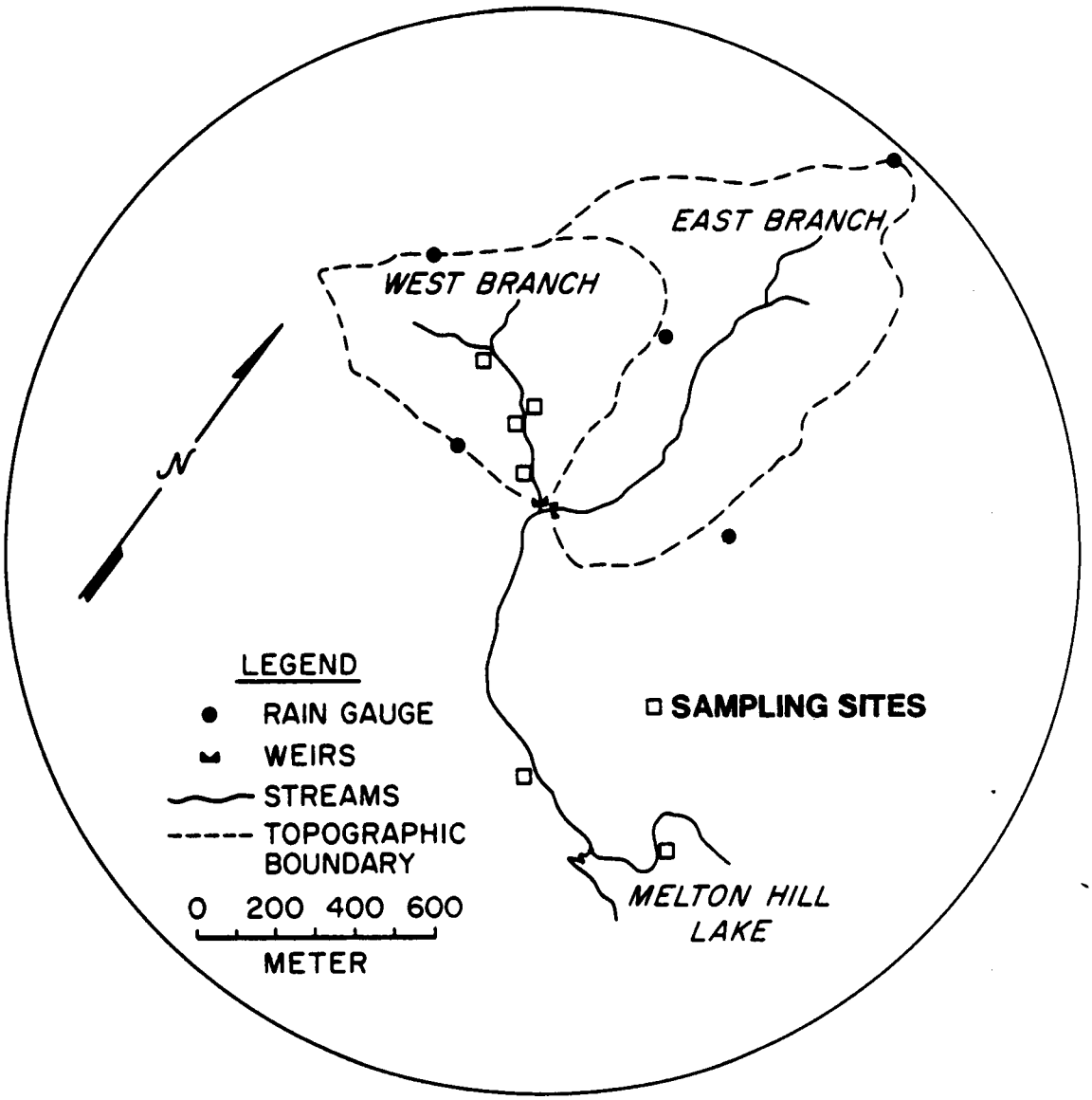


Figure 5. Map of Walker Branch Watershed showing aquatic sampling locations. The second-most upstream box designates the location of the spring.

Laboratory Procedures

Filtration. To distinguish particulate phosphorus from soluble phosphorus (and to prevent extensive exchange of soluble phosphorus into the particulate phase or vice versa during handling), all samples were filtered through 0.4-micron Nuclepore membrane filters immediately after collection. Particularly turbid samples were prefiltered with Whatman glassfiber filters (GF/C or GF/F) to prevent clogging of the membrane filter and to speed up filtration. All filters were washed with at least 100 ml of deionized water immediately prior to use.

Phosphorus Analysis. SRP was determined by the method of Murphy and Riley[10] as outlined by Strickland and Parsons.[6] See Appendix A for exact protocols. Duplicate 50-ml aliquots were analyzed. All glassware was washed with 25% HCl and rinsed twice with deionized water. Ammonium molybdate, sulfuric acid, and antimony potassium tartrate solutions were considered to be stable indefinitely, but were made up fresh approximately every other month. Ascorbic acid solution was made up fresh every five to seven days. Absorbance measurements were made at 885 nm using 10-cm glass cells on a Perkin Elmer Hitachi 200 spectrophotometer or a Perkin Elmer 553 spectrophotometer. Phosphate standard calibration curves were run periodically; the slope changed very little over time.

TSP was determined by a persulfate oxidative technique based on the methods of Menzel and Corwin[11] and Gales et al.[74] The procedure is described in Appendix A. Duplicate samples were run and a mean value calculated. SUP was defined as the difference between TSP and SRP.

Orthophosphate (PO_4^{3-}) was determined by an enzyme assay described by Pettersson.[15] A detailed outline of the procedure used can be found in Appendix A. This analysis was used to obtain a more accurate estimate of PO_4^{3-} concentrations than that based on the measurement of SRP. However, after prolonged problems with enzyme instability, irreproducible results, and inconveniently long analysis times the method was omitted from the monthly analysis routines.

Conductance Measurements. Conductance measurements were made using a Jenway conductivity meter and electrode. Values were recorded in microSiemens.

Analytical Method Development

Outline of Analytical Development Process

I. HPLC Separation of Standards: PO_4^{3-}, IMP, IHP
   A. Optimized Elution Parameters
      1. Gradient
      2. Flowrate
   B. Achieved Desired Resolution of Peaks
II. HPLC Separation of IHP Hydrolysates (IP Intermediates)
   A. Phytase Dephosphorylation of IHP
   B. Determined Elution Positions of Intermediates
      1. Log-Log Plot Unit Number vs Elution Position
      2. Compared Calculated with Actual Elution Positions

III. Determination of IHP in Wheat Bran by HPLC

A. Tested Method on Complex Matrix

B. TCA Extraction of Wheat Bran

IV. Determination of IP in Walker Branch Soil Sample by HPLC

A. Tested HPLC Method on More Complex Matrix

B. Alkaline Bromination and Extraction of Soil Sample

V. HPLC Determination of IP in Walker Branch Groundwater

A. Attempted to Identify DOP Compounds

B. Procedures

1. Collection of 10 liters
2. Filtration: 0.4 μm
3. Cation-Exchange Chromatography
4. Ultrafiltration: YCO5
5. Alkaline Bromination
6. Rotoevaporation

High-Performance Liquid Chromatography

A Varian Vista Series 54 High-Performance Liquid Chromatographic System was used in all developmental work. The Vista 54 contains a model 5000 Liquid Chromatograph (LC) with a single piston reciprocating pumping system and pulse damper to minimize pulsation of flow. The LC was used in the gradient elution mode. The pumping system was designed for pressures up to 350 atmospheres, but during this study pressures ranged from 60 to 81 atm. Vista 54 also contains a Vista 401 Intelligent terminal with CRT and 16K random

access memory, which was used to design gradient elution and flowrate programs as well as to monitor the course of the LC.

A Rheodyne high-pressure rotary manual loop injector was used to inject the sample into the pressurized eluent stream just upstream from the analytical column (or guard column if it was in line). Sample injection volume depended on the size of the sample loop used. In this study a 0.100-ml sample loop was used in all cases. Before each run, the sample loop was flushed with eluent, then with the sample solution, and after each run it was flushed with distilled water. Both rotary sample injection valves and loops were designed to operate within the pressure ranges used in this study.

An Aminex A-27 ion-exchange resin was used as the separation medium. The Aminex A-27 had 8% crosslinkage of divinylbenzene to styrene with quaternary ammonium functional groups attached as the active sites. The average particle size was 15 $\pm$ 2 μm. The resin was supplied and packed by Bio-Rad Laboratories, Richmond, California, into a 250 $\times$ 4.0 mm stainless steel column under the conditions specified for this research.

For samples more complex than commercial standard solutions, a guard column was placed in line upstream of the analytical column to remove any salts and insoluble or particulate matter from the sample prior to entrance onto the analytical column. A Bio-Rad Micro-Guard System was used. The system consisted of a 4 cm $\times$ 4.6 mm disposable cartridge, slurry packed with 10-micron Aminex A-27 packing material, in a Micro-Guard holder.

The eluent used in this study was a linear gradient from 0.10 M NaCl to 0.50 M NaCl with a constant 0.5 mM tetrasodium ethylenediaminetetraacetic acid (Na_4EDTA) solution to maintain a pH of 10.0. The analytical column was

stored with 0.5 M NaCl/0.5 mM Na_4EDTA. Prior to use, the column was slowly ramped down to initial eluent concentrations and equilibrated with 0.10 M NaCl/0.5 mM Na_4EDTA for 30 minutes before injection of the first sample and recycled in the same manner before a second run. Table 1 summarizes the HPLC physical characteristics and operation parameters used in this study.

Phosphorus detection for the HPLC system was done by collection of 0.4-ml (1.0 minute) fractions using an ISCO Retriever III fraction collector set in the "time" mode, followed by wet chemistry phosphorus analysis as performed on the watershed field samples with modifications for lower volume (see

Table 1. HPLC physical characteristics and operating parameters

Parameter	Description
Column	250 × 4.0 mm
Resin	Aminex A-27
Resin particle size	15 ± 2 μm
Injection volume	100 μl
Column temperature	Ambient
Flowrate	0.40 ml/min
Eluent	0.10 M to 0.50 M NaCl with 0.5 mM Na_4EDTA, pH 10
	Gradient mode, time interval: 60 min, hold 20 min
Fraction volume	0.4 ml
Operating pressure	60–81 atm

Phosphorus Analyses in Appendix A). Absorbance measurements were made using 1.0-cm cuvettes on a Perkin-Elmer 553 spectrophotometer. The Aminex A-27 column, HPLC system, and fraction collector were provided by Oak Ridge National Laboratory.

Standard solutions were prepared from reagent-grade chemicals. An ortho-phosphate stock solution was prepared by dissolution of KH_2PO_4 in HPLC-grade high-purity water to a concentration of 50 μg of atomic phosphorus/ml and stored at 4 °C. Myo-inositol hexaphosphate was obtained as the sodium salt and myo-inositol 2-monophosphate as the amine salt from Sigma Chemical Company, St. Louis, Missouri. They were dissolved in HPLC-grade water to the appropriate concentration. The IP standard solutions were stored at 4 °C for only a few days before being discarded and fresh standards prepared.

Enzymatic Dephosphorylation of Inositol Hexaphosphate

Dephosphorylation of IHP was initiated in order to establish the elution positions of the IP intermediates. The enzymatic procedure is a slight modification of that performed by Herbes.[75] The enzyme used, phytase, selectively cleaves off PO_4^{3-} groups; so, if stopped at intermediate stages of reaction, a mixture of IPs may be found. Phytase was obtained from Sigma Chemical Company and kept dessicated and frozen until just before use. A detailed outline of the dephosphorylation procedure used in this study is given in Appendix A.

Extraction of Inositol Hexaphosphate from Wheat Bran

The method of extraction of IHP from wheat bran was based on the procedure of Knuckles et al.[69] The wheat bran was purchased from a local health food store. A detailed outline of the extraction procedure used is given in Appendix A.

Extraction of Inositol Phosphates from Soil: Alkaline Bromination

A modified version of the alkaline bromination procedure used by early soil chemists was used in this study. The method involved a combination of basic extraction of soil organics and oxidative bromination of all organics except the IPs in a single step as described and recommended by Irving and Cosgrove.[58] The soil used for the extraction was from the A-1 horizon of a Claiborn soil, collected from a cove of Walker Branch. The sample was supplied by the Earth Sciences Section of Oak Ridge National Laboratory. A detailed description of the extraction procedure is given in Appendix A. A modification of this procedure was also applied in the final stage of method development to a concentrated ground water sample prior to injection into the HPLC.

Cation-Exchange Chromatography

A 10-liter Walker Branch water sample was passed through a 3.0 $\times$ 21.5 cm id Dowex 50W-X8 cation-exchange column (Na^+ form) at a flowrate of 5 to 10 ml/min before being concentrated by ultrafiltration. This step converted all salts to the most soluble cation (Na^+) form to prevent their precipitation during the concentration step.

Sample Concentration

In order for a water sample from Walker Branch to be analyzed for the presence of IPs by HPLC, it had to be concentrated by approximately 1000-fold. The technique selected to do this was ultrafiltration through an Amicon YCO5 ultrafiltration membrane. The YCO5 membrane retains compounds with a molecular weight above 500. The membrane manufacturer stated that the YCO5 also retained PO_4^{3-}. After initial filtration through 0.4-μm Nuclepore filter to remove particulates, the 10-liter water sample was sequentially concentrated above three different YCO5 membranes of decreasing diameter (150 mm, 76 mm, 43 mm) to a final volume of 8.8 ml. Amicon filter holders, with built-in stirring bars, appropriate to the filter diameter were used. Filtration was induced by N_2 above the membrane at not greater than 55 psi, depending on the flowrate desired. After alkaline bromination of the sample, the final concentration step was performed by rotary evaporation to 1 ml on a Brinkmann Buchi Rotovapor—R evaporator.

Data Processing

Watershed field data were examined using SAS statistical and graphics packages on the DEC PDP-10, an Oak Ridge National Laboratory mainframe computer system. Some simpler manipulations were performed on an Apple IIe personal computer using Omnifile and Omnigraph software packages. The watershed phosphorus data were examined graphically for changes in time within each compartment and changes in space within each month.

CHAPTER IV

RESULTS AND DISCUSSION

Distribution of Soluble Phosphorus in Walker Branch Watershed

The purpose of the watershed field studies was (1) to trace the patterns of soluble phosphorus through adjacent compartments of a hydrologic flowpath in the watershed, (2) to pinpoint locations of change in phosphorus concentration and distribution of biochemical form, (3) to detect any seasonal patterns of soluble phosphorus concentration and distribution, and (4) to postulate what environmental factors influenced the patterns observed.

Overview of Field Studies Results

Figure 6 gives an overview of the results of phosphorus analyses performed on watershed samples throughout the year from June 1984 to May 1985. For this summary of the data, the sampling year was divided into two seasons: a leafed season—June through October of 1984, plus April and May of 1985; and a winter season—November 1984 through March 1985. The seasonal distinction was made after visual examination of the seasonal patterns of soluble phosphorus in each compartment, which showed that the greatest changes in phosphorus occurred between the seasons rather than within them.

The concentrations reported in Fig. 6 are seasonal means. All concentrations reported in this thesis are written as micrograms atomic phosphorus per liter of solution. The precipitation values (rainfall and throughfall) are volume-weighted means by season. The throughfall, soil solution, and groundwater

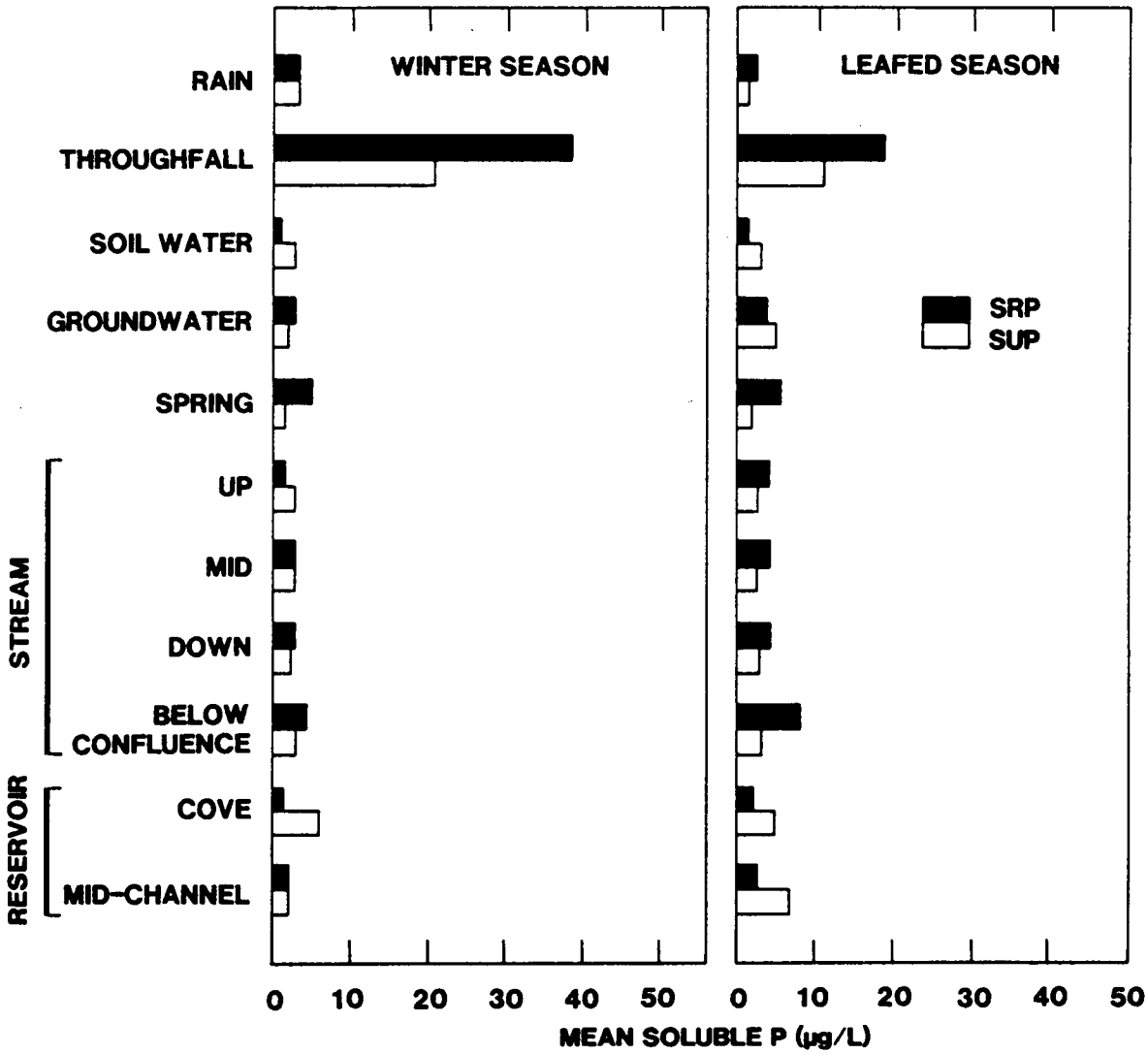


Figure 6. Seasonal mean SRP and SUP concentrations along a hydrologic flowpath in Walker Branch Watershed.

values represent both sample and seasonal means. Due to time constraints on the phosphorus analysis, replicate samples were not collected for each site along the stream and reservoir, so the values for these sites are average phosphorus concentrations observed during the season. Table 2 lists the mean values from which Fig. 6 is drawn and includes a measure of the combined seasonal and sample variation as reflected by the 95% confidence interval. The watershed data, compiled according to the month sampled, appear in Appendix B.

