EXTRACTION AND IDENTIFICATION OF ORGANIC PHOSPHORUS COMPOUNDS OF SOILS

by

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INTRODUCTION

The importance of phosphorus in plant nutrition has been recognized since the middle of the nineteenth century, when the analysis of plant ash showed the presence of phosphates and other inorganic salts, and the use of phosphorus-containing manures and fertilizers in field experiments greatly improved crop yields (59). Since that time, particularly during the last fifty years, phosphorus has arrested the attention of soil scientists who have contributed greatly to our knowledge of it and its soil-plant relationships.

It is now well established that phosphorus occurs in soils in both inorganic and organic combination, the latter often in amounts which account for as much as half of the total soil phosphorus (23, 51, 60, 79). From the standpoint of the nutrition of plants, because phosphorus is absorbed chiefly as the inorganic orthophosphate ion (54), the role of inorganic phosphorus has received by far the more attention. In general the role of organic phosphorus in plant nutrition has been considered of little value because soils high in organic phosphorus are often low in available phosphorus (28, 77). On the other hand, the level of organic phosphorus in some cultivated soils has fallen considerably below that of adjacent virgin land (72), a fact which is not inconsistent with the belief that organic phosphorus through mineralization, can contribute to some extent to the nutrition of plants. The known potential phosphate reserves for the manufacture of phosphorus fertilizers are not unlimited, and consequently soil scientists are becoming quite concerned about our lack of knowledge of the organic phosphorus of soil and of its usefulness in crop production.

Although attempts to identify soil organic phosphorus compounds have not been very fruitful, the probability of identifying more of these compounds in the near future is rather bright because of the current spectacular advances and improvements in analytical instruments and techniques. A knowledge of the organic phosphorus compounds present in soil will do much to further our understanding of the transformations they undergo in the soil and the factors which influence their availability to plants. The chemical behavior of organic phosphorus fractions from soils has suggested the presence of nucleic acid-like compounds (9, 80, 82), phytin (8, 9, 80, 83) and trace amounts of phospholipids (31, 82). Recently, by the use of chromotographic methods, Smith and Clark (67) concluded that perhaps two-thirds of the material which behaves chemically as phytin is admixed material, and Adams et al. (1) concluded that only a small portion of the soil organic phosphorus could be accounted for as nucleic acids. Thus it appears that the organic compounds comprising the major portion of the soil organic phosphorus have yet to be isolated and identified.

Intimately associated with the problem of identification of organic phosphorus compounds are the methods used for extracting these compounds from the soil. The generally accepted extraction procedures have included the use of strongly alkaline solutions at elevated temperatures, but these rather harsh conditions could hydrolyze some compounds and probably cause other chemical changes with the result that the compounds obtained do not actually occur as such in the soil.

This investigation has been devoted to developing a mild extraction procedure for soil organic phosphorus and the use of some analytical techniques to identify some of the extracted compounds.

LITERATURE REVIEW

1. Introduction

Perhaps the first evidence for the occurrence of organic phosphorus in soils was the observation of Mulder (46) in 1844 that it was impossible to prepare fractions of soil organic matter which were free of phosphorus. Since then many papers on soil organic phosphorus have appeared in the literature and the excellent reviews by Pierre (53), Bremner (11) and Black and Goring (7) which have been published in recent years provide a comprehensive picture of the present state of knowledge on the subject. The present review will be restricted to the following:

Differentiation of organic and inorganic phosphorus.

The quantitative determination of organic phosphorus in soils.

Mild procedures for the extraction of soil organic phosphorus.

Organic phosphorus compounds occurring in soils.

2. Differentiation of Organic and Inorganic Phosphorus.

The early methods of differentiating between organic and inorganic phosphorus involved selective extraction wherein it was believed that dilute acids dissolved mainly inorganic phosphates (62) while dilute ammonium hydroxide dissolved mainly organic phosphates (33, 71). However several workers (45, 50, 60, 79) have shown that either acidic or alkaline extractants are capable of dissolving both inorganic and organic phosphates. Once this fact was established, soil chemists recognized the need for a procedure which would give an estimate of both the inorganic and organic phosphorus present in a soil extract. One of the first such procedures was the use of magnesia mixture to precipitate the inorganic phosphorus. Stewart (70) was apparently the

first to apply this technique to soil extracts with but little success. However, Potter and Benton (56) modified the procedure and concluded that the difference between the amount of phosphorus precipitated from the untreated extract and that precipitated from the extract treated to convert the organic into inorganic form was a satisfactory estimate of the organic phosphorus extracted. Although there was evidence of incomplete precipitation of inorganic phosphorus when it was present in small amounts and also of contamination of the magnesium ammonium phosphate with organic phosphorus,Schollenberger (63) and Auten (5) considered that the Potter and Benton method determined the organic phosphorus with a fair degree of accuracy.

At the present time methods for the differentiation between organic and inorganic phosphorus in soil extracts are based on the molybdenum blue reaction. In 1927 Parker and Fudge (49) applied both the Deniges and the Fiske-Subarrow methods to the determination of inorganic phosphorus in soil extracts containing organic phosphorus. These two methods differ mainly in the reagent used to reduce the phosphomolybdate complex. The former, using stannous chloride as the reducing agent, was found to be the more sensitive and was especially recommended for dilute soil extracts. Improvements in the method were made by Truog and Meyer (74) after they made a systematic study of the influence of concentration of sulphuric acid, ammonium molybdate, and stannous chloride on the intensity of color produced. This method was rather limited in its application, since for visual colorimetry it was necessary to have colorless solutions. Dean (20) employed this principle in determining organic phosphorus in alkaline soil extracts which he decolorized with kieselguhr or by treatment with bromine.

Pearson (50) treated his soil extracts with decolorizing charcoal before determining inorganic phosphorus. Wrenshall and Dyer (79) found, however, that complete decolorization is not obtained by the action of bromine, and that the treatment with alkali and bromine is sufficiently drastic to release some phosphorus from organic combination. By the application of photoelectric colorimetry (25) they devised a technique which enabled them to determine inorganic phosphorus in the presence of the colored organic matter.

The molybdenum blue reaction (under the conditions prescribed for phosphorus analysis and in the absence of arsenate) is specific to the inorganic orthophosphate ion. Therefore this reaction will permit a clear differentiation between inorganic and organic phosphorus compounds provided the former is present only in the soluble orthophosphate form, and that the latter is not hydrolyzed by the extraction reagents or the color development reagents during the analysis. If other forms of inorganic phosphorus are present which are resistant to hydrolysis by the analytical method of determining orthophosphate, the estimation of inorganic phosphorus will be low and of organic phosphorus high. On the other hand, if some of the organic phosphorus is hydrolyzed under the experimental conditions, the estimate of inorganic and organic phosphorus will be high and low respectively. Black and Goring (7) also point out that "Soil extracts may contain inorganic phosphorus in the form of suspended clay, and if some of this phosphorus becomes soluble in the acid solution used for developing the molybdenum blue color, it may either augment or diminish the apparent amount of organic phosphorus, according to the circumstances of the particular case". Consequently until the forms of inorganic

phosphorus ions in the soil are more definitely known and the hydrolysis of organic phosphorus compounds can be eliminated, there is some possibility that the estimation of the inorganic and organic phosphorus in soil extracts is not entirely accurate. Perhaps the Martin and Doty (44) modification of the Berenblum and Chain procedure (6) is one of the most satisfactory methods available today. The inorganic molybdophosphoric acid is selectively extracted from the organic phosphorus compounds present in the soil extract. The extraction is completed rapidly (20 seconds), and consequently the possibility of hydrolysis of organic phosphorus compounds is greatly minimized. Interference from color in the soil extract is also greatly decreased, if not entirely eliminated.