Phosphorus in Hydrologic Flowpath Compartments

Beginning with the most upstream compartment of the hydrologic flowpath, rainfall events sampled contained a low but significant concentration of soluble phosphorus relative to the stream concentration. SUP accounted for almost 50% of the TSP in the winter season and over 30% in the leafed season (Fig. 6). There was also a decrease in the seasonal mean concentration of SUP from the winter to the leafed season. However, on the scale of one year, soluble phosphorus concentrations in rainfall were relatively constant from event to event, compared to throughfall soluble phosphorus concentrations. The relative constancy of SRP in rainfall can be seen in Fig. 7. However, as the rain fell through the canopy, phosphorus concentrations changed dramatically. Figure 7 also compares SRP concentrations in rainfall to that in chestnut oak throughfall. In all events except two, both SRP and SUP were released from the canopy into solution. In July 1984, SUP increased from 1.13 μg phosphorus/liter in rainfall to 5.51 μg phosphorus/liter in chestmut oak throughfall. In August 1984, SUP increased from 1.78 μg phosphorus/liter in rainfall to 21.7 μg phosphorus/liter in throughfall. The increase in SUP from

Table 2. Summary of SRP and SUP concentrations along a hydrologic flowpath in Walker Branch Watershed

(Mean values and 95% Confidence Intervals)

	Leafed season					Winter season				
	SRP (P μg/L)		SUP P μg/L			SRP (P μg/L)		SUP (P μg/L)		
Compartment	$\bar{X}$	95% conf. interval	$\bar{X}$	95% conf. interval	*n*	$\bar{X}$	95% conf. interval	$\bar{X}$	95% conf. interval	*n*
Rainfall	2.29[a]		1.05		6	3.44		2.97		6
Throughfall										
Chestnut oak	7.02		8.39		5	31.93		13.81		6
Tulip poplar	30.9		14.2		4	45.31		29.42		6
Soil water	0.83	2.16	2.68	4.26	2	0.79	0.78	2.66	1.34	4
Groundwater	3.51	1.23	4.45	1.84	6	2.91	0.59	1.80	0.56	5
Spring	5.24	0.15	1.18	1.17	7	5.03	0.87	1.07	0.20	5
Upstream	3.63	2.47	1.81	1.04	7	0.99	0.90	2.48	1.56	5
Midstream	3.56	1.07	1.99	0.80	7	2.51	1.17	2.69	3.02	5
Downstream	3.76	0.90	2.43	1.52	7	2.46	1.93	1.87	0.53	5
Below confluence	7.78	3.87	2.76	1.59	7	4.16	0.49	2.61	1.67	5
Reservoir-cove	1.43	0.76	4.08	1.76	7	1.16	1.23	3.63	1.62	4
Reservoir-main channel	1.96	1.12	6.18	2.54	8	1.72	0.97	2.28	1.17	5

[a]Precipitation values are volume weighted means; no confidence interval was calculated.

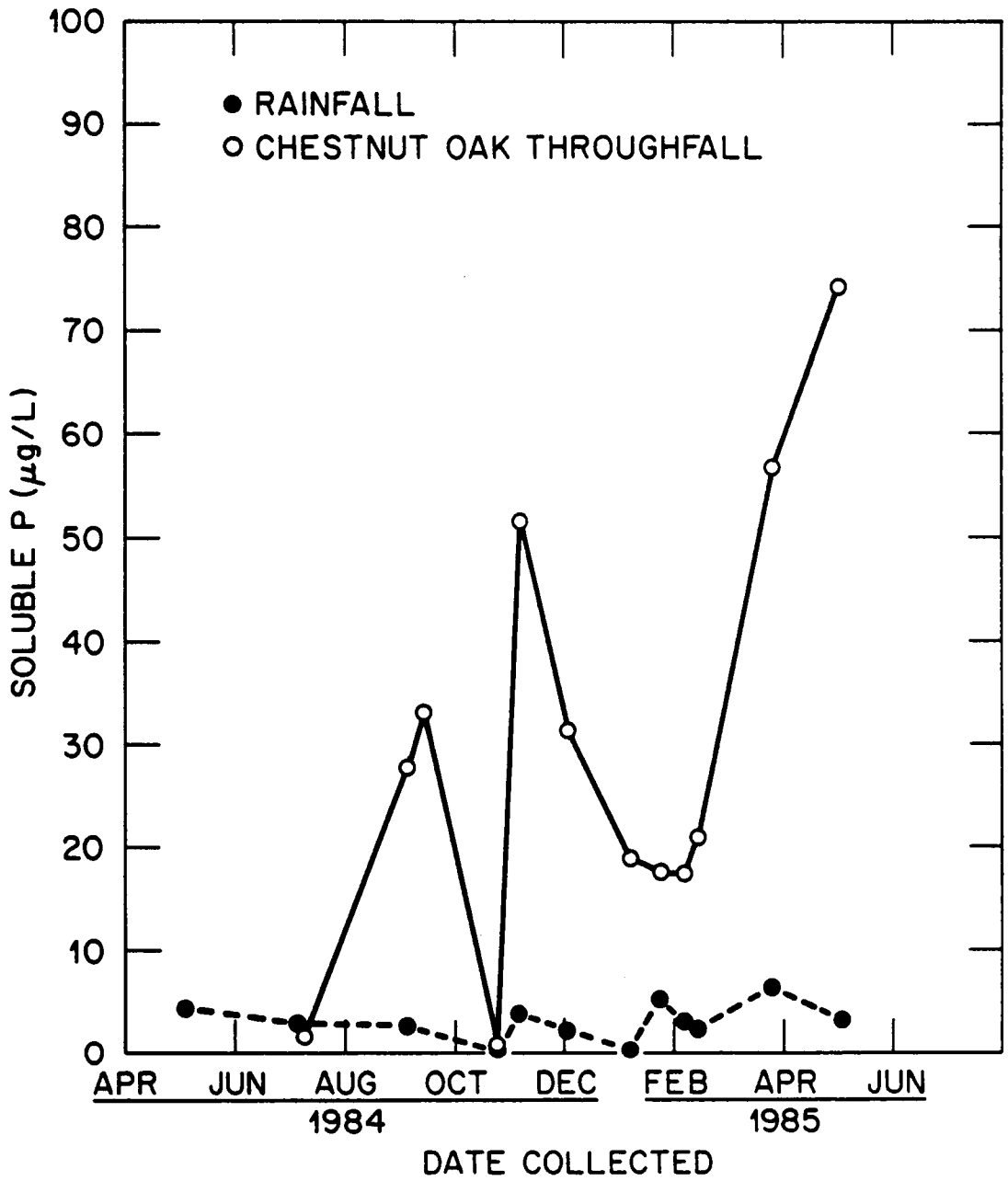


Figure 7. A comparison of SRP concentrations in rainfall and throughfall over one year.

rainfall to throughfall was highest in November when rainfall had 13.74 μg SUP/liter and tulip poplar throughfall contained 189.0 μg SUP/liter. In some months, phosphorus concentrations were an order of magnitude greater in throughfall than in rainfall. The increase in phosphorus concentrations cannot entirely be explained by evaporation occurring between the sample compartments. The higher concentration of soluble phosphorus in throughfall indicates a leaching and/or wash-off of phosphorus from the leaves and branches of the canopy. In July and October of 1984 the opposite appears to have occurred; a slight decrease in phosphorus concentrations from rainfall to chestnut oak throughfall indicated a net uptake of SRP within the chestnut oak canopy (Fig. 7). The tulip poplar canopy showed a similar decline of SRP in the months of May 1984 and 1985 (see Tables B-1 and B-12 in Appendix B for data). These decreases in phosphorus concentration between rainfall and throughfall may be due to biological uptake in the canopy at these times, but the exact cause was not determined.

Because almost the entire watershed is covered with a mixed hardwood canopy, it appears a relatively large input of soluble phosphorus could be transported from the canopy into the aquatic portion of the watershed as a result of this wash-off or leaching process. But as the soil solution results show, only low concentrations of SRP and SUP reached the B-horizon soil layer. Figure 8 shows the change in TSP occurring between the tulip poplar throughfall and the B-horizon soil layer. Without interaction with soil biogeochemical processes, soluble phosphorus concentrations would be expected to increase from throughfall to soil water due strictly to evapotranspiration. The dramatic decrease in concentrations of soluble phosphorus suggests strong retention of

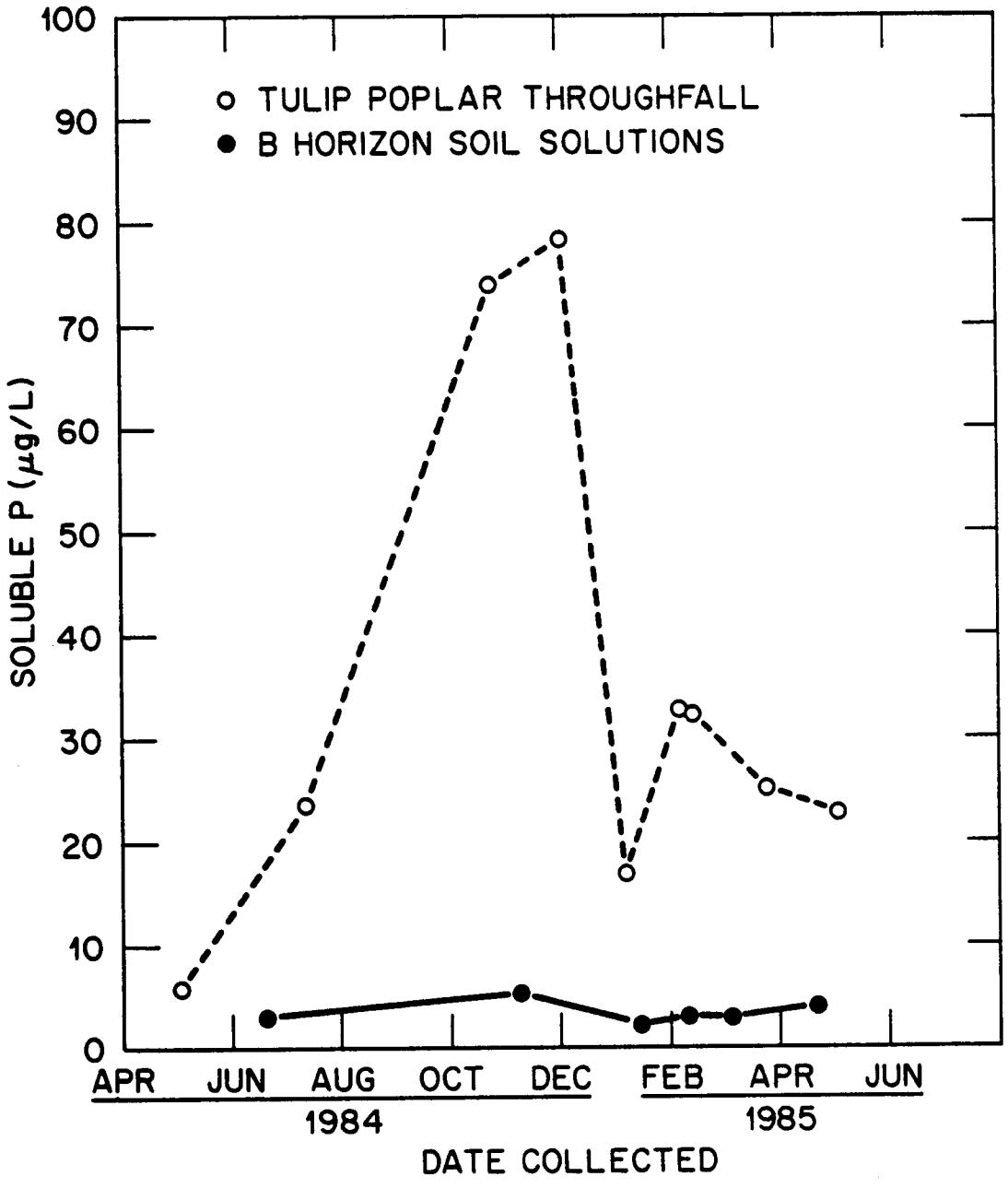


Figure 8. A comparison of TSP concentrations in throughfall and B-horizon soil solutions over one year.

phosphorus by either biological uptake or adsorption to ionic sites on soil particles or a combination of these two processes. It is important to note, however, that because the soil solution samples were collected by another research group, the samples were handled somewhat differently than the remainder of the watershed samples. The samples were left in contact with the sample container (soil lysimeter) for longer periods of time than the other watershed samples. The exact time length, although no more than one month and possibly as short as a few days, and the effect this extra time had on the samples was not determined. However, the soil solution samples showed consistently and dramatically lower phosphorus concentrations than throughfall, which probably could not be explained completely by longer exposure to the sample container.

Because the monthly soil solution samples were collected from the B-horizon only, the location in the soil of greatest phosphorus reduction could not be inferred from the monthly sample means. However, results of the detailed sampling and analysis performed on two sites (see Fig. 4 page 21, for locations of the Tarklin and Fullerton sites) in December 1984 gave a better picture of phosphorus retention within the soil profile. Figure 9 is a summary of these results. The soil solution mean values of 2 to 4 sample replicates for each site were compared to the throughfall data for the dominant canopy type at that site.

It appears that in December the entire loss of SRP occurred within the litter layer, where biological activity is greatest.[1] SRP concentrations dropped from >30 μg phosphorus/liter in throughfall to <2 μg phosphorus/liter in solution that was collected after passing through the litter layer. The SRP concentration then remained fairly constant throughout the remainder of the soil profile. On

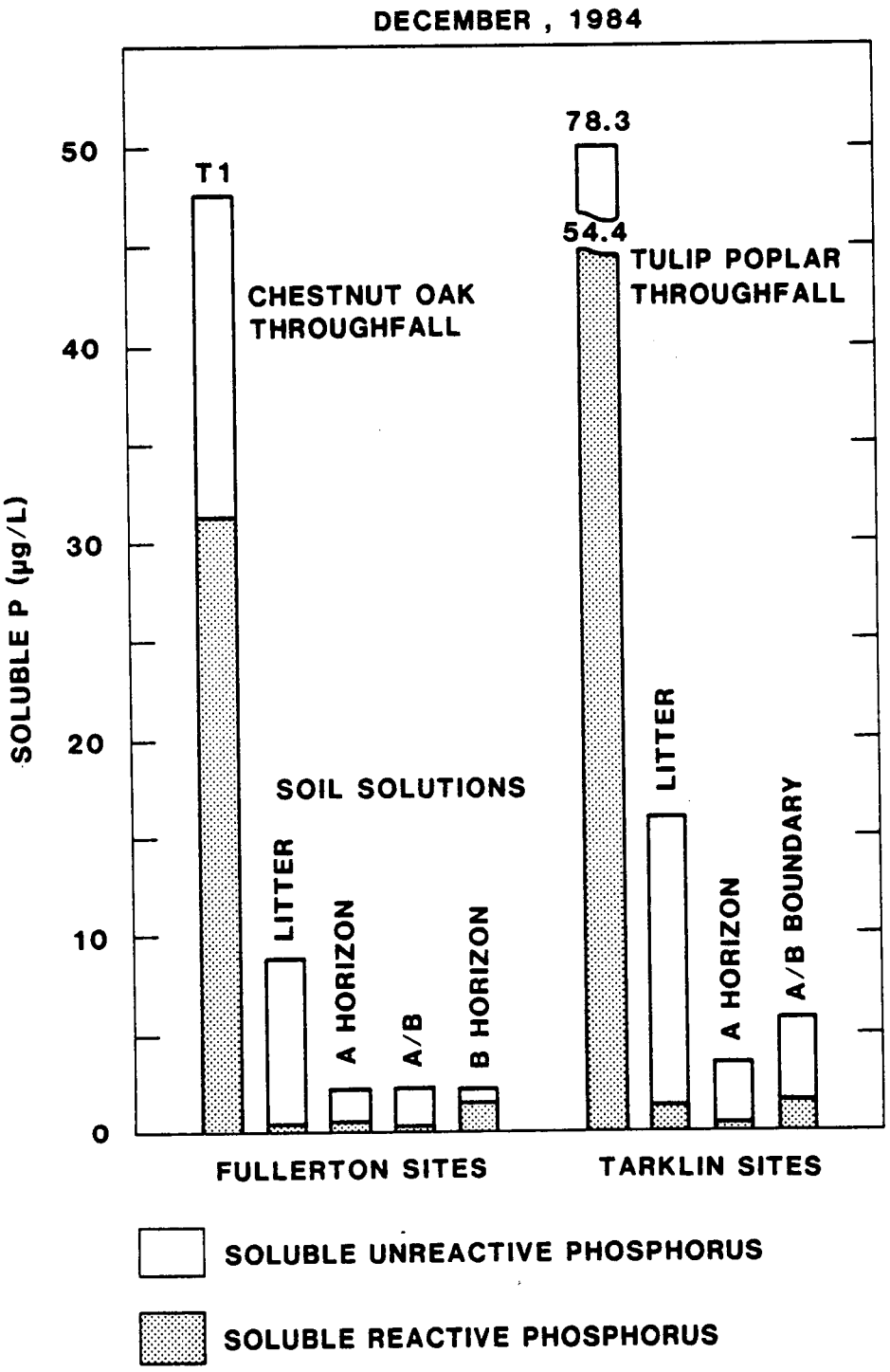


Figure 9. A comparison of soluble phosphorus in throughfall and soil solutions, December 1984. Shaded = SRP; Open = SUP.

the other hand, the retention of SUP seemed to lag behind that of SRP. The greatest decrease did occur in the litter layer, although the uptake was not as dramatic as that of SRP. SUP concentrations continued to decline through the A horizon. There was a statistically significant decrease in SUP concentration from the litter to the A horizon at the Tarklin site.

In similar research on phosphorus dynamics in soils, Wood and Borman[1] reported for Bear Brook Watershed in New Hampshire that biological processes appeared to control phosphorus movement in the upper organic soil layers and that geochemical processes potentially began to dominate phosphorus distribution in the underlying soil layers. Also, work done by M. Silver[23] on Walker Branch leaf litter showed a net uptake of SRP and a net release of SUP as spring water was passed through the litter. Whether these net changes in SRP and SUP were due to biotic or abiotic processes was not determined. One plausible explanation—based on the data presented here and on the research mentioned above—for the rapid decline of SRP in throughfall as it passes through Walker Branch soils, is that SRP was quickly taken up by the biota in the litter layer.

Conflicting processes may have been regulating SUP movement through the soil. Release of SUP in the litter layer, as seen in Silver's experiments, may have occurred simultaneously with geochemical adsorption and biological breakdown of SUP in both the litter and A horizon, resulting in a slower net uptake of SUP than of SRP. However, little work has been done on the retention of SUP by soils, so the processes controlling its concentration in the soil profile can only be speculated. Whatever these processes are, very low concentrations of soluble phosphorus are transported through the soil system into the aquatic

systems of the watershed. It appears that most of the phosphorus in throughfall is retained in the rooting zone of the terrestrial portion of the watershed indicating a very tight phosphorus cycle.

Soluble phosphorus concentrations remained relatively low and constant throughout the remainder of the hydrologic flowpath (Fig. 6). Phosphorus concentrations in groundwater (as determined from four riparian zone wells) were slightly higher than that in soil solutions. The wells showed some amount of variation among themselves and seasonally, although no obvious pattern was evident (see Tables B-1 through B-12 for groundwater well data). Phosphorus concentrations in spring water were relatively constant year round at 5 μg SRP/liter and 1 μg SUP/liter. These data and data on calcium, magnesium, and bicarbonate concentrations in the spring reflect a diffuse aquifer in which water is in equilibrium with the dolomitic bedrock.[76]

Uptake and turnover of phosphorus within the Walker Branch stream channel have been shown to be controlled predominantly by biological mechanisms.[26] So the relatively constant TSP concentrations from upstream to downstream in Walker Branch could be attributed to biological "buffering" of the nutrient. Comparisons of soluble phosphorus concentrations between specific sites along the stream channel suggests that biochemical transformation of soluble phosphorus forms is occurring during the year. Figure 10 compares the SRP concentrations of the upstream site, the spring site, and the midstream site over the year sampled. The upstream site is located ~100 meters above the spring, and the midstream site is located ~2 meters below the spring. The spring SRP concentrations are fairly constant over time as noted before. Upstream SRP appears to follow a seasonal pattern with a maximum in mid-

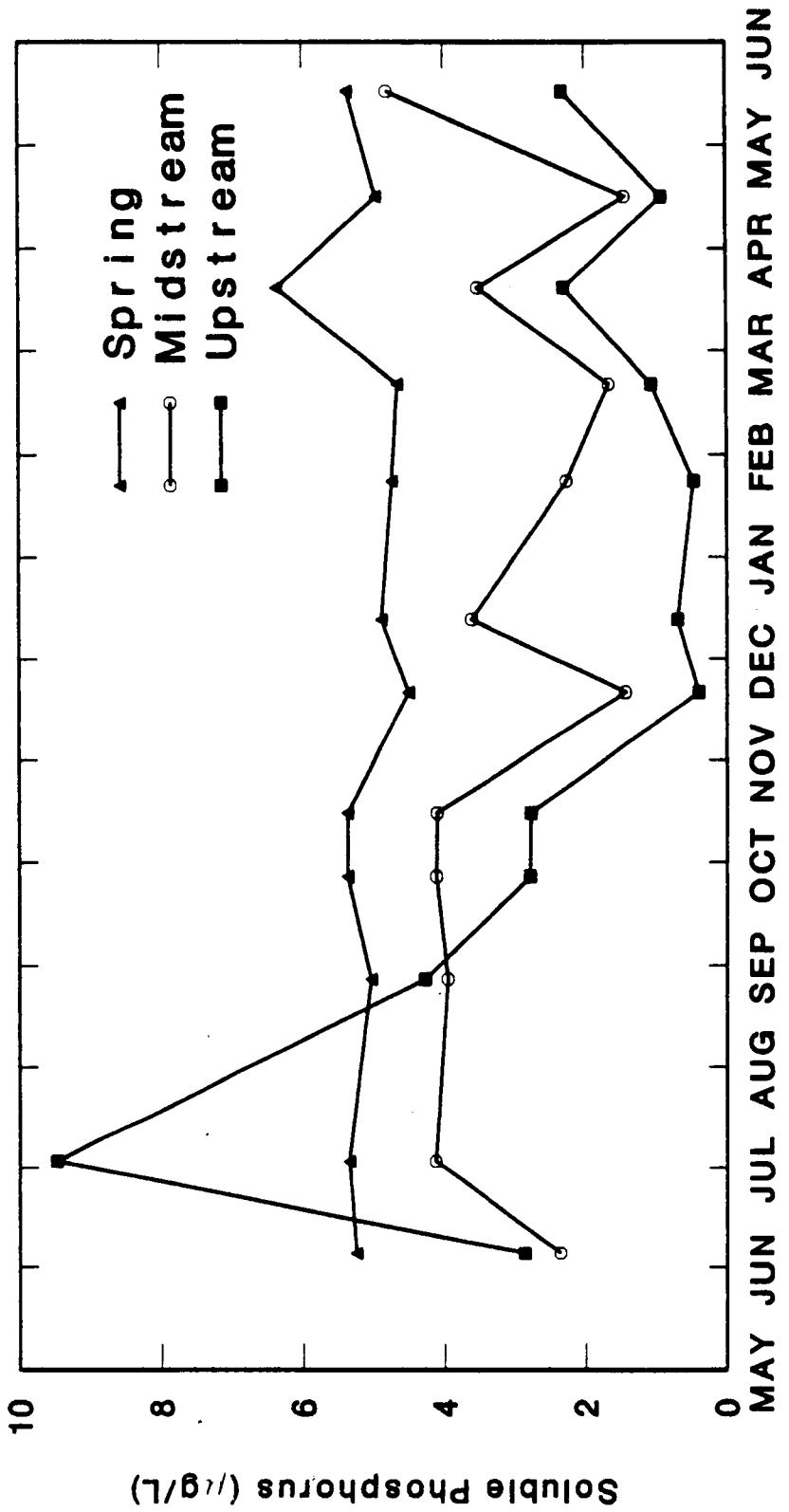


Figure 10. SRP concentrations in upstream, spring, and midstream sites.

summer and a minimum in the fall soon after leaf abcission. This autumn decrease in SRP corresponds to an increase in rates of phosphorus uptake associated with microflora developing on leaf detritus.[29]

The lack of a seasonal pattern in SRP at the midstream site may be due to dilution with the higher and more constant SRP in the spring just up stream, since the spring accounts for approximately 20% of the stream flow at the midstream site. However, in June, July, and August 1984, the midstream site showed lower SRP than both the upstream and spring sites, indicating that the stream bed was accumulating SRP. Rigler[30] performed a series of experiments on streams in Great Britain that supported the hypothesis that SRP accumulated on the stream bed during periods of base flow. Newbold et al.[28] found that particulate organic matter accounted for 95% of inorganic phosphorus uptake in Walker Branch. So a reasonable explanation for the decline in SRP could be that microorganisms are taking up SRP in the stream channel. However, not all sources of water entering Walker Branch were sampled and diffuse sources may have had a lower SRP content than the stream thus, the difference between the 4.1 μg/liter SRP at midstream and the 9.5 μg/liter SRP at upstream during July 1984 could be due in part to dilution of the stream water with water from unmeasured sources entering between the two sites containing less than 4.1 μg/liter SRP. In order to determine the exact cause of the decline in stream SRP, measurements of phosphorus in all known inputs of water between the upstream and midstream sites need to be made and experiments to determine the interaction of stream water with groundwater along that reach of the stream need to be performed.

While SRP appeared to be taken up in the stream channel at some times during the year, SUP was being released. Figure 11 compares SUP at the same three locations as in Fig. 10. Even though SUP was low at all three sites, six out of the twelve months sampled showed higher SUP at the midstream site than in upstream water or spring water, indicating a release of unreactive phosphorus within the stream channel. These results support previous work on Walker Branch showing the release of HMWP compounds, as determined by gel chromatography, from particulate organic matter in stream water.[77] Thus, it is possible that the concentration of DOP (as estimated by both HMWP and SUP) increased from upstream to downstream at several times during the year due to release by particulate organic matter in the stream. Again, however, the argument could be made for dilution of the stream water with unmeasured water sources. If the groundwater wells sampled in Walker Branch Watershed are a good representation of subsurface water that possibly is in constant exchange with the stream water, then the groundwater may be a source of SUP to the stream. Table 2 page 36 shows that mean seasonal SUP in the groundwater wells was higher, although not significantly, than mean SUP in the stream. On the other hand, if the low spring SUP values represent true groundwater that is in contact with the stream or if there were no exchange of water between the stream and a subsurface reservoir, then dilution could not be a factor in the increase of SUP between the upstream and midstream sites. Thus, release of SUP by microorganisms in the stream appears a more plausible explanation.

If there is a uniform release rate of SUP per unit area of stream bottom, the concentration of SUP in the stream water should increase in the down-

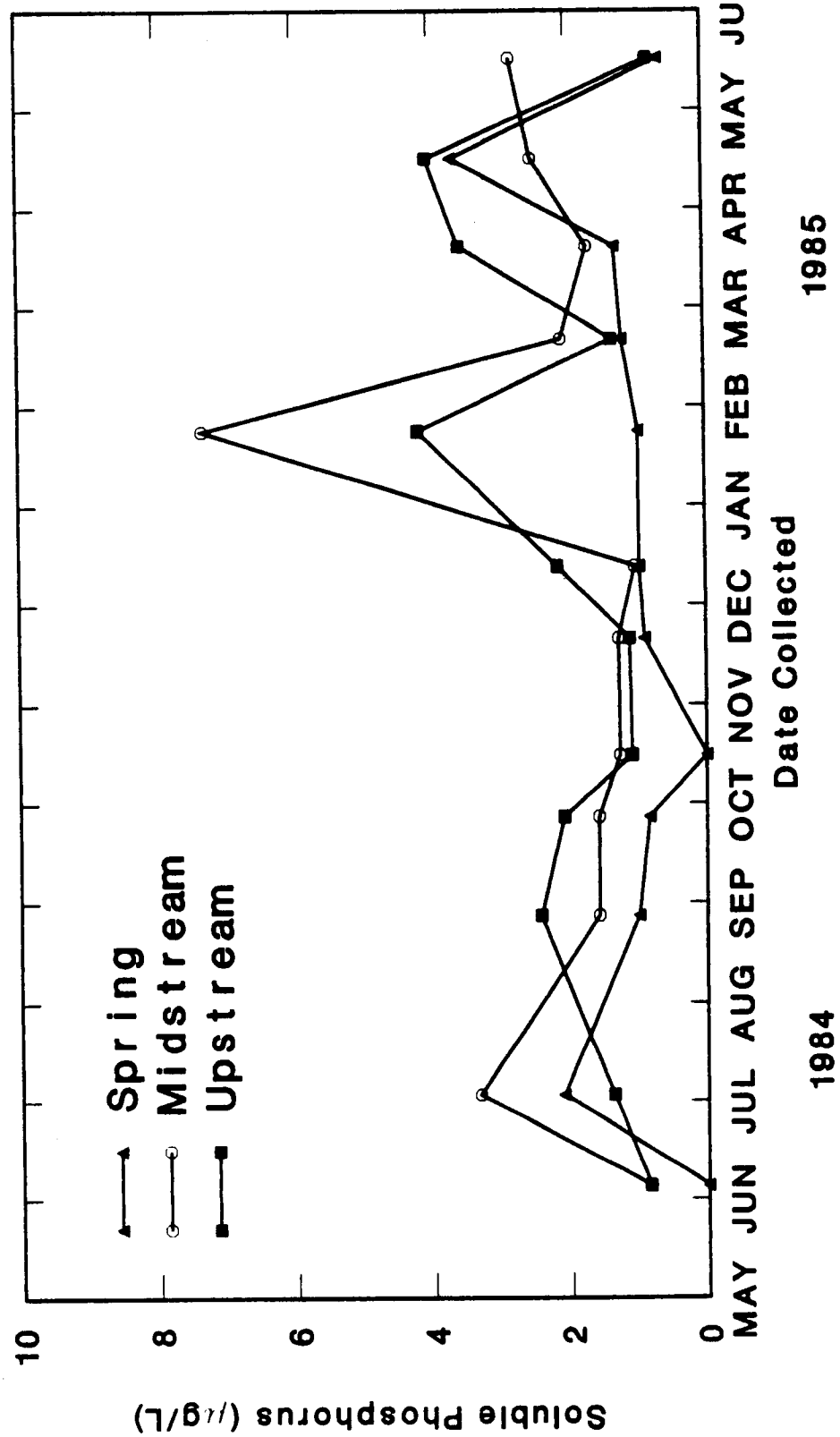


Figure 11. SUP concentrations in upstream, spring, and midstream sites.

stream direction. As Fig. 12a shows, a general trend of increasing SUP in the downstream direction can be seen during the leafed season in Walker Branch. However, this trend is not evident in the mean values for the winter season (Fig. 12b).

Examination of Fig. 6 on page 34 reveals that SRP was the dominant form of soluble phosphorus in Walker Branch. However, in Melton Hill Reservoir and in soil water, the unreactive fraction was the major form. The increase in the fraction of TSP accounted for by SUP from throughfall to soil water was probably due to the high uptake of SRP relative to SUP by the litter layer. In the reservoir, this same change in the dominant biochemical form could be related to the residence time of phosphorus within the system. In the stream, where the residence time of water is relatively short, SUP made up <40% of TSP, calculated as annual means at each stream sample site. In a lentic system, such as the reservoir, the residence time of water (and hence soluble phosphorus) should be much greater than in the stream, thus allowing more interaction with suspended organic particulate matter and more time for release and build up of organic phosphorus compounds.

SUP made up a considerable fraction of the TSP concentration in all of the hydrologic compartments sampled in Walker Branch Watershed. Table 3 summarizes the data by showing the annual mean percentage of SUP at each site sampled. The values range from 17 to 75% SUP. Because SUP is an estimate of dissolved organic phosphorus, a significant fraction of soluble phosphorus could be in the organic form at almost all points in the hydrologic flowpath.

As described in the literature review of this thesis, very little is known about the character, identity, and availability of the DOP component of natural

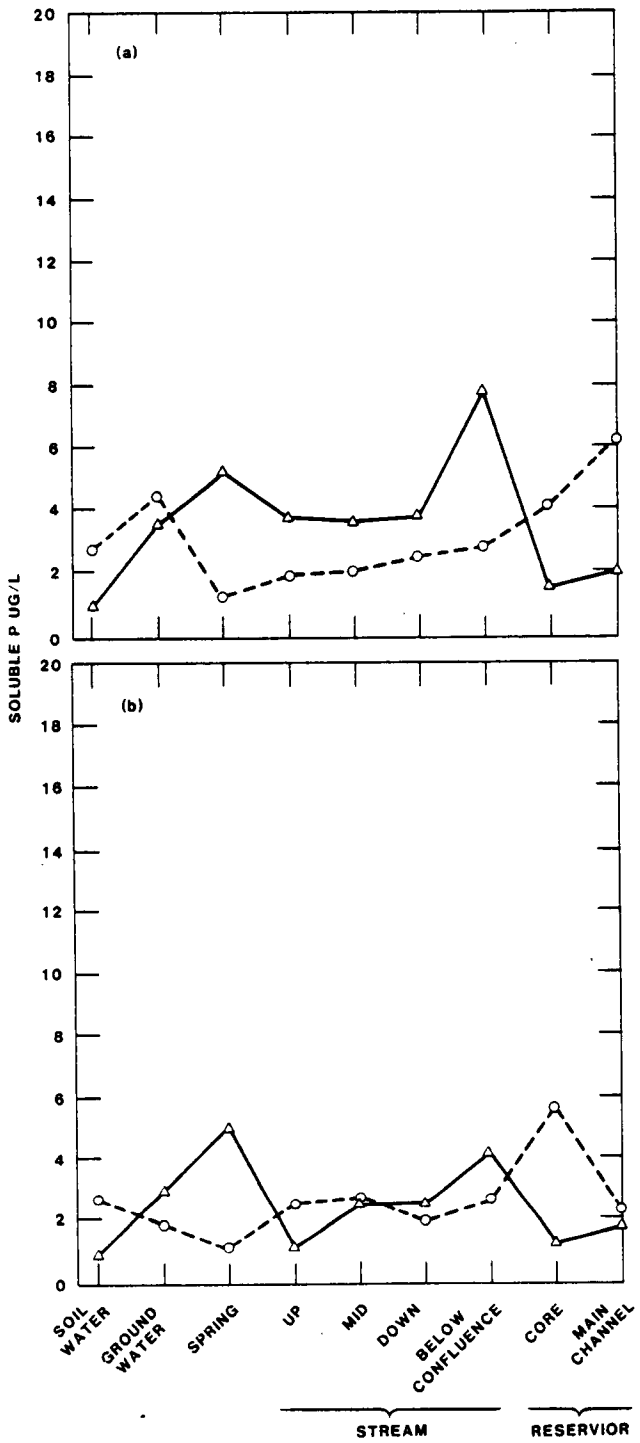


Figure 12. SRP and SUP along a hydrologic flowpath from soil water to reservoir water in Walker Branch Watershed. A) Leafed season, B) Winter season. Triangle = SRP; Circle = SUP.