3. The Quantitative Determination of Organic Phosphorus in Soil.

Two different approaches to the quantitative determination of organic phosphorus in soil are

1) determination of the inorganic phosphorus extracted from a soil before and after some treatment to convert the organic phosphorus in the soil to inorganic, and
2) determination of the inorganic phosphorus present in a soil extract before and after treatment of the extract to convert the organic phosphorus present into the inorganic form.

In both of the above approaches, the increase of inorganic phosphorus brought about by the conversion treatment is taken as a measure of the organic phosphorus. Thus, although the two approaches require somewhat different procedures, they both involve oxidation of organic matter. In the first case this is done by treating the soil before extraction whereas in the second it is done in the extracts.

The first approach can be realized by high-temperature hydrolysis, wet combustion, and dry combustion. High-temperature hydrolysis was used in early studies of organic phosphorus (71), and Black and Goring (7) suggest that the technique is worthy of re-examination for determining soil organic phosphorus. In the wet combustion method the soil organic phosphorus is converted to inorganic by hydrogen peroxide or other oxidizing agents, and the inorganic phosphorus is extracted with weak acids (10, 23, 50). Limitations of this method are incomplete mineralization of the organic phosphorus and subsequent incomplete extraction of the mineralized phosphorus (73) with the result that low values will be obtained. In the dry combustion method inorganic phosphorus is determined in extracts of comparable ignited and non-ignited samples of soil. This method has been used since the early investigations of organic phosphorus were undertaken and because of its simplicity its use has been continued to the present day. Some possible defects in the method have been pointed out from time to time and improved techniques have been reported periodically. Early workers did not use the same acid strength for extracting the soil before and after conversion of the organic phosphorus but Stewart (71) recognized this obvious defect and he used the same extraction technique for both samples. Other possible sources of error were an increase in the acid solubility of naturally occurring iron and aluminium phosphates by ignition (29), and the incomplete extraction of the organic phosphorus when converted to inorganic (73). In an effort to minimize these possible errors Legg and Black (41) employed a strong extracting solution (concentrated hydrochloric acid) and investigated the influence

of different ignition temperatures. In arriving at the conditions for their ignition procedure they used as a basis of comparison the results from an improved extraction procedure proposed by Mehta et al. (45) which will be discussed later in this review. Legg and Black realized that ignition methods can give high results because of increased solubility of the inorganic phosphates on ignition or low results because of incomplete mineralization of the organic phosphates on ignition. They studied these two variables at different ignition temperatures and selected 240° C as being that temperature at which the positive error of the former variable equalled the negative error of the latter. The organic phosphorus content arrived at by their procedure,in which comparable samples of soil are extracted by concentrated hydrochloric acid before and after ignition for one hour at 240° C, may be taken directly as an estimate of organic phosphorus in the soil. Saunders and Williams (60) have reported an ignition procedure whereby samples of soil ignited at 550° C for one hour and nonignited soil are extracted with 0.2 N sulphuric acid for 16.5 hours. Their results by ignition were somewhat higher than those of an extraction method they proposed. Since the soils and the extraction methods used in developing the above mentioned ignition procedures were not the same the relative merits of the ignition procedures are unknown and to the writer's knowledge no comparative studies have been made.

The second approach to the quantitative determination of soil organic phosphorus involves extraction with one or more reagents designed to remove all the organic phosphorus without changing any of it to the inorganic form. When organic phosphorus is extracted from

the soil and estimated from the increase in inorganic phosphorus upon decomposition of the organic compounds, there is little question that the results represent an estimate of organic phosphorus extracted. In addition to providing an estimate of the soil organic phosphorus, extraction methods also provide a means of separating the organic phosphorus compounds from the inorganic material of the soil, and this is of course a necessary step in any studies to identify phosphorus compounds occurring in the soil. Obvious limitations of the extraction methods are incomplete extraction of the organic phosphorus and hydrolysis of labile organic compounds by the extraction reagents. These two limitations will result in low estimates of organic phosphorus and for any given soil if one procedure extracts more organic phosphorus than other procedures, it must be considered more accurate than the others.

Extraction procedures usually involve a dilute acid pretreatment which removes very little organic phosphorus, but because of the removal of cations the organic phosphorus compounds are quite readily removed by the subsequent alkaline extraction. Both Schollenberger (63) and Pearson (50) used 0.1 N hydrochloric acid as preliminary extractant. Schollenberger then extracted the soil with 1.1 N ammonium hydroxide at room temperature, but Pearson found that extracting the soil with 0.5 N ammonium hydroxide for 18 hours at 90°C removed about twice as much organic phosphorus. Wrenshall and Dyer (79) changed the strength of both the acid and alkaline extractants. They reported that the soil organic phosphorus was stable under their extraction procedure and that the total soil phosphorus was almost completely extracted by the two treatments with 4 N hydrochloric acid

and 3 N ammonium hydroxide.

The superiority of sodium hydroxide as the alkaline extracting agent was reported by Bower (8) although Schollenberger (63) had previously found no advantage in using the alkali hydroxides instead of ammonium hydroxide. Mehta et al.(45) and Saunders and Williams (60) studied the use of sodium hydroxide as an extractant and both investigations resulted in extraction procedures which are improvements over the procedures that they previously used. Larger amounts of organic phosphorus were obtained in the extracts, presumably because extraction was more complete and at the same time precautions were taken to minimize hydrolysis of the phosphorus compounds.

In a series of experiments Mehta et al.(45) found that if, in the Pearson method (50), 0.5 N sodium hydroxide was substituted for 0.5 N ammonium hydroxide, more organic phosphorus was extracted. Re-extraction of the ammonium hydroxide extracted soil with hot sodium hydroxide yielded additional organic phosphorus, but re-extraction of the sodium hydroxide extracted soil with ammonium hydroxide did not. Re-extraction of the ammonium hydroxide extracted soil with ammonium hydroxide and the sodium hydroxide extracted soil with sodium hydroxide yielded no appreciable additional quantities of organic phosphorus. From these experiments and subsequent studies on the strength, duration and temperature of acid pretreatment and the hydrolysis of soil organic phosphorus compounds they concluded that 1) sodium hydroxide is a superior extractant to ammonium hydroxide; 2) substantial loss of organic phosphorus by hydrolysis may occur during hot sodium hydroxide extraction; 3) of the organic phosphorus extracted by two consecutive sodium hydroxide treatments at room temperature, that in the second

extract is less susceptible to hydrolysis at high temperature; 4) after initial extraction with cold sodium hydroxide, subsequent extraction with 0.5 N sodium hydroxide at 90° C can be continued as long as 8 hours without appreciable loss by hydrolysis, whereas with 1 N sodium hydroxide the loss is appreciable in 4 hours; 5) concentrated hydrochloric acid could be used advantageously as the acid pretreatment. Consequently their recommended extraction procedure is briefly as follows: the soil receives a concentrated hydrochloric pretreatment for 10 minutes on a steam plate, a 0.5 N sodium hydroxide extraction at room temperature, followed by a second 0.5 N sodium hydroxide extraction at 90° C. In this way they were able to extract more organic phosphorus than when employing methods used by previous workers.