Table 3. Annual mean percent SUP along a hydrologic flowpath of Walker Branch Watershed

Compartment	Mean % SUP	Standard Deviation	*n*
Rainfall	44.9	23.9	12
Throughfall	42.4	23.0	12
Soil water	74.1	16.0	6
Groundwater	46.1	13.5	10
Spring	16.6	11.4	12
Stream water			
Upstream	50.5	25.9	12
Midstream	40.4	17.3	12
Downstream	41.0	16.7	12
Below confluence	30.5	15.9	12
Reservoir water			
Reservoir-cove	74.9	14.6	13
Reservoir-main channel	69.2	16.0	14

waters. To understand the role that DOP plays in nutrient cycling in aquatic ecosystems, specific compounds first need to be identified and characterized. The known compounds can then be used in experimental studies to determine (1) their availability to the biota, (2) their uptake and turnover rates, and (3) whether they are a source or sink of available phosphorus in the aquatic system. The first step toward understanding the role of DOP in aquatic systems is developing analytical tools leading to the identification of specific DOP compounds. The remainder of this study deals with the development of an analytical method to separate specific DOP compounds, the IPs, by HPLC.

Analytical Method Development

Preliminary Method Development

A column packed with Aminex A-27 anion-exchange resin was chosen to separate the homologous series of IPs based on the separation of standard IPs Hixson[44] achieved using this resin. Due to the technological advances in chromatographic hardware in the past eight years, a much smaller column was used in this study than in Hixson's to achieve a similar separation, but with much shorter elution times. The column was packed by Bio-Rad Laboratories under the pH and eluent specifications of this study. The pressure limit of the column was 82 atm, so the flowrate was optimized to decrease retention times without exceeding the column pressure limit. The optimum flowrate was 0.4 ml/min. A mobile phase similar to that which Brazell et al.[71] used to separate polyphosphates was chosen for this study. Sodium chloride solutions of various concentrations but with a constant 0.5 mM Na_4EDTA concentration were

tested for the elution of standard IPs and PO_4^{3-}. Initial isocratic runs using NaCl/Na_4EDTA as the eluent indicated, by the percent recovery data obtained from standard elutions and by the absence of unknown intermediate peaks, that on-column hydrolysis of the IPs to the intermediate IPs and PO_4^{3-} would not be a problem. There was little indication of on-column hydrolysis throughout the rest of the study.

Separation of Standards

In optimizing the mobile phase concentration, 0.2 M NaCl (0.5 mM Na_4EDTA was used in all cases) was found to elute, in separate runs, both PO_4^{3-} and IMP within 12 minutes and only one minute between their peak maxima. Isocratic elution with 0.5 M NaCl resulted in both PO_4^{3-} and IMP eluting unresolved in the void volume. However, IHP was retained on the column using 0.5 M NaCl, eluting in under 70 minutes. The next step was to try a more dilute solution to achieve different retention for PO_4^{3-} and IMP. NaCl (0.1 M) retained both standards indefinitely; the concentration had to be increased to elute them. A linear gradient from 0.1 M to 0.5 M NaCl over a 30-minute time interval with the final eluent concentration held constant for at least 60 more minutes was tested on the individual standards. The resulting chromatograms are shown in Fig. 13. IHP was resolved from IMP and PO_4^{3-}, the latter two of which appear to be slightly separated. Separation of a standard mixture showed, however, that the IMP and PO_4^{3-} peaks were not adequately resolved (see Fig. 14). Resolution was calculated as 2 times the difference in the retention times of the two peaks divided by the sum of the baseline peak widths. An adequate resolution would be at least 1.0. The gradient was

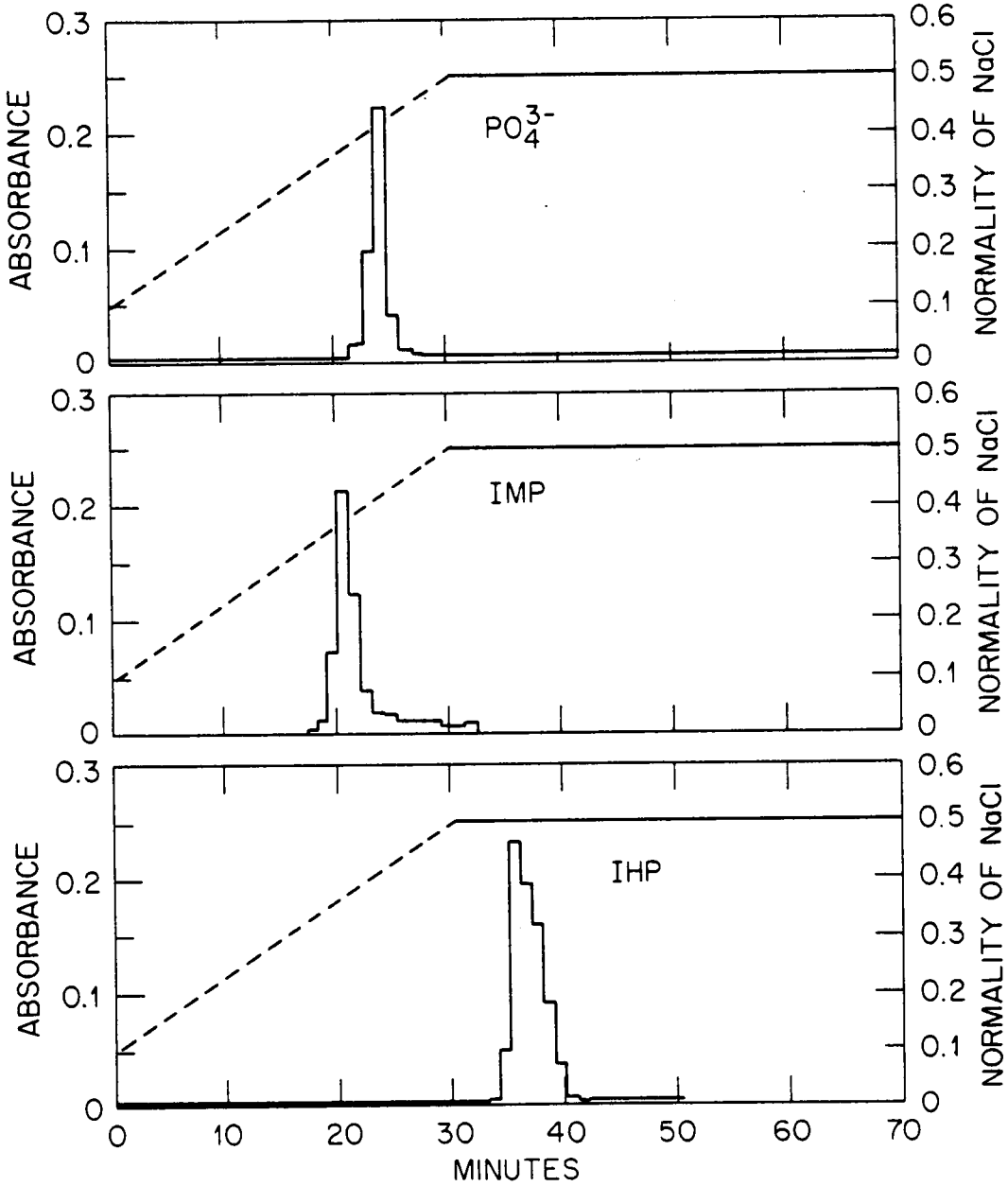


Figure 13. HPLC Elution of PO_4, IMP, and IHP Standards. Gradient: 0.1 to 0.5 M NaCl in 30 mins, hold 60 mins. Injection: 100 ul. Concentrations: 25, 31, 68 mg P/L respectively.

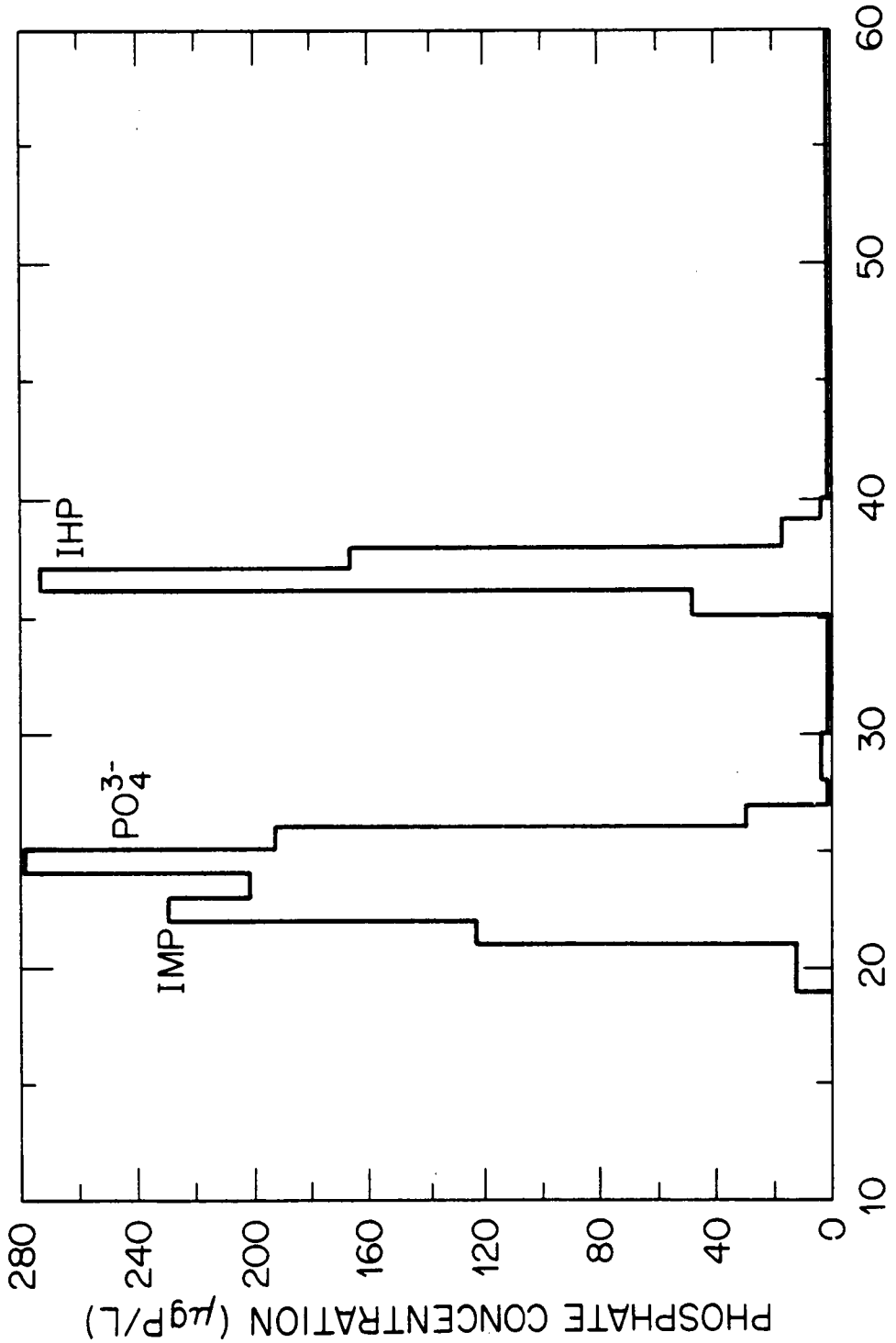


Figure 14. HPLC separation of PO_4, IMP, and IHP standard mixture. Same parameters as Figure 13. Resolution of PO_4 and IMP is 0.5.

extended to a 60-minute time interval with the final concentration (0.5 M NaCl) held for an additional 20 minutes. Figure 15 shows that a 60-minute gradient program was sufficient to resolve IMP and PO_4^{3-} with a resolution of 1.14. IHP was retained such that there was potential room for the elution of intermediate IPs without overlap. Mass balance calculations for the standard IPs eluted from the column are summarized in Table 4. Recovery of PO_4^{3-} was generally good, >90%, and recovery of IMP from the column was sufficient, >83%. Lower recoveries of IHP (64% and 70%) were probably due to permanent adsorption to the column rather than to hydrolysis, which would be indicated by >100% PO_4^{3-} recoveries. The 60-minute linear gradient program was used for the remainder of the HPLC work.

Separation of Inositol Hexophosphates Hydrosylates

An HPLC separation of commercially available phosphate esters of myo-inositol (IMP and IHP) and PO_4^{3-} was achieved using a 60-minute gradient from 0.1 to 0.5 M NaCl (with 0.5 mM Na_4EDTA) and the final concentration held constant for 20 more minutes. However, elution positions of the intermediate IPs was still uncertain. Because the di, tri, tetra, and pentaphosphate esters of myo-inositol are not available commercially, they were prepared through enzymatic hydrolysis of IHP by phytase. Phytase is reported to cleave phosphate groups sequentially from IHP.[78,40] The end products of the reaction are PO_4^{3-} and inositol. A reaction mechanism has been proposed by Tomlinson and Ballou.[78] If the reaction is halted prior to completion, a mixture of IPs and PO_4^{3-} should be produced.

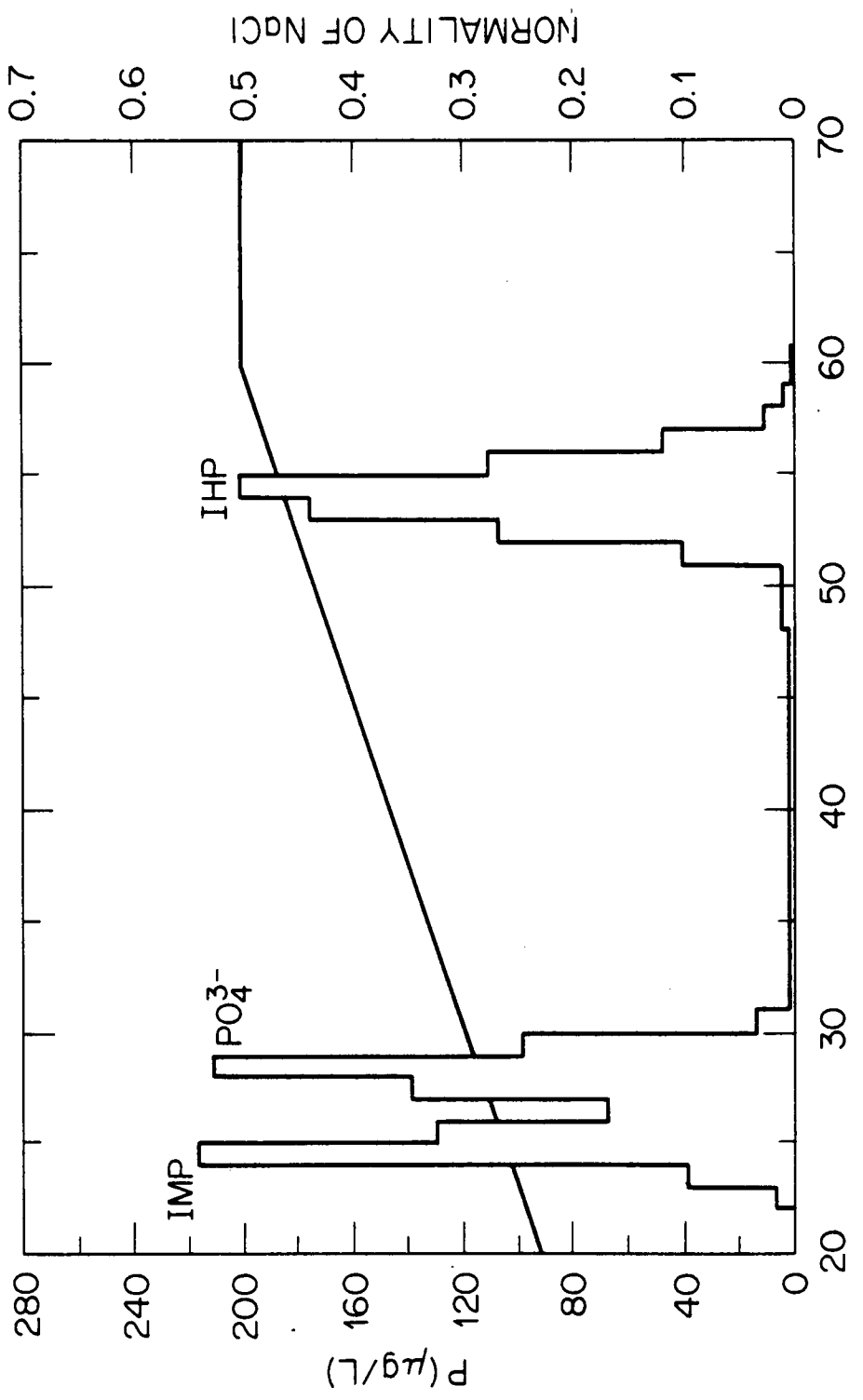


Figure 15. Sixty-minute gradient elution of IP standard mixture. Concentrations: 25, 28, 34 mg P/L PO_4, IMP, and IHP respectively. Resolution of PO_4 and IMP is 1.14.

Table 4. Summary of mass balances of commercial standards eluted by the HPLC method

Component	Injected DOP (μg)	Injected PO_4 (μg)	Eluted DOP (μg)	Eluted[a] PO_4 (μg)	% Component Recovery
Replicate A					
PO_4		2.50		2.26	90.5
IMP	2.75	0	2.75	0	100.0
IHP	3.40	0	2.17	0	63.8
Replicate B					
PO_4		2.50		2.27	90.9
IMP	2.75	0	2.30	0	83.8
IHP	3.40	0	3.48	0	102.0
Standards in sodium acetate buffer[b]					
PO_4		4.00		3.99	99.7
IMP	1.00	0	0.96	0	95.7
IHP	4.00	0	2.82	0	70.4

[a]Mass of DOP eluted within a single standard peak.
[b]See Figure 17b for chromatogram.

Enzyme hydrolysis of IHP was performed (see Appendix A for details of procedure) resulting in three different product mixtures. The reaction in two of the reaction vessels was stopped after 40% and 47% hydrolysis of IHP phosphorus to PO_4^{3-}. An aliquot of these two mixtures was filtered and examined by HPLC, first individually (see Figs. 16a and b for the chromatograms), then combined into one hydrolysate mixture (Fig. 17a, scale magnified). The third reaction vessel had 18% hydrolysis and an HPLC separation showed only the IHP and PO_4^{3-} peaks. For comparison, a standard mixture containing the commercial standards was eluted under the hydrolysis reaction conditions except for the presence of phytase (see Fig. 17b for the chromatogram). Phytase was examined by HPLC to determine the elution position of any possible phosphorus contamination (Fig. 18). Duplicate chromatograms of all runs shown confirmed the elution positions ($\pm$ 1 min) of each peak discussed. A difference of up to 1 minute in the elution times of allegedly identical peaks can be attributed partially to mis-timing during the simultaneous, manual initiation of the gradient program and the fraction collector, and injection of the sample into the eluent stream.

All the hydrolysate mixture chromatograms show a distinct PO_4^{3-} peak at approximately 30 minutes retention time (Figs. 16 and 17). The 40% hydrolysis mixture (Fig. 16a) shows a peak at 59 minutes, which corresponds to IHP as seen in the standard separation shown in Fig. 17b. The shift in the retention time of the IHP peak compared to that in the earlier standard elution chromatogram shown in Fig. 15 (shift from 55 min in first standard run to 59 min under hydrolysis conditions) is probably due to the different chemical environ-

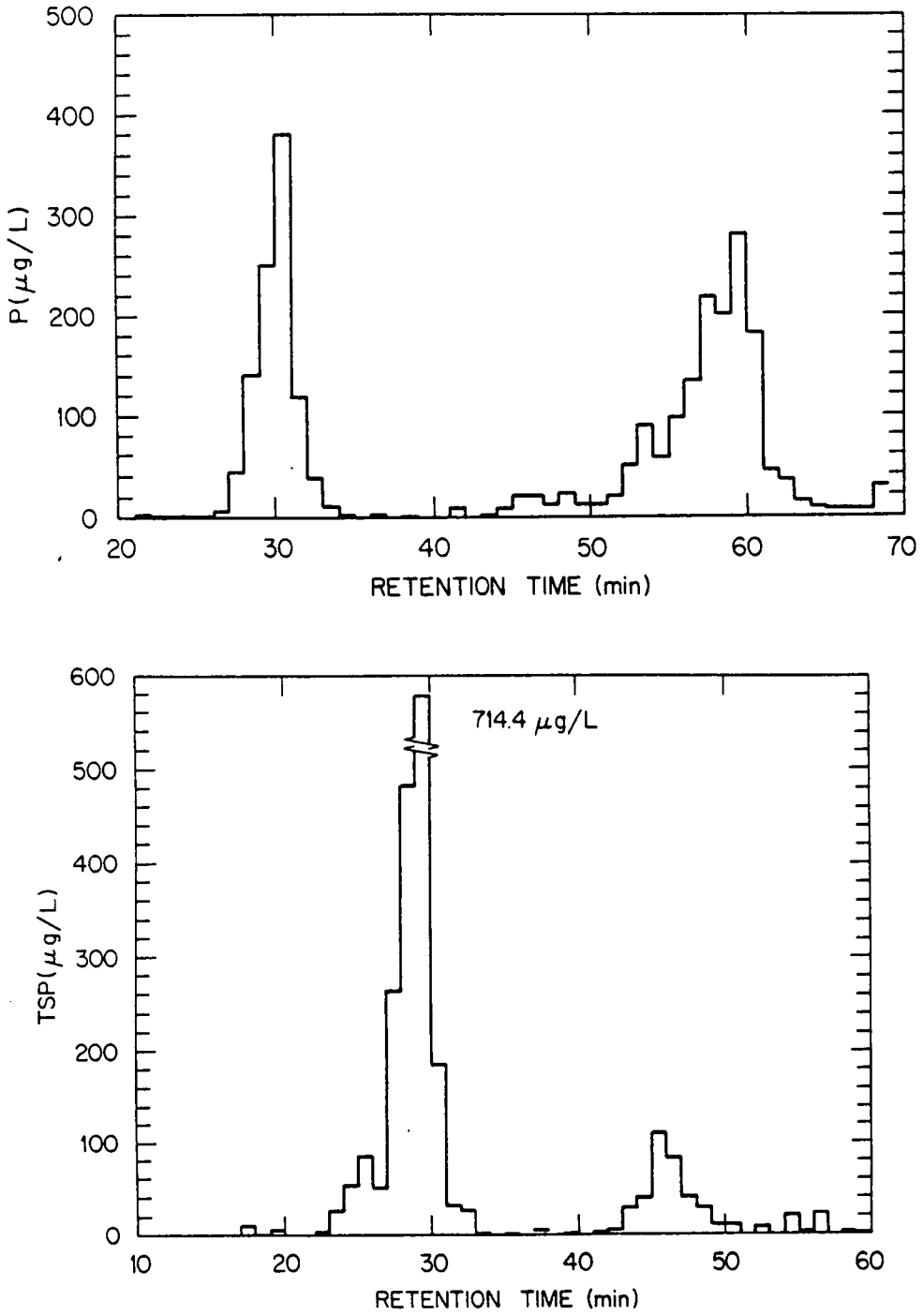


Figure 16. Chromatograms of IP hydrolysate mixtures. A) 40 % Hydrolysis of IHP, B) 47% Hydrolysis of IHP.

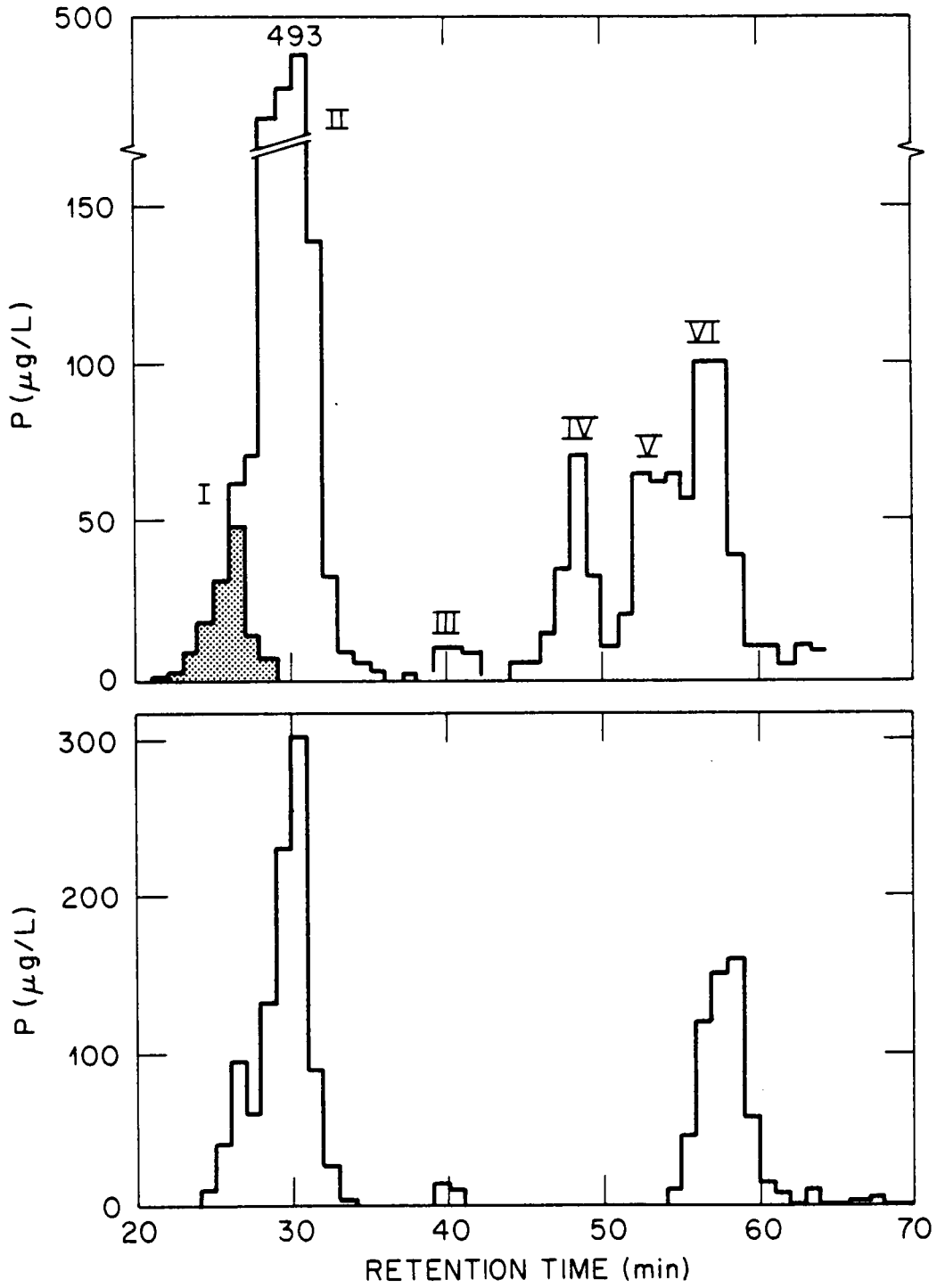


Figure 17. Chromatograms of A) The combined hydrolysate mixture and B) A standard IP Mixture. Concentrations of standards: 40, 10, 40 mg P/L of PO_4, IMP, IHP respectively. Shaded peak distinguishes SUP from SRP.

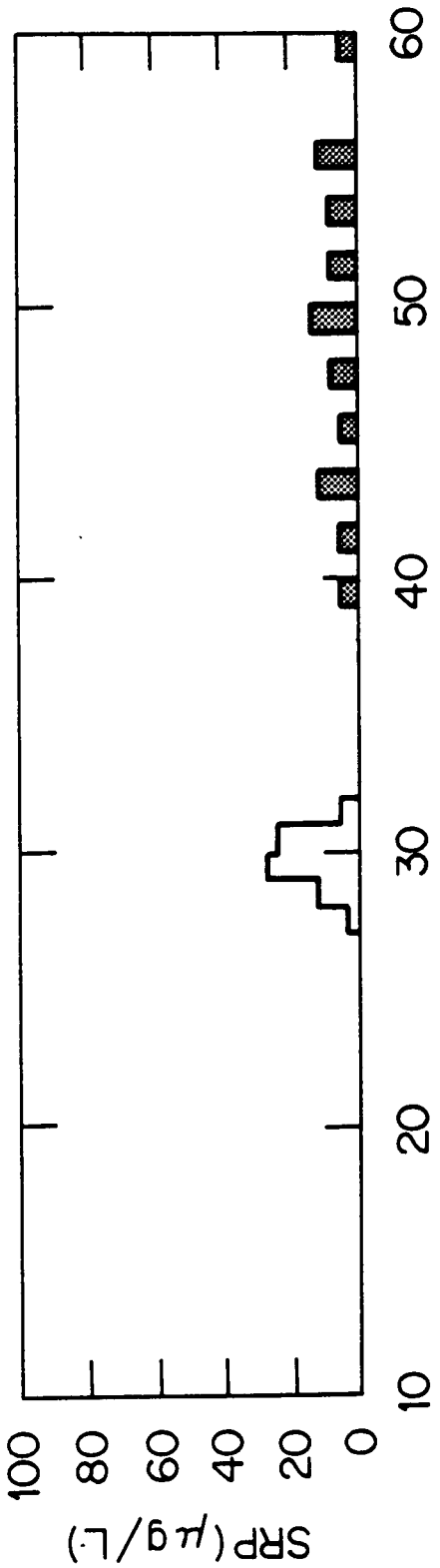


Figure 18. Chromatogram of phytase control. Shading indicates SUP concentrations.

ment experienced in the presence of the enzymatic reaction buffer solution, sodium acetate (pH 5.0).

The chromatogram of the 47% hydrolysis mixture (Fig. 16b) does not show an IHP peak, but a peak corresponding to IMP was eluted as well as a peak eluting between the retention times of standard IMP and IHP. The combined hydrolysate mixture (Fig. 17a) separated into five (possibly six) different components. Peaks corresponding to IMP, PO_4^{3-}, and IHP were recognized, labeled I, II, and VI, respectively, with two (possibly three) intermediate peaks distinguished. The peak labeled III was not greater than 2× the background noise, so no attempt at its identification was undertaken, even though a peak with similar retention time was seen in the standard elution chromatogram (Fig. 17b). The loss of resolution of PO_4^{3-} and IMP in the hydrolysate mixture was probably due to the extremely high concentration of PO_4^{3-} resulting from hydrolysis of IHP. However, the IMP peak is still clearly distinguishable (and indicated by shading in Fig. 17a) by analyzing for both SRP and TSP in each fraction.

Since the IPs are a family of a homologous series of compounds, a linear relationship between the log of the elution position and the log of the number of units in the homologous series is expected.[72,44] Figure 19 is a linear plot of the expected elution positions of the intermediate IPs based on the elution positions of IMP and IHP under hydrolysis reaction conditions (IMP and IHP as identified in the combined hydrolysate mixture). Table 5 compares the elution times for the IP intermediates calculated from the log-log plot to the elution times of the components in the hydrolysate mixture. The agreement of the calculated elution times for inositol tetraphosphate and pentaphosphate with the measured

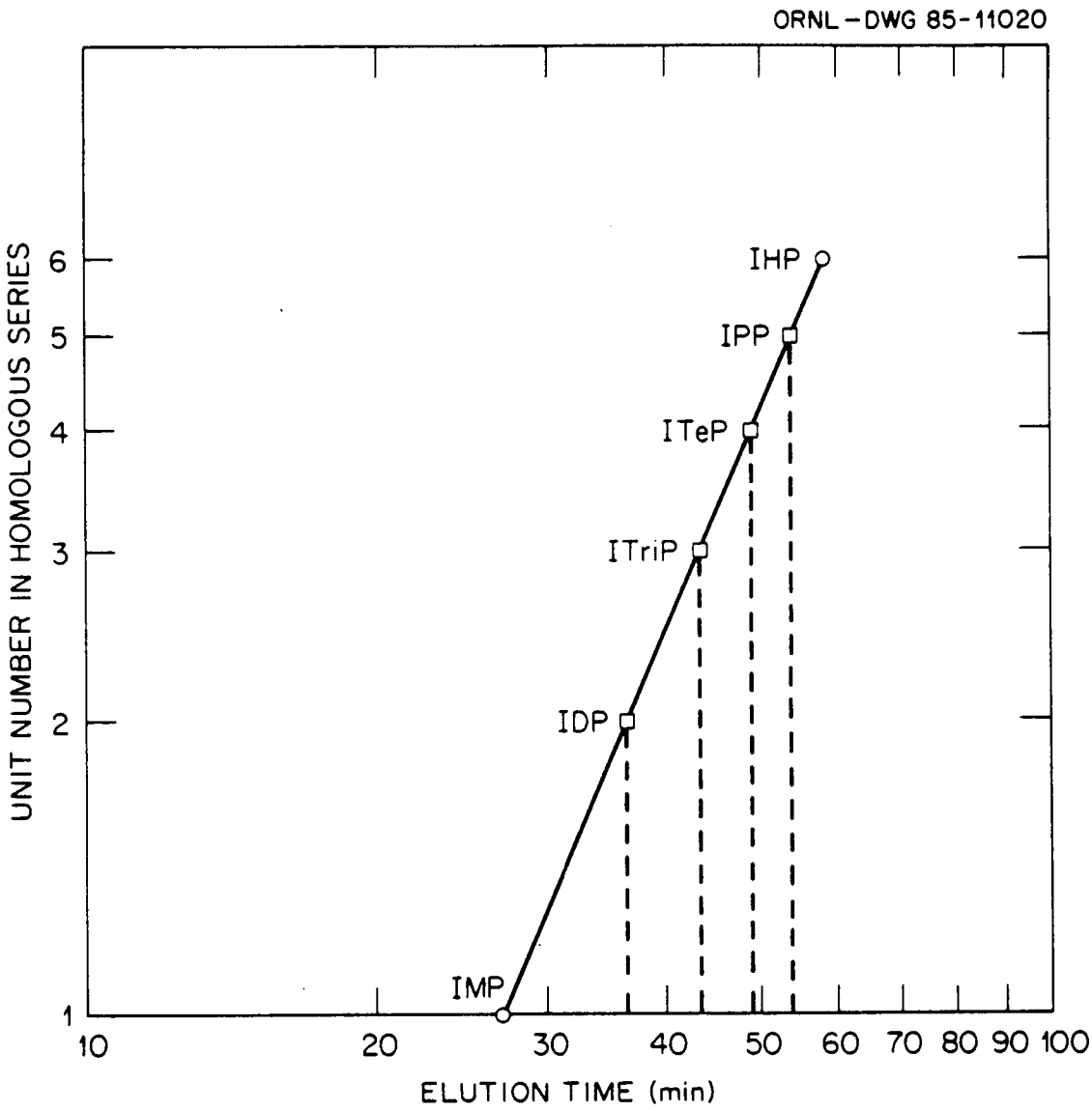


Figure 19. Log-Log plot of unit number in homologous series versus elution time on Aminex A-27 resin.

Table 5. Measured and calculated elution positions for IHP, IMP, intermediate inositol phosphates and orthophosphate on Aminex A-27

Compound	Measured elution time (m)	Calculated[a] elution time (m)	Unit number in homologous series
Orthophosphate	31		
IMP	27		1
IDP		36.4	2
ITriP		43.1	3
ITetP	49[b]	48.9	4
IPP	54[b]	53.8	5
IHP	58		6

[a]Calculated values from Log-Log plot.
[b]Elution times measured for peaks IV and V of hydrolysate mixture.

elution times of peaks IV and V respectively in Fig. 17a provides supporting evidence for the presence of these intermediates in the hydrolysate mixture and suggests that the IP intermediates may be separated by this analytical method, even though baseline resolution was not achieved. The absence of inositol di- and triphosphate in the hydrolysate mixture may have been due to the mechanism of hydrolysis but does not rule out the possibility of their separation by the proposed HPLC method.