Prior to selecting the conditions for their extraction procedure for the determination of soil organic phosphorus, Saunders and Williams (60) studied existing procedures and concluded that 1) the Pearson method gave low results because of incomplete organic phosphorus extraction and also because of hydrolysis due to a hot extraction procedure; 2) a hot 0.1 N hydrochloric acid pretreatment was superior to the 4 N hydrochloric acid pretreatment of Wrenshall and Dyer (79); 3) any increase in the extractive power of 0.1 N sodium hydroxide at 90° C is counteracted by hydrolysis of the phosphorus and a cold extraction is preferable. Briefly, their extraction procedure consists of a hot 0.1 N hydrochloric acid pretreatment for 1 hour followed by a cold 0.1 N sodium hydroxide extraction for $16\frac{1}{2}$ hours. Two such sodium hydroxide extractions are considered to be adequate.

4. Mild Procedures for the Extraction of Organic Phosphorus

It is to be noted that the extraction procedures mentioned above are designed for the quantitative estimation of soil organic phosphorus and are of necessity rather harsh. In keeping with the objective of maximum removal of organic phosphorus certain precautions have been taken to minimize hydrolysis, nevertheless the procedures are sufficiently harsh to question the advisability of their use for a study of organic phosphorus compounds occurring in soil. If these methods of extraction are used, chemical changes in the soil compounds, such as hydrolysis, may occur and justifiable doubt may be cast as to whether compounds identified from such extracts actually occur as such in the soil. Consequently, if one is to attempt to identify the forms of organic phosphorus in soils, the use of mild extraction procedures which will release a large amount of organic phosphorus without chemical alteration appears to be a prerequisite. For the extraction of soil organic matter the need for mild procedures has been emphasized (12) and investigations of mild extractants have been reported.

The observation of Dion and Mann (24) that pyrophosphate was much more efficient in the extraction of manganese from soil than orthophosphate led Bremner and Lees (13), who were interested in mild extractants for soil organic matter, to test the extractive capacities of neutral solutions of sodium salts of organic and inorganic acids. Sodium pyrophosphate, sodium oxalate, sodium fluoride and sodium citrate were found to be the most effective of the reagents tested, although less organic matter was extracted than by caustic alkali. These results, together with those of Heintz and Mann (36), which showed that

solutions of hydroxy acids were almost as effective as pyrophosphates, indicated that the organic matter is intimately associated with metallic cations and that its solubility in neutral reagents is largely determined by the nature of this association. Further, the efficiency of a neutral salt extractant appeared to depend on the ability of its anions to remove interfering metals either as insoluble precipitates or as soluble co-ordination complexes (12). Martin and Reeve (61) reported the use of aqueous or aqueous-acetone solutions of the chelating agents cupferron, 8- hydroxyquinoline (oxine), and acetyl-acetone for the extraction of organic matter from the B horizon of podsolic soils, and Coffin (19) using a two phase system oxine-benzene: aqueous soil suspension succeeded in removing 75% of the organic matter from the B horizon of a podsol. Oxine had been used previously by some workers in soil phosphorus studies to block refixation of the phosphorus during extraction (30, 60, 76), but Saunders and Williams found that because of its interference organic phosphorus could not be determined.

5. Organic Phosphorus Compounds Occurring in Soil.

Investigations of the organic phosphorus compounds of soil have provided evidence for the presence of phytic acid and its phospho derivatives, nucleic acid-like compounds and small amounts of phospholipids.

Studies conducted during the past twenty-five years have established the presence of phytic acid, in which six molecules of phosphoric acid are combined with inositol as inositol hexaphosphate, and lower phosphate esters of inositol. Yoshida (83) obtained inositol and phosphoric acid upon acid hydrolysis of soil organic phosphorus preparations, and Dyer et al. (27), found that a part of the organic phosphorus in a

podsol soil was precipitated as a ferric salt having a phosphorus/iron ratio corresponding to that of ferric phytate. Bower (8) found that a part of the organic phosphorus in soils could be precipitated as a ferric salt, and showed by determining the inositol/phosphorus ratio of the precipitate that the organic phosphorus so isolated was an inositol hexaphosphate. Bower also found that additional organic phosphorus in the filtrate from the ferric phytate precipitate could be precipitated as a calcium salt under alkaline conditions, and he obtained evidence that it contained inositol phosphoric acid esters having fewer than six phosphoric acid groups. Anderson (3) using a paper chromatographic method for separating inositol phosphates found inositol hexaphosphate but only a very small quantity of tetra-and/or triphosphates in three soils of Scotland. He could not detect either the mono- or diphosphates.

Smith and Clark (68) developed a chromatographic technique employing an anion exchange resin by which inositol phosphates could be separated. They employed the method to separate the compounds in the 'phytate fraction' of soil extracts and found that some components of the extracts of each soil were eluted in well defined zones simultaneously with inositol penta- and hexaphosphates (67). Inositol tetraphosphate was not found and since mono-, di- and triphosphates of inositol could not withstand the soil extract clarification procedure, information on the presence of these compounds in soil was lacking. They also found an organic phosphorus compound in soils that was more strongly absorbed on the resin than the penta- and hexaphosphates. The incorporation of P32 into these compounds when soil was incubated in the presence of radioactive orthophosphate suggested a microbiological

synthesis of phytin compounds in the soil.

Caldwell and Black (16) sought further evidence for the microbiological syntheses of phytin compounds. They selected different soil parent materials, quartz sand, clays, and clay-sand mixtures which contained no measurable amount of inositol hexaphosphate, incubated them for several months after inoculating with soil microorganisms and supplying organic and inorganic nutrients. They found an appreciable accumulation of organic phosphorus. Inositol hexaphosphate and a supposed isomer of inositol hexaphosphate (the material more strongly absorbed on the resin than inositol hexaphosphate) amounted to 7% of the total organic phosphorus accumulated. The supposed isomer of inositol hexaphosphate gave positive chemical tests for inositol but it would not support the growth of two assay organisms known to require mesoinositol. They concluded that about 50% of the soil inositol phosphate is of microbiological origin.

With the advent of chromatography improvements have been made in the methods for determining inositol phosphates in soil. The earlier estimates obtained by Wrenshall and Dyer (72) and Bower (8, 9) are now considered high because of the impurities found in the "phytic fraction" of soil extracts by Smith and Clark (67). Caldwell and Black (17) using a similar technique (15) found that the inositol hexaphosphates averaged 39 p.p.m. or 17% of organic phosphorus in 49 soils. Anderson (3) found they contributed about 30% to the total organic phosphorus in three soils examined.