High Pressure Liquid Chromatography Determination of Inositol Hexophosphate in Wheat Bran

Once the analytical method had been shown to separate available IP standards and hydrolysis produced intermediate IPs, its ability to isolate and detect IPs in a complex matrix was tested on a wheat bran extract. Wheat bran was chosen because of its known high concentration of IHP (1 to 7% IHP by weight)[60,62,63,67,68] and because of the relative ease of the IHP extraction procedure (Appendix A). After desication, a sample of wheat bran was extracted with trichloroacetic acid (TCA), filtered (0.4 μm), and an aliquot analysed for SRP and TSP. The extract contained 1.3 mg/liter SRP and 29.6 mg/liter TSP (mean values of duplicate assays), so DOP is estimated by difference to be 28.3 mg/liter. Injection of a 100 μl aliquot of the wheat bran extract onto the HPLC under the conditions stated in Table 1 page 29 resulted in the chromatogram shown in Fig. 20. A replicate chromatogram showed very similar results.

Wheat bran IHP eluted within one minute of the elution time of standard IHP shown in the chromatogram of Fig. 17b where pH conditions were similar

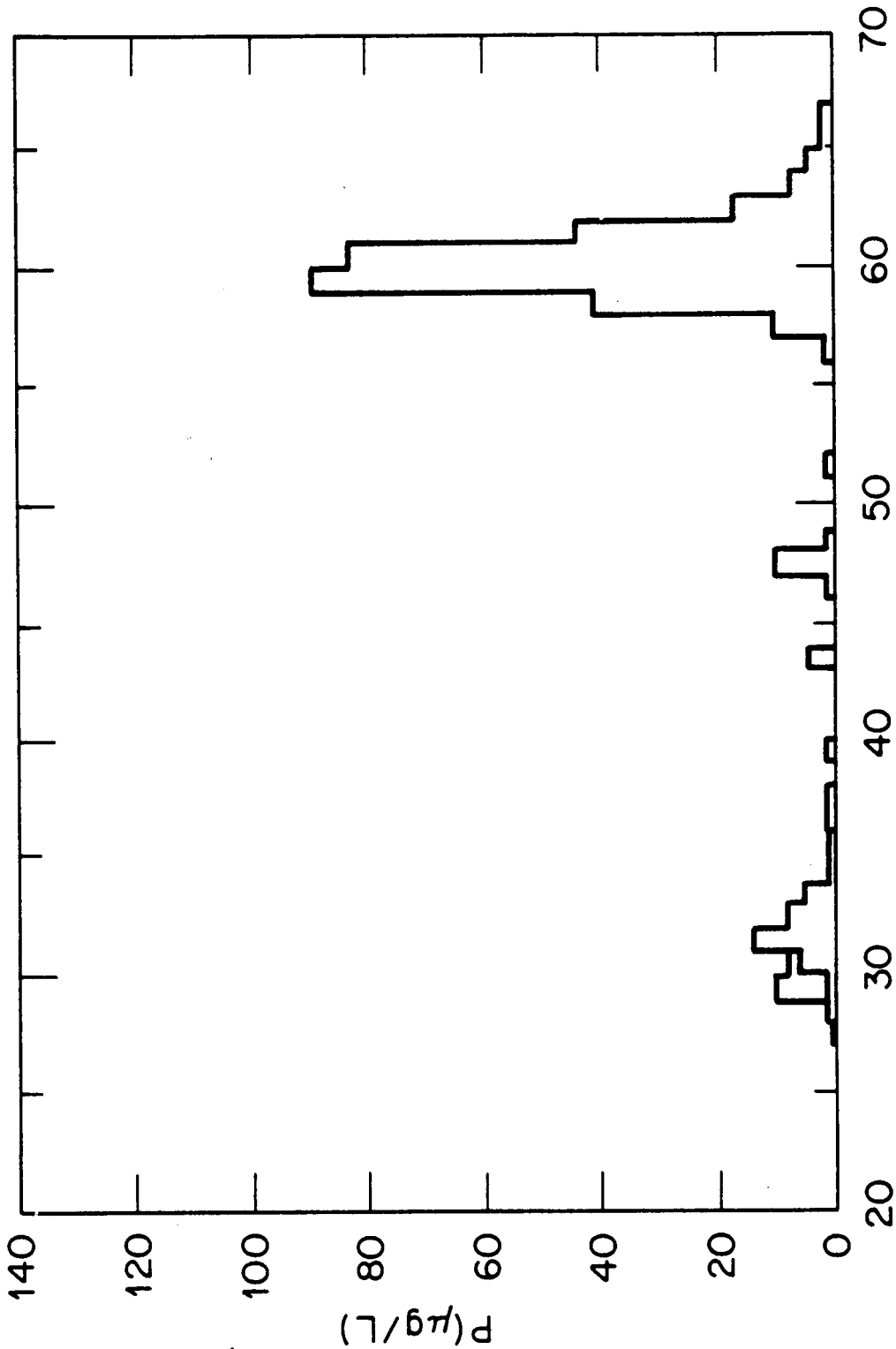


Figure 20. Chromatogram of wheat bran extract.

to that of the bran extract. A difference of one minute in retention times could be attributed to mis-timing of the simultaneous initiation of the eluent program, the fraction collector, and injection of the sample. A small PO_4^{3-} peak was recognized in the two replicate chromatograms. The small organic phosphorus peak that eluted prior to the PO_4^{3-} peak may be IMP.

Percent recovery calculations comparing eluted phosphorus to injected phosphorus are summarized in Table 6. It appears that some hydrolysis of IHP to PO_4^{3-} may have occurred, some time between the preliminary phosphorus analysis and elution of the extract, as indicated by the incomplete recovery of DOP and a high (>100%) recovery of SRP. However, on a mass basis, the increase in SRP is only a small fraction of the DOP loss. Also, the DOP recoveries of the wheat bran extract elution are similar to the IHP recoveries seen in the standard elutions summarized in Table 4 page 57. It is also possible that some other organic phosphorus compound in the extract accounted for a fraction of the initial DOP but was not eluted from the HPLC column. After analysis of SRP and TSP the wheat bran extract was refrigerated overnight before injection onto the HPLC. If hydrolysis was responsible for the increase in SRP, it may have occurred either during overnight storage prior to injection or during the HPLC analysis; exactly which one is unclear. In any case, the extraction procedure and wheat bran matrix appeared to have little effect on the ability of the HPLC analytical method to separate IP fractions as shown by the consistent elution times of IMP, PO_4^{3-}, and IHP.

The results of the wheat bran IHP studies indicated that the proposed analytical method could potentially be used to separate and identify IHP in foods.

Table 6. Summary of mass balances on wheat bran extract

Component	Injected P (μg)	Eluted P (μg)	P % Recovery
Replicate A			
SRP	0.13	0.19	145.0
DOP[a]	2.83	1.96[b]	70.6
Replicate B			
SRP	0.13	0.17	130.0
DOP[a]	2.83	1.51[c]	64.2

[a]DOP was estimated by the difference between TSP and SRP.

[b]90% of DOP eluted as single IHP peak.

[c]83% of DOP eluted as single IHP peak.

But more important in this study, the wheat bran test showed that the method could selectively distinguish an IP within a complex matrix.

High Pressure Liquid Chromotography Analysis of Inositol Phosphates in Soils

To test further the IP separation technique, a Walker Branch watershed soil sample was extracted by a method adapted from the procedure recommended by Irving and Cosgrove[58] (see Appendix A for details of the extraction). The procedure called for a NaOH extraction of the soil, concurrent with the oxidative bromination of all organic matter except the IPs, including the oxidation of other organic phosphorus compounds in the soil. Even though alkaline bromination has been considered a sufficient means for removing non-IP material from environmental samples,[58] problems of incomplete oxidation have been reported.[43] However, the IPs should be the only organic phosphorus compounds detected in the HPLC eluent.

An aliquot of the soil extract was concentrated from ~30 ml to 5 ml by rotary evaporation and 100 μl was eluted according to the proposed HPLC method. The resulting chromatogram is shown in Fig. 21. Chromatograms of an unconcentrated aliquot and a second concentrated aliquot (not a replicate) of the soil extract can be found in Figs. 22 and 23. A distinct PO_4^{3-} peak eluted at 29 minutes simutaneously with a smaller unreactive peak, possibly IMP or any organic phosphorus compound that may have not been oxidized by the alkaline bromination procedure. In the chromatogram of the unconcentrated soil extract (Fig. 22) this peak was resolved from PO_4^{3-} and eluted at the posi-

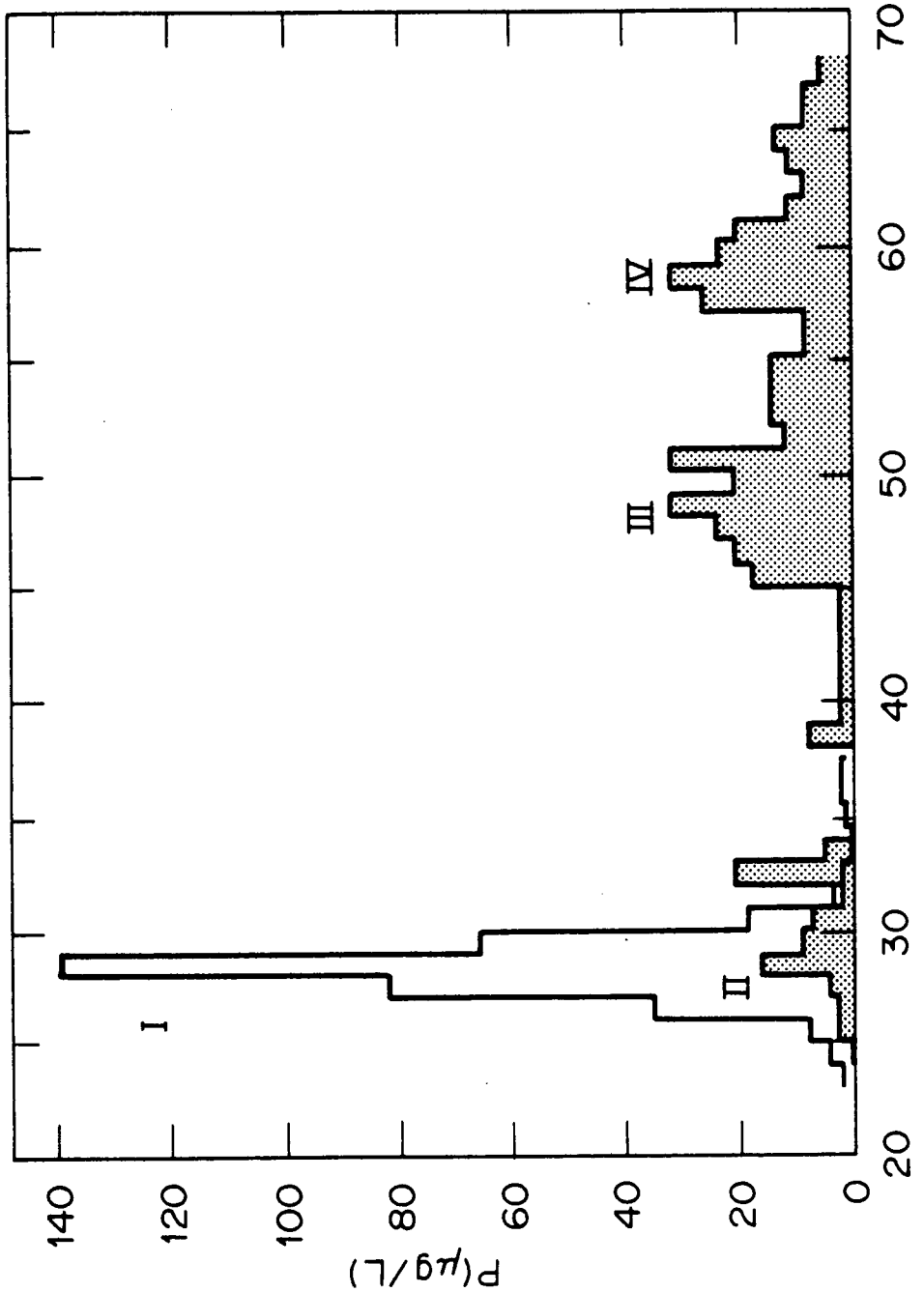


Figure 21. Chromatogram of concentrated Walker Branch soil extract. Initial SRP = 14.8 mg/L, SUP = 31.7 mg/L. Shading indicates SUP concentration.

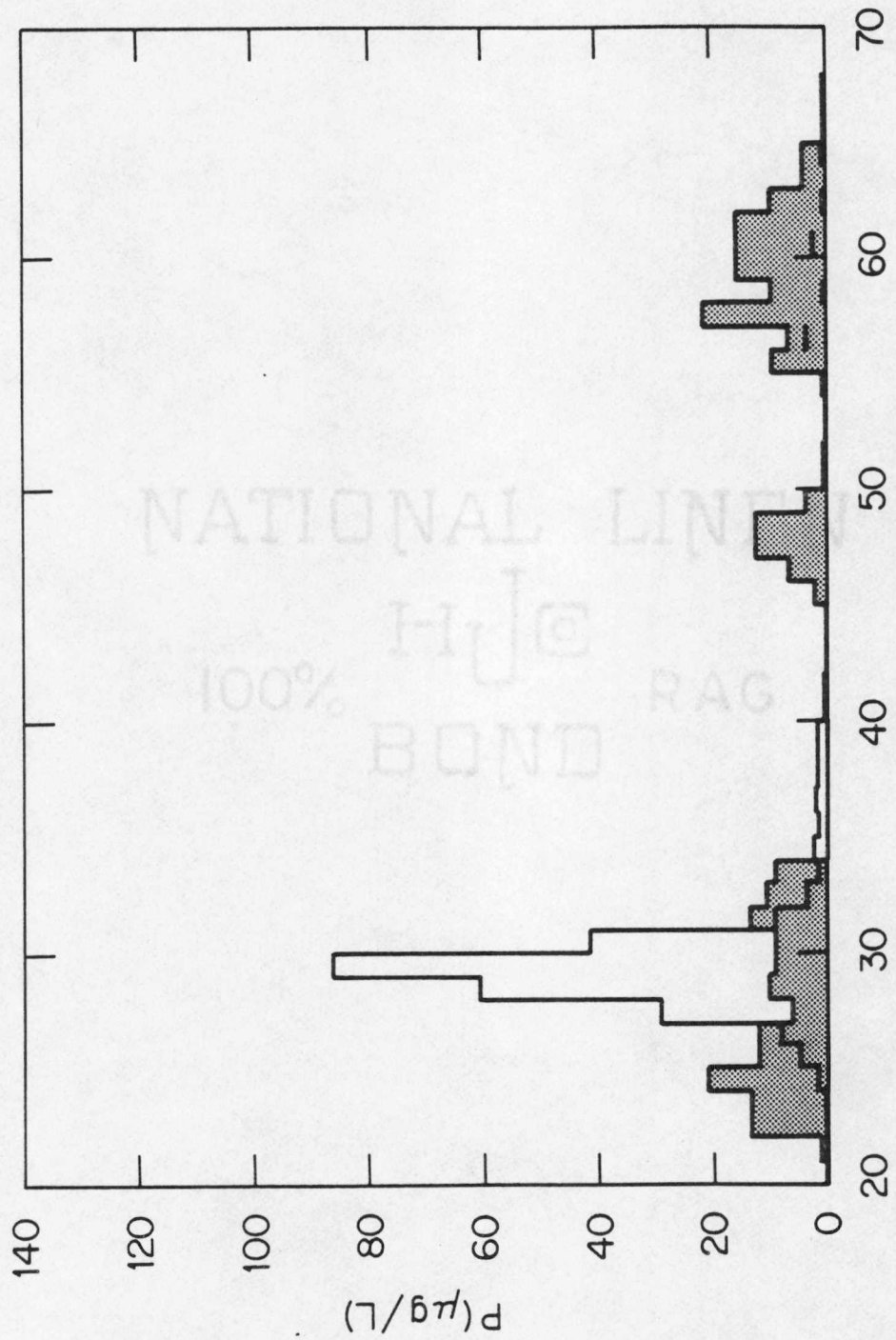


Figure 22. Chromatogram of unconcentrated Walker Branch soil extract. Initial SRP = 9.1 mg P/L, SUP = 21.3 mg P/L. Shading indicates SUP concentration.

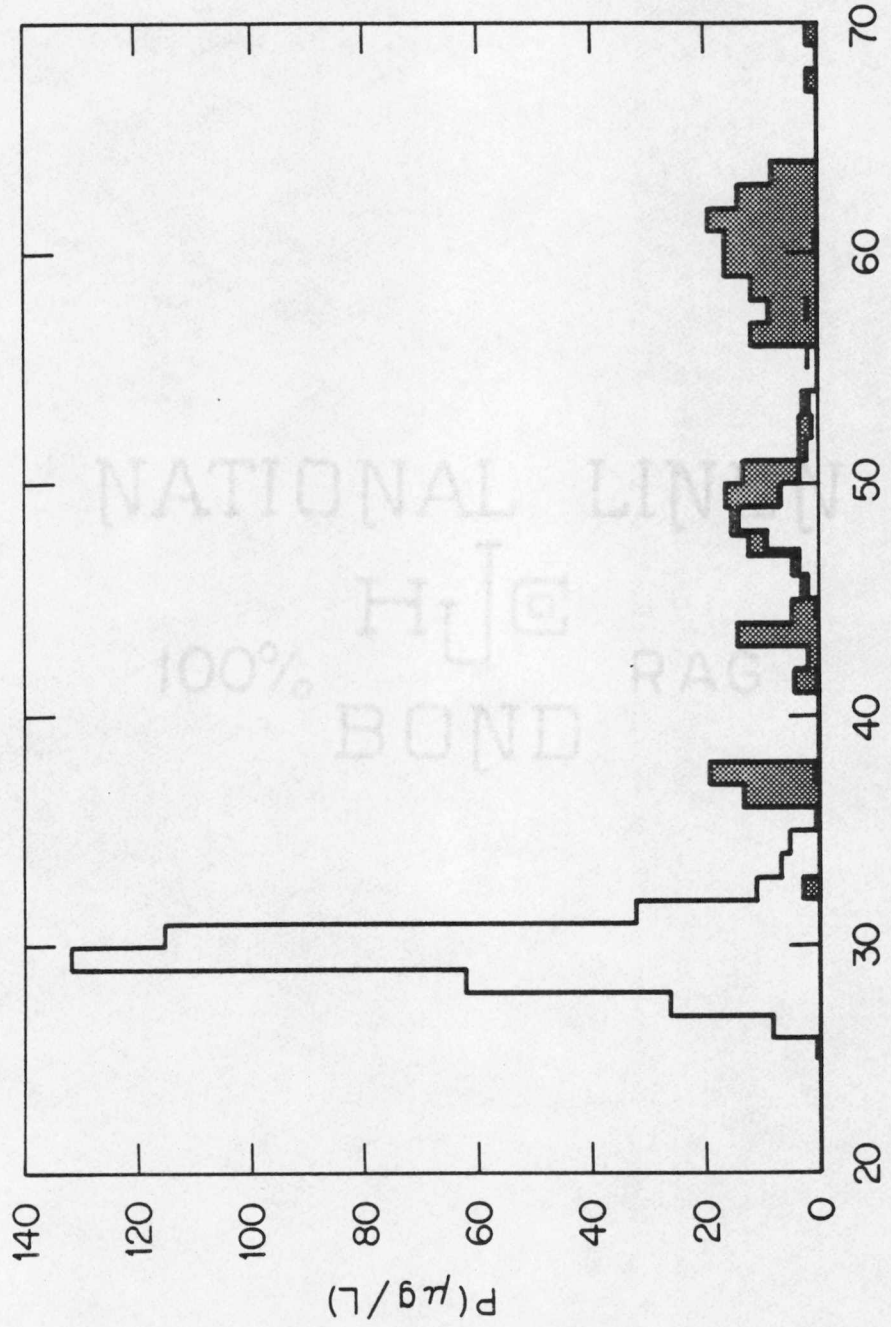


Figure 23. Chromatogram of second concentrated Walker Branch soil extract. Shading indicates SUP concentration.

tion of standard IMP. Three peaks eluted with retention times corresponding to those calculated (Table 5) for inositol tetra, penta, and hexaphosphate.

The purpose of the soil extraction was to test the HPLC separation on a matrix approaching the complexity of natural waters and not to quantitate IPs in soils. So the results given, although not quantitative, do suggest the possibility of isolating the IPs from natural systems by alkaline bromination followed by HPLC separation leading to their identification in natural waters.

Determination of Inositol Phosphates in Walker Branch Water

An attempt was made to isolate IPs in a Walker Branch groundwater sample using the HPLC technique. Because the minumum detectable concentration was ~5 mg DOP/liter and mean watershed SUP concentrations were less than 5 μg phosphorus/liter it was necessary to concentrate the water by a factor of greater than 1000 prior to injection onto the HPLC column.

Ultrafiltration through sequential YCO5 membranes with a final concentration step of rotary evaporation was chosen as the concentration procedure after preliminary studies of ultrafiltration on a water sample. The results of the preliminary studies using YM2 and YM5 membranes (molecular weight cutoffs of 2000 and 5000 respectively) as well as the YCO5 membrane to concentrate a Melton Hill Reservoir sample are sumarized in Table 7.

For the HPLC analysis, a 10-liter Walker Branch groundwater sample was collected (combined collection from well #2 and well #3), filtered (0.4 μm Nuclepore), cation exchanged to Na^+ form, then ultrafiltered to a 8.8 ml retained volume. The sample was then brominated (see Appendix A for details

Table 7. Summary of preliminary ultrafiltration studies

Sample or Retentate	Volume (mL)	Mass of P (μg)		
		TSP	SRP	SUP
Original	9300	28.83	23.25	5.58
YM5	17	5.53	1.14	4.40
YM2	7.8	11.92	1.66	10.26
YC05	10.1	7.12	2.78	4.34
Total P (μg)		24.57	5.58	19.0
% P recovered		85.2	24.0	34.0

	Concentration Factors		
	Initial	Final	Concentration Factor
TSP mass concentration	3.1 μgP/L	705 μgP/L	227X
Volume concentration	9300 mL	34.9 mL	266X

Source: Minear R.A. Unpublished results. Oak Ridge National Laboratory, Oak Ridge, Tennessee.

of procedure) to destroy any interfering organics. Following alkaline bromination, the 11.8-ml sample was rotary evaporated down to 1.5 ml.

Initial phosphorus analysis indicated 6.36 μg/liter SRP and 1.69 μg/liter SUP in the 10-liter sample. Phosphorus analysis after ultrafiltration and before alkaline bromination showed a concentration factor of 52.2× for SUP and only 17.9× for SRP. Phosphorus concentrations after alkaline bromination were higher than before the oxidative procedure. Reasons for this increase are unclear. It was possibly due to contamination or interference from the reagents used (the analysis was not blank corrected) or to degradation by the bromination reaction of some highly refractory phosphorus compounds not detected in the persulfate digestion of the TSP analysis. Phosphorus analysis was not performed after evaporation due to the low sample volume. Table 8 summarizes phosphorus analyses performed during the concentrating procedure. A 100-μl aliquot of the concentrated groundwater sample was injected onto the HPLC, followed by phosphorus analysis of the collected fractions. No SRP or SUP was detected in any of the fractions collected in two replicate runs of the concentrate. Possible explanations for the negative results are that the concentration procedures were not sufficient for the phosphorus to be detected in any one peak. Ultrafiltration did not retain SUP as expected, possibly because of membrane leakage, or the breakdown of SUP at room temperature to PO_4^{3-} which was retained even less. It is possible also that there was significant loss of phosphorus in the transfer from the alkaline bromination reaction vessel to the round bottom flask of the rotovapor, or that phosphorus adsorbed to the rotovapor flask. A combination of any of the above reasons may have prevented the

Table 8. Summary of phosphorus analyses during groundwater concentration

Stage of Concentration completed	Concentration (μg/L)		Concentration factor (Cf/Ci)		Sample volume (ml)
	SRP	SUP	SRP	SUP	
Collection	6.36	1.69			10,000
0.4 μ filtered					
Ultra-filtration	114.1	88.2	17.2	52.2	8.8
Alkaline bromination	202.4	462.6	31.8	273.7	11.8
Rotary evap.					1.5

detection of IPs in the groundwater if they were present. A second analysis of Walker Branch water was not attempted.

Because the HPLC technique had been shown to be effective in (1) separating commercial standards, (2) separating an IHP hydrolysate mixture, (3) isolating IHP in a wheat bran extract, and (4) separating a mixture of IPs in a soil extract, it is felt that there is sufficient evidence to recommend that the technique be used for futher characterization of DOP compounds in natural waters. Obviously, additional work is necessary to evaluate methods for concentrating water samples prior to injection onto the HPLC.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

The primary ecological implication of this investigation is that soluble phosphorus does undergo transformation in concentration and biochemical form along a hydrologic flowpath in a forested stream watershed, specifically the Walker Branch Watershed. It was shown that as water moved along a hydrologic flowpath, interactions with biotic and abiotic factors within each compartment resulted in changes in the form and concentration of soluble phosphorus in the water. Dramatic changes occurred between the rainfall and throughfall compartments and the throughfall and soil water compartments, indicating leaching or wash-off of phosphorus from the canopy and biological uptake and geochemical adsorption of phosphorus compounds in the litter. These changes affected the concentration—and hence the availability—of phosphorus to organisms in downstream aquatic systems by allowing only low concentrations of soluble phosphorus to be exported from the soil system. Export of phosphorus from the terrestrial component of the watershed is particularly important in this case because autotrophic and heterotrophic processes in the stream are phosphorus limited.

The seasonal pattern exhibited by SRP concentrations at the upstream site and the loss of SRP and the release of SUP within the stream channel between the upstream and midstream sampling sites supported previous evidence for biological control of phosphorus dynamics in the stream. Transformation of soluble phosphorus from predominantly SRP in the stream to predominantly SUP in

the reservoir and the indication of a significant fraction of organic phosphorus at all points in the watershed emphasized the need to understand better the role DOP plays in aquatic phosphorus dynamics. This need was the rationale behind the analytical development phase of the current investigation.

The primary result of the analytical development phase of this project was that ion-exchange HPLC can potentially be used as a separation tool for the IPs in environmental samples. Separation was achieved on a standard mixture and on a hydrosylate mixture of IPs. The elution positions of intermediate IPs were estimated from a log-log plot of unit number in the homologous series versus elution position. IHP was isolated from IMP and PO_4^{3-} in an extract of wheat bran. The HPLC technique was then tested on an alkaline brominated extract of a Walker Branch soil sample. Four peaks were eluted with retention times corresponding to those observed or calculated for inositol mono-, tetra-, penta-, and hexaphosphate.

An attempt was made to demonstrate the presence of IPs in Walker Branch groundwater using the HPLC method. No phosphorus compounds were detected during HPLC elution of a groundwater concentrate, probably because concentrating the sample was ineffective. The problems with the concentration procedure may have been due to leakage of the ultrafiltration membranes or to breakdown of organic phosphorus at room temperature during the long procedure to the less retained SRP form.

In spite of inconclusive results with the groundwater sample, the method has been shown to be effective in separating the IPs in other environmental matrices (i.e., soil and grain). These results indicate that with more refinement

of the concentration procedures, the HPLC technique potentially could be used in the characterization of DOP compounds in natural waters.

Conclusions

Specific conclusions of this thesis research are as follows:

1. Soluble phosphorus undergoes transformation in concentration and biochemical form between adjacent compartments of a hydrologic flowpath in a forested watershed.

2. SUP accounts for a significant fraction of TSP in each compartment of the watershed; hence, soluble organic phosphorus may be an important factor in the phosphorus cycle.

3. Ion-exchange HPLC is a feasible separation tool for the homologous series of inositol phosphates.

4. A reliable means for concentrating DOP in natural waters needs to be developed in order to continue characterization of this fraction of soluble phosphorus.

CHAPTER VI

RECOMMENDATIONS FOR FURTHER STUDY

Continued investigation of the transport of soluble phosphorus along the major hydrologic flowpaths in watersheds can lead to better understanding of the upstream-downstream connection of ecological systems, specifically in the area of nutrient dynamics and availability. This project collected substantial amounts of data on soluble phosphorus concentrations and conductivity along a flowpath in the watershed and over time. Continued comparisons among the data from this project and with data such as stream flow and rainfall amounts from other projects on Walker Branch could result in a better understanding of the processes controlling soluble phosphorus in Walker Branch.

Refinement of the analytical methods for the determination of the various soluble phosphorus forms should continue. Techniques to achieve a better estimate of soluble inorganic phosphorus concentrations should be developed. The alkaline phosphatase enzyme inhibition assay for orthophosphate (PO_4^{3-}),[15] with several modifications to improve reproducibility and reduce analysis times, may be a satisfactory tool in PO_4^{3-} determination.

Continued refinement of the HPLC separation technique may improve overall performance of the characterization tool. An obvious hindrance to exploratory research is the use of slow and tedious analytical procedures. The detection system used for the HPLC method was wet chemical analysis of the collected fractions (0.4 ml), up to 70 fractions per chromatogram. Each fraction was split and analyzed for both SRP and TSP, resulting in up to 140 different samples to be processed per HPLC run. Because the IPs do not show a unique absorbance

or fluorescence pattern and refractive index detectors are not sensitive enough to approach detection limit requirements, wet chemical analysis appears to be the best choice at this time for phosphorus detection in HPLC.

One means to reduce phosphorus analysis times for HPLC was demonstrated by Brazell et al.[71] in their application of HPLC flow injection analysis (FIA) to the determination of polyphosphoric acids in phosphorus smokes. The FIA system was used as an on-line, post-column detector for condensed phosphates. Molybdenum blue reagent was pumped into the post column eluent stream, which then passed through a reactor coil to hydrolyze the polyphosphates. The eluent/reagent stream then passed through a 10-nm flow cell for absorbance measurements. Determinations for total phosphorus could be made at a rate of 25 samples per hour.

Flow injection analysis could potentially be adapted for use as an HPLC detector of organic phosphorus compounds as well. An organic oxidizer must be added to the post-column flowpath to release organically bound phosphorus so it can react with the molybdenum reagent. An FIA detector system could dramatically improve the HPLC analysis times, therefore allowing more widespread use of the separation technique on natural water samples in attempts to detect the presence of IPs.

An additional modification to enhance sensitivity of the HPLC detection system could be the application of laser-induced thermal lensing effect to molybdenum blue colorimetry. Fujiwara et al.[79] used thermal lensing to determine phosphorus at the parts per trillion level in seawater. Thermal lensing is an important way to monitor small absorbance, so its application to the pro-

posed HPLC method would alleviate some of the preconcentration problems seen in the application of the method to a groundwater sample.

In the process of compound identification, HPLC is designed as a separation tool to be used in conjunction with other analytical techniques. In the continuing challenge to characterize DOP in natural waters, other analytical methods need to be developed to complement the HPLC separation technique. Methods such as ^{31}P-FT-NMR could be used to verify the identity of the phosphorus compounds eluted in the HPLC fractions. Again, however, low concentrations will pose a serious problem. Thus, the most pressing analytical problem is the development of a reliable means of concentrating DOP that will not alter its form and will raise phosphorus levels to those needed for detection.

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APPENDICES

APPENDIX A

ANALYTICAL PROCEDURES

Phosphorus Analyses

Soluble Phosphorus Analyses[10,11,74]

Reagents

1. Sulfuric Acid Solution—140 ml conc. H_2SO_4 was added to 900 ml deionized (d.i.) water. The solution was cooled and stored in glass.

2. Ammonium Molybdate Solution—Reagent grade ammonium molybdate crystals (15 g) were dissolved in 500 ml d.i. water and stored in plastic out of direct sunlight. The solution was considered to be stable indefinitely.

3. Antimony Potassium Tartrate Solution—Antimony potassium tartrate (0.34 g) was dissolved in 250 ml d.i. water and stored in a plastic container. The solution was considered stable for months.

4. Ascorbic Acid Solution—Reagent-grade ascorbic acid (5.4 g) was dissolved in 100 ml d.i. water. The solution was made up weekly.

5. Mixed Reagent—Solutions 1, 2, 3, and 4 above were mixed in the order given with the following ratios to result in the volume needed: 125:50:25:25. The mixed reagent was made fresh each day.

Procedure

A. Soluble Reactive Phosphorus—An aliquot (5.0 ml) of the mixed reagent was added to the sample (50.0 ml) and mixed thoroughly. After 10–30 minutes, the absorbance of each sample was measured against distilled water at 885 nm with a spectrophotometer.

B. Total Soluble Phosphorus—H_2SO_4 (24 N, 0.5ml) was added to a 25.0-ml sample in a 125-ml Erlenmeyer flask. Then 0.2 g potassium persulfate was added. The mixture was heated for 30 minutes in an autoclave at 120 °C (15–20 psi), then allowed to cool. One drop of phenolphthalein was added and the pH adjusted until pink with 1 N NaOH, then to colorless with strong acid. The solution was cooled and diluted to 50.0 ml.

Standard Calibration Curves

Standard Calibration Curves were plotted for standard orthophosphate concentrations within the concentration ranges of samples. New standards were analyzed and curves calculated every few months.

Modifications for HPLC Fraction Phosphorus Analyses

Reagents

The reagents used were the same as those given above.

Procedure

1. Each 0.4-ml fraction was diluted to 5.0 ml with d.i. water.

2. For TSP analysis— a 2.5-ml aliquot from each diluted fraction was placed in a 16 × 100 mm test tube. Strong acid (24 N, 50 μl) and ~0.1 g potassium persulfate were added to the test tube. The tube was covered and autoclaved (same as above), then allowed to cool. The solution was neutralized with ~0.45 ml 4-N NaOH using phenolphthalein as an indicator. One drop of strong acid brought it to colorless. About 2.05 ml d.i. water was pipeted into the test tube to make 5.0 ml, and mixed well. Mixed reagent (0.5 ml) was added and absorbance read in a 1-cm cell.

3. For SRP analysis—to the remaining 2.5 ml of the fraction was added 0.25 ml mixed reagent. Time (15–30 minutes) was allowed for color development, and absorbance was measured at 885 nm in a 1-cm cell.

NOTE: All TSP absorbance readings were doubled before calculating concentrations from standard calibration curves.

Enzyme Assay for Orthophosphate in Natural Waters[15]

Reagents

1. Alkaline Phosphatase: (frozen), 14 mg protein/ml, 26 units/mg protein, 1 unit will hydrolyze 1 micromole/minute. A stock solution (2.5 units/ml) was prepared by diluting the enzyme (34.3 μl) to 5.0 ml with 0.1-M Trizma base solution (pH 8.3). It was kept frozen. A working concentration solution (0.025 units/ml) was prepared by diluting 50 μl of stock solution to 5.0 ml with 0.1-M Trizma (pH 8.3). This solution was refrigerated.

2. Substrate: 4-methylumbelliferyl Phosphate (MUP), mol wt. 256.2, was kept frozen, and was desiccated before weighing. The stock solution was prepared by dissolving solid substrate in 0.1-M Trizma (pH 8.3) to achieve 2.5 mmol/L. The solution was kept frozen. Serial dilutions were made until a working concentration of 0.5 μmol/L substrate in Trizma base was attained. The working solution was prepared daily.

A blank was prepared from 5.0 ml d.i. water and 0.5 ml of 0.1-M Trizma. A Turner Model 111 fluorometer with a Corning 7-60 primary filter and Wratten 8 and 48 as secondary filters was used to measure fluorescence. A 30$\times$ window and a temperature control sample door were used for each reading.