Evidence for the presence of nucleic acids in soil has been obtained by the detection of their hydrolysis products, namely phosphoric acid, pentose sugars, and purine and pyrimidine bases, in the hydrolysates

of soil organic phosphorus (66, 80, 82). Bower (9) found that nucleic acid could be separated from phytin and its derivatives (except the monophosphate) by precipitating the latter with calcium. Under similar conditions he found that alkaline soil extracts could be fractionated into an insoluble calcium precipitate and filtrate - the nucleic acid fraction - which contained less than one third of the organic phosphorus extracted. Dephosphorylation studies using alkaline sodium hypobromite and enzymes from corn roots and wheat bran showed that the two fractions behaved like phytin and nucleic acid, respectively. Potter and Snyder (57) and Wrenshall et al. (81) using acid and alkaline hydrolysis, respectively showed that the rates of dephosphorylation of soil organic phosphorus preparations were slower than those of nucleic acid. Recently, Adams et al. (1) using ion exchange chromatography were unable to find more than 1 p.p.m. and 6 p.p.m. of ribonucleic acid in soils containing 575 p.p.m. and 327 p.p.m. respectively. In addition they were unable to detect purine bases in soil hydrolysates, and they concluded that very little nucleic acid exists in the soil. It is therefore apparent that the 'nucleic acid' fraction of soil requires much more investigation in order to determine its components.

Phospholipids, which are essential constituents of cells, have also been shown to be a part of soil organic phosphorus (66, 82). Organic phosphorus has been found in alcohol and ether extracts of soils, and solubility in alcohol and ether is a property characteristic of phospholipids. Furthermore choline, a product of hydrolysis of lecithin, has been isolated from soil extracts. The quantity of this form of organic phosphorus found in soils has never been high and Wrenshall and McKibbin (82) reported only 0.3% of the organic phosphorus in two soils examined to be ether soluble.

EXPERIMENTAL MATERIALS AND METHODS

1. Soils

Two soils were used in this investigation, namely the Greensboro loam (18) and the St. Bernard clay loam (40). The former was developed on till derived from impure limestone, and in general is well supplied with total phosphorus but this is difficultly available and a large amount of it is held in organic form. The latter soil was developed on calcareous till and is also well supplied with total phosphorus. The calcium content is much higher in the St. Bernard soil and this is reflected in its higher pH. The soil samples were obtained from the top six inches of unimproved pastures and that portion passing through a 2 m.m. screen was retained for use in this study. The general nature of these soils is indicated in Table 1.

Table 1

Soil Characteristics

	Greensboro	St. Bernard
Texture	loam	clay loam
pH	4.5	7.0
Calcium oxide	0.5%	3.0%
Total phosphorus*	564 p.p.m.	1363 p.p.m.
Organic phosphorus**	353 p.p.m.	522 p.p.m.
% Organic phosphorus	62%	38%

* Sherman method (65).
** Saunders and Williams method (60).

Throughout this study the ammonium form (52) of the soils was used except where otherwise noted, and soils which have received no pre-treatment will be designated 'normal' soils.

2. Methods Used to Estimate Inorganic and Organic Phosphorus in Solution.

Two methods of estimating inorganic orthophosphate in solution were used:

1) Martin and Doty modification (44) of the Berenblum and Chain method (6).

2) Dickman and Bray method (22).

The Martin and Doty method was preferred for determining inorganic orthophosphate particularly in highly colored soil extracts, and the Dickman and Bray method, because of its simplicity, was particularly useful in determining the total phosphorus content of solutions after digestion to convert all the phosphorus to the orthophosphate form.

The digestion was carried out by adding 1 ml. of 72% perchloric acid to an Erlenmeyer flask containing an aliquot of the solution to be analyzed for total phosphorus. The flask was heated on a hot plate until the solution was reduced to small volume and white fumes of perchloric acid began to evolve. The digested sample was then either used in its entirety or brought to volume and a suitable aliquot used, depending on the amount of phosphorus present, for the estimation of total phosphorus. The digested sample or aliquot was neutralized with dilute ammonium hydroxide to the *p*-nitrophenol end-point before making the colorimetric phosphorus analysis. Total phosphorus values as estimated by the two colorimetric procedures following perchlorate digestion were in agreement.

The estimation of organic phosphorus in solution was invariably arrived at by the difference between the estimates of total and inorganic phosphorus in solution.

3. Methods Used to Estimate the Phosphorus Content of Soil.

The total phosphorus contents of the soils were estimated by the method outlined by Sherman (65). The total organic phosphorus contents of the soils were estimated by the extraction procedure of Saunders and Williams (60).

4. Methods Used for Extraction of Organic Phosphorus from soil

a. Ammonium fluoride extraction.

One of the methods used for extracting soil organic phosphorus was that outlined by Hamilton (35). The extracting solution was 0.9 N ammonium fluoride, adjusted to pH 5.3 with hydrochloric acid. The procedure used is as follows: 4 gm. of soil were placed in Erlenmeyer flasks to which 28 ml. 0.9 N ammonium fluoride solution were added. The flasks were placed on a rotary shaker for one minute after which the extracts were filtered before removing aliquots for the estimation of phosphorus.

b. Sodium hydroxide extraction.

The sodium hydroxide extraction procedure was similar to but not identical with that used by Anderson (3). It is as follows: 250 g. of soil were placed on a large Buchner funnel and leached with 2 liters of 0.5 N hydrochloric acid and 2 liters distilled water to remove chlorides. The soil was then transferred to a large beaker, 1 liter of 1 N sodium hydroxide solution added and placed on a steam bath for four hours. The soil and sodium hydroxide extract were separated by centrifugation and the soil residue was washed with 1 liter distilled water to remove any soluble phosphorus compounds.

c. Oxine extraction.

The general procedure employed in the extraction of organic

phosphorus by oxine (8 - hydroxyquinoline) is as follows: To a suitably-sized flask the soil and water were added and the pH of the suspension adjusted to the desired value with either dilute ammonium hydroxide or dilute acetic acid. A 2.5% (w/v) solution of oxine in benzene was added and the flask placed on a mechanical rotary shaker. The benzene acted as a reservoir for oxine and as an accumulator of metallic complexes of oxine, the latter, like oxine, being much more soluble in benzene than in water. The pH was checked periodically with a Beckman Model G pH meter and maintained within ± 0.2 of the required value. At the completion of the shaking the liquid phases were separated in a separatory funnel and the benzene washed several times with water to remove soil and entrapped aqueous extract. The aqueous phase and combined washings then were centrifuged until clear for subsequent studies.

5. Estimation of Constituents in the Benzene Phase of Oxine Extracted Soil.

On the recommendation of Coffin (19), digestion of the benzene phase was accomplished as follows: to the residue from a sample of the extract after evaporation over a steam bath were added 15 ml. conc. HNO_3, 15 ml., 72% $HClO_4$, and 1 drop $VOSO_4$ solution containing 0.15 mg. vanadium. This mixture was heated on a hot plate at approximately 200° C until the volume had been reduced to a few drops. The digests were then made to volume with water and used for the estimation of phosphorus, iron and aluminum. Iron was determined by the acid thiocyanate method of Houlihan and Farina (37), while the aluminon method described by Robertson (58) was employed for the estimation of aluminum.

6. Methods Used to Separate Organic Phosphorus from Other Organic Matter of Extracts.

It was found that a partial separation of the organic phosphorus from other organic matter was necessary before subjecting the organic phosphorus in the sodium hydroxide and oxine extracts of soils to chromatographic analysis. Two different procedures for effecting this separation were used.

a. Acidification.

This is a technique widely used on alkaline extracts in soil organic matter studies to precipitate humic acids. In the present study the objective was to eliminate a considerable part of the organic matter but still retain a large amount of organic phosphorus. By the addition of hydrochloric acid the soil extracts were adjusted to pH 1.5 and the precipitated organic matter was removed by centrifugation.

b. Sodium hypobromite treatment.

The procedure was essentially that used by Smith and Clark (67). The soil extract was acidified to pH 0.5 with hydrochloric acid and the precipitated organic matter was centrifuged off and discarded. Sodium sulphate was added to the supernatant solution to make it 4% sodium sulphate, and the pH was adjusted to 1.7 with sodium hydroxide. Several milliliters of ferric chloride solution (55 mg./ml.) were added, after 24 hours the precipitated material was centrifuged off and added to a beaker containing 25 ml. of 0.5 N sodium hydroxide. It was placed on the steam bath, 10-15 ml. of hypobromite solution (bromine added to 5 N sodium hydroxide) were added and when no further decrease in the yellow color occurred the resulting precipitate was filtered off and

discarded. To the clear filtrate calcium chloride solution (50 mg./ml.) was added until no further precipitation resulted and the precipitate was collected on a Buchner funnel. It was dissolved with dilute hydrochloric acid, the resulting solution adjusted to pH 1.7, ferric chloride added and the iron precipitate was centrifuged off and retained. It was decomposed by adding a sodium hydroxide solution, the resulting ferric hydroxide was discarded and the clear solution was passed through a column of Dowex-50 in the hydrogen form to remove metallic ions. The sodium hypobromite treatment was also applied to a hydrochloric acid soil extract but the procedure was begun with the addition of sodium sulphate.

7. Anion Exchange Chromatography of Organic Phosphorus.

a. Column chromatography.

Separation of organic phosphorus fractions was realized by using a column of De-Acidite resin as the adsorbent and hydrochloric acid solutions as the eluting agents as outlined by Smith and Clark (68). De-Acidite is a weakly basic anion exchange resin of the aliphatic amine type (47). 24 cm. long columns of De-Acidite in the sulphate form placed in a 50 ml. burette were used for all chromatographic runs.

b. Removal of chloride from soil extracts.

In order to get samples of soil extracts free from chloride, which interfered with chromatographic separations, the following procedure was followed. The solution to be chromatographed (after treatment to remove organic matter) was passed through a 15 cm. long column of Dowex-50 in the hydrogen form. The cation-free eluate was then placed over solid sodium hydroxide in a vacuum desiccator (2)

and evaporated to dryness. The removal of hydrogen chloride was complete in one to two days. It was noted that this procedure caused a small amount of hydrolysis of organic phosphorus. The residue was then taken up in a suitable volume (usually 10 ml.) of 0.01 N sulphuric acid and the solution added to a column of De-Acidite anion exchange resin for chromatographic separation.

c. Elution procedures.

The concentration of the hydrochloric acid eluting solution was increased during each chromatographic run. This was accomplished by two different procedures.

1) Stepwise elution. In this procedure the hydrochloric acid eluting agents were increased in steps by using 500 ml. of 0.85 N, then 250 ml. of 1.30 N, and finally 250 ml. of 3.0 N. These were stronger solutions than were used by Smith and Clark (67) but they had the advantage of reducing the time required for a chromatographic separation (14).

2) Gradient elution. The procedure as outlined by Grande and Beukenkamp (32) was followed. Gradient elution was begun with 0.01 N hydrochloric acid in the reservoir. As the eluent entered the chromatographic column, 3.0 N hydrochloric acid entered the mixing flask in an equivalent amount because it was tightly stoppered to maintain 1 liter of solution within the mixing flask throughout the run. Because the passage of distilled water or dilute hydrochloric acid through De-Acidite causes it to swell so much that it practically stops the flow of eluent, gradient elution using more concentrated hydrochloric acid was employed. The 0.01 N hydrochloric acid solution was replaced with a 0.85 N solution, but the 3.0 N solution was retained in the

reservoir. The former type of gradient elution will be referred to as type A and the latter as type B. The strength of the eluent entering the column at any time was calculated according to the formula given by Grande and Beukenkamp. The calculated normality of the eluent at any time is given in Fig. 1 for both type A and type B gradient elutions. Samples of the gradient, type A, eluent were titrated to check the curve and agreement was good as can be seen from the plotted experimental values.

8. Method Used for Estimation of Organic Phosphorus in Elution Peaks.

Because the total phosphorus in many chromatographic fractions was very low, suitable aliquots were added directly to 20 m.m. x 150 m.m. test tubes which were used for the extraction step in the Martin and Doty procedure (44). 0.3 ml. of 72% perchloric acid was added and the tubes were inserted in a beaker of concentrated sulphuric acid on a hot plate. When white fumes of perchloric acid began to evolve the heating was stopped and the tubes were allowed to cool. Following neutralization to the p-nitrophenol end-point the samples were brought to a volume of 8 ml., 10 ml. of isobutanol-benzene solvent and 2 ml. of the molybdate reagent were added. The test tubes were shaken immediately for 20 seconds and when the two phases separated 5 ml. of the organic phase were added to a 25 ml. volumetric flask for color development. The percent transmission was read from an Evelyn colorimeter using a 660 millimicron filter. With a curve prepared from standard orthophosphate solutions which were treated similarly to the unknowns, estimates of the total phosphorus present were made.

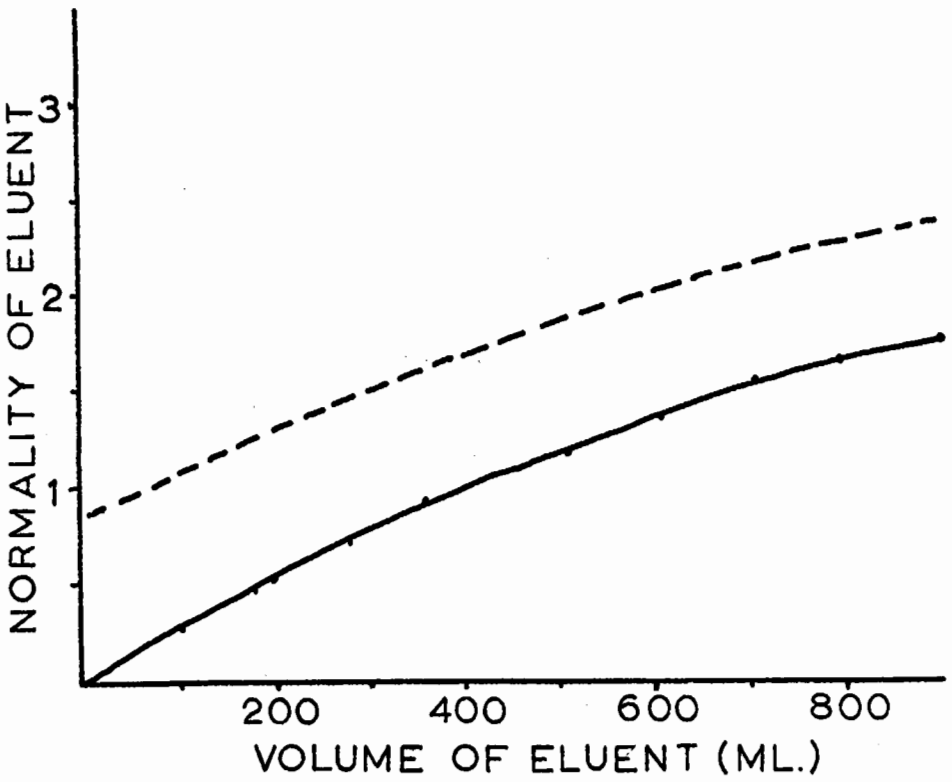


Fig. 1. Normality of the hydrochloric acid eluent.