Procedure

The temperature was maintained at $\sim$20 °C. A 5.0-ml water sample or standard was placed in a 12 $\times$ 75 mm disposable culture tube. The fluorometer was zeroed with the blank solution. 0.4 ml substrate (MUP) was added to sample; the solution was mixed and then the fluorescence measured for the zero time reading. 10 μL of alkaline phophatase was added and a stop watch started; the solution was mixed gently, and the fluorescence was recorded every 15 seconds for 3 minutes.

Calculations: The reaction rate = the slope of the fluorescence vs time line.

Calibration Curve: 1/rate was plotted against PO_4^{3-} concentration of standards. A new curve was plotted for every sample batch.

Standards: 1 to 10 μg phosphorus/l concentration range

Replication: 3 replicates for each standard and sample

Phytase Dephosphorylation of Phytic Acid[75]

Reagents

1. Acetate Buffer (pH 5.0): a 0.2-M sodium acetate solution was pH adjusted to 5.0 with glacial acetic acid.

2. Sodium Salt of myo-Inositol Hexaphosphate: the salt was dissolved in d.i. water to attain 100 mg phosphorus/l in the reaction vessel.

3. Phytase Solution (0.04 units/ml): 8.0 mg of phytase (desiccated and frozen) was dissolved in 10.0 ml twice-deionized water. The enzyme contained 0.05 units/mg Phytase, and 1 unit will hydrolyze 1 micromole phosphorus/minute from 50 mg/l phosphorus IHP.

Reaction

The following were mixed in a 20-ml test tube: (1) 1.25 ml acetate buffer, (2) 0.75 ml IHP stock (687 mg phosphorus/l), (3) 0.10 ml phytase solution, and (4) 2.90 ml twice-deionized water. The reaction mixture was incubated at 55 °C for 16 hours. Extent of reaction tests were taken periodically by removing 1.0-ml aliquots for SRP and TSP analysis. The reaction was terminated by refrigeration of the reaction tubes. The hydrolysate mixtures were filtered before HPLC analysis.

Wheat Bran Extraction Proceedure[69]

Procedure

1. Wheat bran (1.000 g dried and ground to pass 20-mesh screen) was weighed into a 50.0-ml Erlenmeyer Flask.

2. 25.0 ml Trichloroacetic acid [(TCA) 3% in d.i. water] was added and the solution was shaken with a wrist-action mechanical shaker for 1/2 hour at 25 °C.

3. In 40-ml centrifuge tubes, the sample was centrifuged at an average 44,000 g for 1/2 hour.

4. A 1.0-ml aliquot of the clear supernatant was pH adjusted by adding 9.0 ml of 0.1-M NaCl/0.5-mM Na_4EDTA (HPLC eluent).

5. The diluted sample was filtered through 0.4-μm Nuclepore membrane filter.

6. 1.0-ml aliquots were removed to analyze for SRP and TSP.

7. The sample was stored overnight in refrigerator if necessary or frozen for not more than one week before HPLC analysis.

Method of Inositol Phosphate Determination in Soils[58]

Reagents

0.1-M HCl, Br_2 solution, solid NaOH pellets, $CaCl_2$, cation-exchange resin: Dowex 50W-X8 (H^+ form), 50–100 mesh.

Solutions

Calcium Chloride Solution—8.6 g anhydrous $CaCl_2$ in 20.0 ml H_2O; hypobromite solution—6 ml Br_2 + 12.0 g NaOH + 100 ml H_2O (prepared in hood with rubber gloves and protective clothing).

Extraction procedure

1. An organic soil sample was collected, air dried, and passed through a 2-mm sieve. 10.0 g of the sample was weighed into a 125-ml Erlenmeyer flask.

2. The soil sample was extracted twice with 25.0 ml 0.1-M HCl for 15 minutes each at room temperature to remove cations. The solution was discarded, and the soil washed with d.i. water (25 ml).

3. The soil was suspended in 100 ml of hypobromite solution at 0 °C. Oxidation proceeded in the hood for 1-1/2 hours at room temperature. Then the solution was boiled for 5 minutes on a hot plate.

4. The supernatant was poured into a 250-ml Erlenmeyer flask. The soil was removed from the original flask and put into centrifuge tubes using 25-ml d.i. water and centrifuged twice at 100 g for 10 minutes; the washings were added to the supernatant flask.

5. 20.0 ml $CaCl_2$ solution was added to the supernatant flask to allow precipitation of calcium phosphate salts. The calcium phosphate salts were filtered (or centrifuged) and washed with (2 × 25 ml) d.i. water and allowed to dry, then weighed.

6. A portion of the salts was redissolved by shaking with ~50 ml H_2O and excess Dowex cation-exchange resin. The solution was then filtered through Whatman No. 1 paper (suction) and the filtrate retained. The soil extract was filtered again through a 0.4-μm Nuclepore membrane before HPLC analysis.

Modification of Alkaline Bromination Procedure for Water Sample

1. To a ~10 ml concentrated water sample (concentrated by ultrafiltration) 0.2 g NaOH was added, and the test tube cooled in an ice bath. Pure Br_2 solution (0.1 ml) (not hypobromite solution used above) was added.

2. Oxidation proceeded at room temperature for 1-1/2 hours; the solution was boiled for 5 minutes, then cooled. About 5.0 ml conc. NH_4OH and 0.20 g sodium metabisulphite were then added.

3. An aliquot of the oxidized water sample was then analyzed for phosphorus and the remainder was vacuum concentrated and filtered before HPLC analysis.

APPENDIX B

SAMPLING DATA

Table B–1. Samples collected during June 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Ortho (μg/L)	Conductivity (μS)
Rainfall	RA	5.50	4.39	1.11	4.91	0.0
Thrufall	T2	6.01	1.17	4.84	6.99	0.0
Upstream	WB1	3.72	2.87	0.85	2.71	0.0
Spring	WB2	5.25	5.25	0.00	2.52	0.0
Midstream	WB3	3.21	2.36	0.85	1.80	0.0
Downstream	WB4	5.16	4.06	1.10	2.75	0.0
Below confluence	WB5	6.10	6.27	0.00	4.29	0.0
Res. cove	RC	10.94	3.38	7.56	1.97	0.0
Res. main	RM	4.31	0.91	3.40	1.68	0.0

Table B–2. Samples collected during July 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Ortho (μg/L)	Conductivity (μS)
Rainfall	RA	4.05	2.92	1.13	4.58	0.0
Thrufall	T2	23.59	12.96	10.63	31.69	0.0
Thrufall	T1	7.13	1.62	5.51	3.46	0.0
Soilwater Means	SW	3.01	1.33	1.68	0.0	0.0
Groundwater Means	GW	12.81	6.50	6.31	0.0	0.0
Upstream	WB1	10.86	9.48	1.38	7.82	0.0
Spring	WB2	7.46	5.35	2.11	5.38	0.0
Midstream	WB3	7.46	4.13	3.33	3.96	0.0
Downstream	WB4	7.46	4.13	3.33	3.90	0.0
Below confluence	WB5	13.78	9.48	4.30	12.97	0.0
Res. cove	RC	3.08	1.46	1.62	2.60	0.0
Res. main	RM	4.21	1.62	2.59	1.22	0.0

Table B–3. Samples collected during August 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Ortho (μg/L)	Conductivity (μS)
Rainfall	RA	4.48	2.70	1.78	0.00	0.0
Thrufall	T1	49.61	27.74	21.87	0.00	0.0
Groundwater	GW	7.60	3.17	4.43	3.02	0.0
Means						
Upstream	WB1	6.71	4.28	2.43	6.00	0.0
Spring	WB2	6.04	5.04	1.00	6.16	0.0
Midstream	WB3	5.54	3.96	1.58	4.17	0.0
Downstream	WB4	5.87	4.70	1.17	4.75	0.0
Below confluence	WB5	7.21	5.71	1.50	7.38	0.0
Res. cove	WB6	3.37	1.03	2.34	4.81	0.0
Res. main	RM	15.56	3.37	12.19	7.07	0.0

Table B–4. Samples collected during September 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Ortho (μg/L)	Conductivity (μS)
Rainfall	RA	0.00	0.00	0.00	0.00	0.0
Thrufall	T1	60.80	33.09	27.71	0.00	0.0
Groundwater						
Well 1	WE1	9.22	3.54	5.68	1.29	0.0
Well 2	WE2	5.38	3.37	2.01	0.00	0.0
Well 3	WE3	14.72	7.21	7.51	0.00	0.0
Well 4	WE4	4.54	1.95	2.59	0.00	0.0
Upstream	WB1	4.88	2.79	2.09	4.94	0.0
Spring	WB2	6.21	5.37	0.84	4.04	0.0
Midstream	WB3	5.70	4.12	1.58	4.56	0.0
Downstream	WB4	6.04	4.54	1.50	4.56	0.0
Below confluence	WB5	9.38	7.12	2.26	6.78	0.0
Res. cove	RC	4.20	1.28	2.92	0.00	0.0
Res. main	RM	9.55	1.70	7.85	0.00	0.0

Table B–5. Samples collected during October 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Ortho (μg/L)	Conductivity (μS)
Rainfall	RA	3.04	0.45	2.59	12.72	0.0
Thrufall	T1	5.04	0.87	4.17	3.72	0.0
Thrufall	T2	73.99	52.87	21.12	0.00	0.0
Groundwater						
Well 1	WE1	13.55	2.04	11.51	1.99	0.0
Well 2	WE2	6.56	3.87	2.69	5.12	0.0
Well 3	WE3	25.74	9.88	15.86	12.68	0.0
Well 4	WE4	7.38	1.87	5.51	2.19	0.0
Upstream	WB1	3.87	2.78	1.09	2.52	0.0
Spring	WB2	5.29	5.37	0.00	4.17	0.0
Midstream	WB3	5.38	4.11	1.27	4.14	0.0
Downstream	WB4	5.37	2.78	2.59	3.59	0.0
Below confluence	WB5	20.06	16.81	3.25	15.97	0.0
Res. cove	RC	5.04	1.70	3.34	1.77	0.0
Res. main	RM	10.05	4.29	5.76	3.96	0.0

Table B–6. Samples collected during November 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)	Ortho (μg/L)
Rainfall	RA	17.53	3.79	13.74	0.0	9.0
Thrufall	T1	86.52	51.45	35.07	0.0	70.9
Thrufall	T2	404.10	215.10	189.00	0.0	262.9
Soilwater	SW	5.21	1.46	3.75	0.0	15.0
Means						
Groundwater						
Well 1	WE1	3.92	2.57	1.35	0.0	3.2
Well 2	WE2	2.42	2.42	0.00	0.0	3.0
Well 3	WE3	9.90	6.68	3.22	0.0	8.3
Well 4	WE4	1.82	1.44	0.38	0.0	2.2
Upstream	WB1	1.52	0.40	1.12	0.0	0.4
Spring	WB2	5.41	4.51	0.90	0.0	3.5
Midstream	WB3	2.72	1.44	1.28	0.0	1.8
Downstream	WB4	2.72	1.22	1.50	0.0	1.9
Below confluence	WB5	6.31	4.66	1.65	0.0	5.1
Res. cove	RC	5.56	2.72	2.84	0.0	2.8
Res. main	RM	3.02	1.22	1.80	0.0	1.4

Table B–7. Samples collected during December 1984

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)	Ortho[a] (μg/L)
Rainfall	RA	4.81	2.27	2.54	0.0	2.9
Thrufall	T1	47.63	31.24	16.39	0.0	58.8
Thrufall	T2	78.32	54.36	23.96	0.0	0.0
Groundwater						
Well 1	WE1	2.87	1.52	1.35	0.0	0.0
Well 2	WE2	3.76	3.17	0.59	0.0	0.0
Well 3	WE3	9.30	6.16	3.14	0.0	0.0
Well 4	WE4	1.52	1.07	0.45	0.0	0.0
Upstream	WB1	2.87	0.70	2.17	0.0	0.0
Spring	WB2	5.86	4.89	0.97	0.0	0.0
Midstream	WB3	4.66	3.62	1.04	0.0	0.0
Downstream	WB4	4.06	1.97	2.09	0.0	0.0
Below confluence	WB5	6.01	4.29	1.72	0.0	0.0
Res. cove	RC	5.56	0.32	5.24	0.0	0.0
Res. main	RM	4.51	0.84	3.67	0.0	0.0

[a]Orthophosphate measurements were discontinued due to unreliable results.

Table B–8. Samples collected during January 1985

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)	Ortho (μg/L)
Rainfall	RA	0.47	0.32	0.15	14.5	0.0
Thrufall	T1	23.68	18.80	4.88	42.0	0.0
Thrufall	T2	16.94	9.30	7.64	55.0	0.0
Soilwater	SW	2.12	0.11	2.01	30.5	0.0
Means						
Groundwater						
Well 1	WE1	2.78	1.98	0.80	215.0	0.0
Well 2	WE2	4.96	3.51	1.45	192.0	0.0
Well 3	WE3	6.27	3.80	2.47	194.0	0.0
Well 4	WE4	4.67	2.13	2.54	234.0	0.0
Upstream	WB1	4.66	0.47	4.19	137.0	0.0
Spring	WB2	5.71	4.74	0.97	261.0	0.0
Midstream	WB3	9.60	2.27	7.33	230.0	0.0
Downstream	WB4	4.36	2.12	2.24	239.0	0.0
Below confluence	WB5	8.26	3.69	4.57	244.0	0.0
Res. cove	RC	13.98	0.31	13.67	266.0	0.0
Res. main	RM	2.35	1.40	0.95	255.0	0.0

Table B–9. Samples collected during February 1985

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)
Rainfall	RA	4.96	3.09	1.87	34.8
Snow/rain	SN	3.30	2.34	0.96	21.3
Thrufall	T1	19.03	17.31	1.72	101.8
Snowthru—chesoak	SN1	26.67	20.83	5.84	40.1
Thrufall	T2	32.81	26.89	5.92	67.9
Snowthru—tupop	SN2	32.35	20.52	11.83	95.7
Soilwater	SW	3.02	0.92	2.10	50.0
Means					
Groundwater					
Well 1	WE1	2.42	0.77	1.65	188.1
Well 2	WE2	6.31	3.39	2.92	178.1
Well 3	WE3	5.86	2.79	3.07	147.0
Well 4	WE4	3.01	1.37	1.64	167.4
Upstream	WB1	2.42	1.07	1.35	104.8
Spring	WB2	5.86	4.66	1.20	254.0
Midstream	WB3	3.76	1.67	2.09	154.0
Downstream	WB4	2.86	1.59	1.27	165.2
Below confluence	WB5	5.11	3.76	1.35	202.0
Res. cove	RC	3.32	0.62	2.70	255.0
Res. main	RM	4.36	2.16	2.20	259.0

Table B–10. Samples collected during March 1985

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)
Rainfall	RA	6.86	6.29	0.57	36.7
Thrufall	T1	71.28	56.84	14.44	53.8
Thrufall	T2	25.19	16.84	8.35	60.4
Soilwater means	SW	2.90	1.14	1.76	39.7
Groundwater means	WE	5.60	3.37	2.23	193.3
Upstream	WB1	5.88	2.31	3.57	144.8
Spring	WB2	7.67	6.37	1.30	263.0
Midstream	WB3	5.23	3.53	1.70	210.0
Downstream	WB4	7.67	5.40	2.27	216.0
Below confluence	WB5	8.16	4.42	3.74	186.7
Res. cove	RC	5.56	1.82	3.74	891.0
Res. main	RM	5.72	2.96	2.76	289.0

Table B–11. Samples collected during April 1985

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)
Soilwater	1–6	3.78	0.26	3.52	22.5
Soilwater	2–6	2.75	0.85	1.90	94.9
Soilwater	3–6	4.67	0.11	4.56	31.6
Soilwater	4–6	4.67	0.26	4.41	31.6
Soilwater	5–6	4.08	0.11	3.97	50.4
Groundwater					
Well 1	WE1	2.90	1.07	1.83	186.7
Well 2	WE2	8.50	3.27	5.23	168.3
Well 3	WE3	8.64	4.16	4.48	169.9
Well 4	WE4	4.54	1.21	3.33	170.2
Upstream	WB1	4.96	0.92	4.04	117.2
Spring	WB2	8.64	4.96	3.68	230.0
Midstream	WB3	3.94	1.44	2.50	139.0
Downstream	WB4	7.76	1.95	5.81	156.0
Below confluence	WB5	9.82	4.45	5.37	162.2
Res. cove	RC	6.88	1.07	5.81	236.0
Res. main	RM	5.55	0.63	4.92	240.0

Table B–12. Samples collected during May 1985

Location	Sample No.	TSP (μg/L)	SRP (μg/L)	SUP (μg/L)	Conductivity (μS)
Rainfall	RA	8.79	2.90	5.89	25.6
Thrufall	T1	101.50	74.23	27.27	30.8
Thrufall	T2	22.66	3.35	19.31	34.3
Groundwater					
Well 1	WE1	4.40	3.12	1.28	265.0
Well 2	WE2	6.16	4.64	1.52	173.7
Well 3	WE3	15.61	7.08	8.53	224.0
Well 4	WE4	3.60	2.56	1.04	231.0
Upstream	WB1	3.12	2.32	0.80	127.8
Spring	WB2	6.00	5.36	0.64	197.8
Midstream	WB3	7.60	4.80	2.80	213.0
Downstream	WB4	5.68	4.16	1.52	225.0
Below confluence	WB5	7.28	4.64	2.64	235.0
Res. cove	RC	6.00	0.88	5.12	225.0
Res. main	RM	5.04	0.47	4.57	228.0

VITA

Julie E. Segars was born in Smyrna, Georgia, on April 13, 1961. She attended elementary school in that city. After moving to Winder, Georgia, in 1973, she graduated from Winder-Barrow High School in June 1979. The following August she entered Shorter College in Rome, Georgia, and in May 1983 she received a Bachelor of Science degree in Natural Science with majors in Chemistry and Environmental Science. In the fall of 1983, she accepted a teaching assistantship from the Chemistry Department of The University of Tennessee, Knoxville and began study toward a Master's degree. In the spring of 1984, she accepted a graduate reasearch assistantship from Oak Ridge National Laboratory. The Master of Science degree with a major in Chemistry was awarded in December 1985.

The author is a member of The American Society of Limnology and Oceanography. She will be employed by Lockheed Corporation of Las Vegas, Nevada.