Solid line - gradient elution, type A.

Broken line - gradient elution, type B.

The Dickman and Bray procedure (22) was used a great deal to estimate total phosphorus in samples which were digested with perchloric acid as outlined above. Total phosphorus values as estimated by the two colorimetric procedures following perchlorate digestion were in agreement.

9. Estimation of Inositol.

The microbiological assay as outlined by Norris and Darbre (48) was used for the estimation of inositol in chromatographic fractions of organic phosphorus separated from commercial phytic acid and soil extracts. A symmetrical (3 + 3) dose design as proposed by Wood (78) was used so that a statistical check on the assay could be performed.

In the initial runs Difco Bacto Malt extract and Difco Bacto yeast extract were used instead of the malt extract (Muntona Ltd.) and the yeast extract (Norman Evans & Rais Ltd.) recommended by the authors. Also a strain of Schizosaccharomyces pombe from the culture collection of the Bacteriology Department, Macdonald College, was used in addition to the strain used by Norris and Darbre. These two strains will be referred to as strain A and strain B respectively.

In the initial runs it was considered essential to check on the susceptibility of the two strains of the yeast organisms to salt (sodium chloride) since the Norris and Darbre hydrolysis procedure for total inositol, when applied to small dilute fractions from the chromatographic column, would result in much higher salt concentrations than Norris and Darbre encountered. Accordingly standard solutions of inositol (1 γ/ml.) were prepared in which the sodium chloride content varied from zero to 0.3 N. The results given in Table 2 show that

when strain A was used as the assay organism salt in low concentrations augmented the apparent inositol content, but above a concentration of 0.2 N the apparent inositol content was decreased. With strain B, however, increased concentrations of salt progressively decreased the apparent inositol content. Strain B had a greater growth response to inositol than strain A. High assay blanks were noted in these assays and according to Norris and Darbre the Difco Bacto yeast extract is responsible.

Table 2

Apparent inositol content of standard inositol solutions containing several levels of sodium chloride.

Sodium chloride concentration	Inositol found	
	Strain A	Strain B
0.0	1.00 γ/ml.	1.00 γ/ml.
0.02 N	1.05 "	0.94 "
0.05 N	1.00 "	0.82 "
0.10 N	1.07 "	0.81 "
0.15 N	1.03 "	0.71 "
0.20 N	0.99 "	0.71 "
0.25 N	0.99 "	0.60 "
0.30 N	0.93 "	0.44 "

In an effort to obtain smaller assay blanks which might improve the precision of the method, malt extract (Muntona, Ltd.) and yeast extract (Norman Evans & Rais Ltd.) were obtained. Assays were again run using both strains of the organism and the results are given in Table 3.

Table 3

Apparent inositol content of standard inositol solutions containing several levels of sodium chloride. Fiducial limits in brackets.

Sodium chloride concentration	Inositol found (γ/ml.)	
	Strain A	Strain B
0.0	1.00	1.00
0.1 N	0.90 (0.83, 0.98)	0.96 (0.91, 1.00)
0.2 N	0.93 (0.87, 0.99)	0.91 (0.88, 0.96)
0.3 N	0.87 (0.80, 0.94)	0.86 (0.81, 0.92)

Under the above experimental conditions when the recommended brands of malt extract and yeast extract were used, strain B was not more sensitive to salt than strain A. In addition, strain B showed a greater growth response to the inositol dose and the assay results obtained from it showed greater precision as shown by the narrower fiducial limits. It was therefore decided to use strain B as the assay organism for estimating total inositol in chromatographic fractions from commercial phytic acid and soil extracts. It was also found that the hydrochloric acid in the hydrolysates could be easily removed by evaporating the hydrolysates to dryness over solid sodium hydroxide in a vacuum desiccator (2). In this manner sodium chloride which inhibits the growth of the assay organism was eliminated. The inositol residue was taken up in the required amount of distilled water to give an estimated inositol concentration of 1 γ per ml. for subsequent assay.

The technical assistance and helpful advice of Dr. W. E. Vanstone in carrying out these microbiological assays are gratefully acknowledged.

EXPERIMENTAL RESULTS AND DISCUSSION

1. Results of Study of Methods for Estimation of Inorganic Phosphorus in the Presence of Fluoride and Boric Acid.

Hamilton (35) extracted soil with 0.9 N ammonium fluoride solution adjusted to pH 5.3 with hydrochloric acid. Inorganic phosphorus and total phosphorus (after appropriate digestion of the extract) were estimated by the Truog and Meyer method (74) and the Martin and Doty method (44). Boric acid as recommended by Kurtz (39) was included in both methods to prevent the interference of fluoride ions. He found that these two methods failed to give good agreement for the inorganic phosphorus contents of the extracts. The Martin and Doty method always gave higher values although the values for total phosphorus as estimated by both methods were very similar. Since it had been reported that the molybdate ion exerts a catalytic effect on the hydrolysis of organic phosphate bonds (42, 75), Hamilton suggested that the higher values for inorganic phosphorus by the Martin and Doty method might be due to the hydrolysis of labile organic phosphorus compounds, since the concentration of molybdate ion in that method is about ten times higher than in the Truog and Meyer method. Several experiments were performed by Hamilton to investigate the validity of the values obtained by the two methods, and although there were no conclusive data to indicate the superiority of either method for determining inorganic phosphorus, the method of Truog and Meyer was adopted. The choice seemed to be based only on the fact that hydrolysis of organic compounds would increase the inorganic phosphorus values, and since the methods did not agree for inorganic phosphorus but did for total phosphorus, suspicion was cast on the method giving the higher value which, in this case, was that of

Martin and Doty. There is no mention in his thesis that he considered the possibility of the continued interference of fluoride in the Truog and Meyer method after he modified it as Kurtz (39) did the Dickman and Bray method (22) to eliminate such interference.

Difficulties, apart from those associated with any particular method, were encountered in the present study when it was found that the standard curves dropped off considerably from a straight line relationship when phosphorus concentrations were plotted against absorbances. This was particularly pronounced when the phosphorus concentration exceeded 0.20 p.p.m. The stannous chloride that was being used at that time for preparing the reducing agent required in these colorimetric procedures was analysed to determine its stannous content (64) and it was found to be only 85% stannous. Such a poor quality of stannous chloride was shown by Smith et al. (69) to be unsuitable for the colorimetric determination of phosphorus. Stannous chloride from another source was analysed and found to be in the order of 95% stannous. This product, in contrast to the former, gave only a small amount of turbidity when dissolved in 10% (v/v) hydrochloric acid solution, and the standard curves obtained by the use of this reagent were good.

A limited number of ammonium fluoride soil extracts were prepared in the present study and inorganic and total phosphorus in the extracts were estimated by the Dickman and Bray method and the Truog and Meyer method. Both methods were modified by the use of boric acid to eliminate the interference of the fluoride ion. Calibration curves for both methods were prepared from a standard phosphorus solution containing potassium dihydrogen phosphate. Calibration curves were also prepared

for the same standard phosphate solution plus the highest amount of fluoride, with and without boric acid, which was being encountered in analysing the ammonium fluoride soil extracts. This amounted to 2 ml. of 0.9 N ammonium fluoride solution in 50 ml. final volume for color development. The fluoride caused serious interference in both methods when boric acid was omitted, but with boric acid the calibration curve for the Dickman and Bray method was, for all practical purposes, identical to the calibration curve obtained when fluoride was absent. In the Truog and Meyer method, however, boric acid only partially removed the fluoride interference and this is illustrated by the calibration curves in Figure 2. Although a straight line relationship between absorbance and concentration of phosphorus was found to exist, the values obtained, when 2 ml. of 0.9 N ammonium fluoride were present in the sample, were only 71% of the values when fluoride was absent. Half of the above amount of fluoride gave values about 74% of the fluoride free calibration curve. Therefore the Truog and Meyer procedure is not suitable for routine use on fluoride-containing extracts even when boric acid is employed.

The values obtained for the inorganic phosphorus content of the St. Bernard and Greensboro soils employing the extraction procedure of Hamilton (35) are given in Table 4. 4 grams of soil (without pretreatment) were extracted with 28 ml. of 0.9 N ammonium fluoride for one minute. 1 ml. and 2 ml. aliquots of these extracts were used for color development in 50 ml. volumetric flasks by both methods and the appropriate calibration curves were used to estimate the inorganic phosphorus.

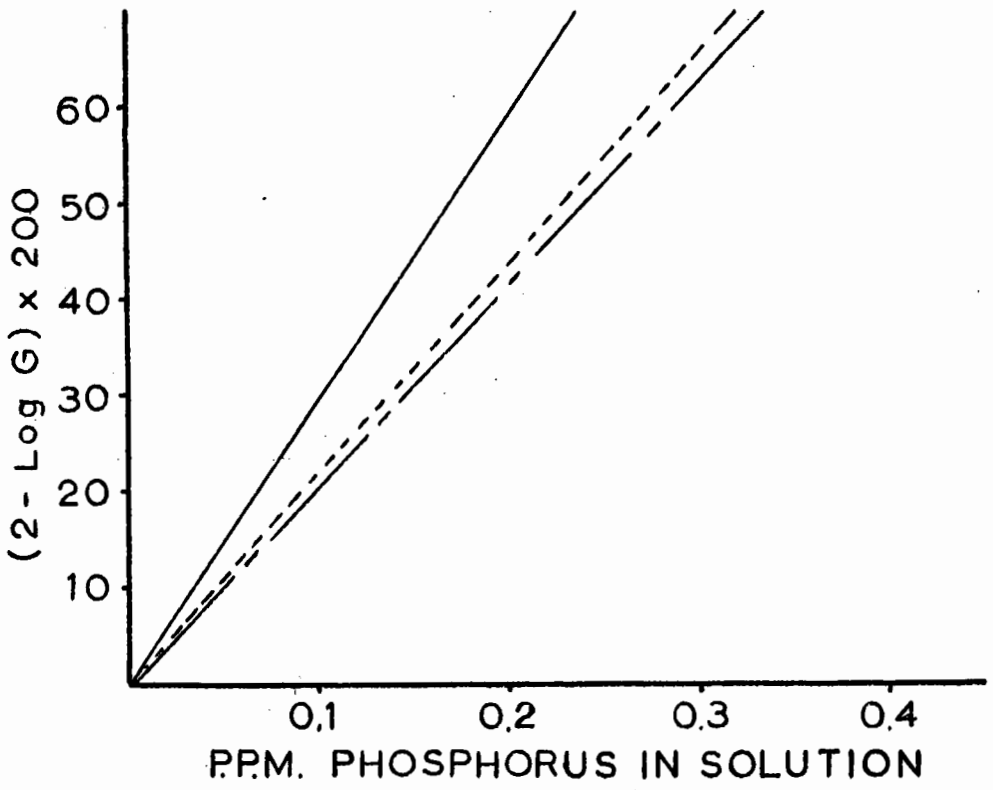


Fig. 2. Phosphorus calibration curve for Truog and Meyer method.

Solid line - no fluoride present.

Regular broken line - 1 ml. 0.9N NH_4F + boric acid.

Irregular broken line - 2 ml. 0.9N NH_4F + boric acid.

TABLE 4

Inorganic phosphorus removed from soil by 0.9 N ammonium fluoride

(P as p.p.m. air dry soil)

Soil	Colorimetric Method	
	Dickman and Bray	Truog and Meyer
	p.p.m.	p.p.m.
St. Bernard	62.5	64.0
Greensboro	69.6	71.0

Using the Truog and Meyer procedure, Hamilton reported the removal of 47 p.p.m. and 42 p.p.m. of inorganic phosphorus from the St. Bernard and Greensboro soils respectively by a similar extraction. If these values are only 71% of the inorganic phosphorus extracted by ammonium fluoride, the actual values would be 66 p.p.m. and 59 p.p.m. respectively. These values are in much better agreement with the values given in Table 4.

A further examination of data presented by Hamilton, in which he compared the inorganic phosphorus values of ammonium fluoride soil extracts by the Martin and Doty method and the Truog and Meyer method seems appropriate at this time. Data in the first two columns of Table 5 are reproduced from Hamilton's thesis. If 1 or 2 ml. of 0.9 N ammonium fluoride soil extract were used for the colorimetric determination, the Truog and Meyer values are only 71% to 74% of the actual inorganic phosphorus content of the extracts. The recalculated values in the third column are based on such a level of fluoride interference.

TABLE 5

Inorganic phosphorus removed from soil
by 0.9 N ammonium fluoride (Hamilton)

P as p.p.m. air dry soil

Extract	Martin and Doty	Truog and Meyer	Recalculated Truog and Meyer	
A	128.2	96.2	130	135
B	42.4	30.5	41.2	43.0
C	168.4	122.5	166	173
D	40.3	28.7	38.8	40.0

For extracts A and C which contained a high concentration of inorganic phosphorus, it is likely that smaller aliquots were used. Nevertheless considerable fluoride interference would still be present. The range of recalculated values for the Truog and Meyer method compare very well with Martin and Doty values and it is evident that the interference of fluoride was present but not recognized at that time.

2. Results of Study of Estimation of Total Phosphorus in Solution by the Martin and Doty Method in the Presence of Sulphate.

Since the Martin and Doty (44) procedure was used for estimating inorganic phosphorus in aqueous solutions of soil extracts and in fractions from chromatographic analyses, it was considered desirable to use it also for estimating the total phosphorus in these solutions after digestion to convert all the phosphorus to the orthophosphate form.

The digestion at that time was carried out by adding 3 ml. of a mixture in 2:1:1 proportions by volume of HNO_3:H_2SO_4:H_2O respectively

to an Erlenmeyer flask containing an aliquot of the solution to be analyzed for total phosphorus. The flask was heated on a hot plate until the solution was reduced to small volume, then 3 or 4 drops of 72% perchloric acid were added and heating continued until the white fumes disappeared. The digested sample was then either used in its entirety or brought to volume and a suitable aliquot used, depending on the amount of phosphorus present, for the estimation of total phosphorus. The digested sample or aliquot was neutralized with dilute ammonium hydroxide to the *p*-nitrophenol end-point before making the colorimetric phosphorus analysis.

It was soon found that the estimates of total phosphorus in extracts containing relatively small amounts of phosphorus were in error because they were often lower than the estimates of the inorganic phosphorus content. Although care was taken to see that no digestions were allowed to go to dryness, the possibility of phosphorus being volatilized was considered. Accordingly a standard orthophosphate solution was used to check the digestion procedure by varying the intensity of digestion as measured by the volume of sample remaining in the Erlenmeyer flask. The intensity of digestion varied from no heating to heating until the flasks were almost dry. It was found that the intensity of digestion was not critical in so far as volatilization was concerned as long as the flasks were not permitted to go to dryness. In every other case however, even where no heating was used, the estimates of phosphorus were appreciably low. This finding suggested that some component in the acids used for the digestion interfered with either the development of the blue color of the reduced phosphomolybdate complex or the extraction of the phosphomolybdate complex by the

isobutanol-benzene from the aqueous phase, and the following experiments were run to learn if there was any interference from single anions.

To 25 mm. x 200 mm. test tubes containing 25 γ of orthophosphate were added singly various amounts of sulphuric, nitric, or perchloric acid. The tubes were not heated but were neutralized with dilute ammonium hydroxide to the p-nitrophenol end-point and the volume was adjusted to 15 ml. Inorganic phosphorus was then determined by the Martin and Doty procedure. The concentrations of the anions in p.p.m. in the 20 ml. of aqueous phase (i.e. 15 ml. of sample and 5 ml. of sulphuric acid-molybdate reagent) are given in Table 6 together with the p.p.m. of phosphorus that were estimated colorimetrically to be in the sample. The 20 ml. aqueous phase of the Martin and Doty procedure that is solvent extracted, because of the addition of the molybdate sulphuric acid solution, contains 48,000 p.p.m. of sulphate ions. The data presented in Table 6 show that both sulphate and nitrate ions can cause serious interference. The critical level is in the vicinity of 70,000 p.p.m. for sulphate ions and 20,000 p.p.m. for nitrate ions, or in the latter case 70,000 p.p.m. for the sum of sulphate and nitrate ions. No critical level was found for perchlorate ions even when present in much larger amounts than are necessary to digest samples containing organic phosphorus. With the highest concentration of perchlorate ions a heavy precipitate occurred when neutralized with ammonium hydroxide which persisted during the extraction procedure, nevertheless complete recovery of the phosphorus was realized.

TABLE 6

Influence of anions in the estimation by the Martin and Doty procedure of the apparent phosphorus content of a standard phosphate solution.

Concentration of anions in the aqueous phase. p.p.m.	Ml. of conc. acid added to sample to give this concentration.	Phosphorus found. p.p.m.	% Recovery
48,000 SO_4	0	0.200	100.0
56,600 "	0.100	0.200	100.0
58,800 "	0.125	0.200	100.0
69,600 "	0.250	0.200	100.0
80,400 "	0.375	0.175	87.5
91,200 "	0. 50	0.150	75.0
177,600 "	1.50	0.012	6.0
307,200 "	3.00	0.000	0.0
48,000 $SO_4^=$ + 4,600 NO_3^-	0.10	0.200	100.0
48,000 " + 11,500 "	0.25	0.200	100.0
48,000 " + 23,000 "	0.50	0.197	98.5
48,000 " + 69,000 "	1.50	0.192	96.0
48,000 " + 1,380,000 "	3.00	0.167	83.5
48,000 " + 57,250 ClO_4^-	1.00	0.202	101
48,000 " + 114,500 "	2.00	0.200	100
48,000 " + 229,000 "	4.00	0.198	99

In the volumetric method for the estimation of phosphorus in fertilizers it is well established that when sulphate is present it may form a part of the ammonium phosphomolybdate precipitate and lead to high results (4). For this reason different conditions of precipitation for phosphatic materials containing sulphate are recommended. In order to learn whether the interference of sulphate in the Martin and Doty procedure was due to an incomplete extraction of the phosphomolybdate complex into the isobutanol-benzene phase or due to poor color development a further experiment was undertaken.

Two standard orthophosphate solutions were used which would give 0.200 p.p.m. and 0.300 p.p.m. phosphorus in the final colored solution. The Martin and Doty procedure was carried out in the normal manner except that the amount of sulphuric acid added to the ethyl alcohol was increased 2, 4, 8, and 16 times as shown in Table 7. The calculations for the column headed "Maximum amount of sulphate ions in final colored solution" are based on the unlikely assumption that all the sulphate in the aqueous phase is extracted into the isobutanol-benzene phase. On this basis the sulphate content in the final colored solution where only 87.5% of the phosphorus is recovered in Table 6 is 46,000 p.p.m.

The data in Table 7 show that sulphate can be increased four-fold in the ethanol reagent to give 140,000 p.p.m. sulphate ion in the final colored solution without any ill effects on the color development. It is therefore concluded that the reason for sulphate interference in the Martin and Doty procedure is that it prevents the complete transfer of the phosphomolybdate complex from the aqueous phase to the organic phase, and not that it interferes with the reduced phosphomolybdate complex color development.

TABLE 7

Influence of increased amounts of sulphuric acid in the ethanol reagent on the apparent phosphorus content of standard phosphate solutions.

Ml. conc. H_2SO_4 in 100 ml. H_2SO_4-ethanol mixture	Maximum amount of sulphate ions in final colored solution.	Phosphorus found	% Recovery	Phosphorus found	% Recovery
	p.p.m.	p.p.m.		p.p.m.	
2 ml. (normal reagent)	40,800	0.200	100.0	0.300	100.0
4 "	74,000	0.200	100.0	0.300	100.0
8 "	140,300	0.200	100.0	0.300	100.0
16 "	272,900	0.193	96.5	0.288	96.0
32 "	538,100	0.183	91.4	0.276	92.0

3. Soil Organic Phosphorus Extraction Experiments.

a. Development of a new method for the extraction of organic phosphorus from soil - Oxine extraction.

Most procedures for the extraction of soil organic phosphorus include a treatment with one or more reagents designed to remove all the organic phosphorus without changing any of it to the inorganic form. Usually the soil receives an acid pretreatment to remove cations, followed by an alkaline extraction with ammonium or sodium hydroxide to remove the greater part of the organic phosphorus. Such procedures are rather harsh and may hydrolyze and/or cause other chemical changes. Such changes are undesirable whether the extracts are being used to estimate the soil organic phosphorus content or to study the nature and

identity of the compounds extracted so that the need for milder extraction procedures is indicated.

The extraction capacities of mild neutral salts of organic and inorganic acids for the removal of soil organic matter have been reported (12, 13, 24, 36) and although less effective than caustic alkali, they are worthy of consideration because of the mild conditions of extraction. Oxine and other organic chelating agents have been used for the extraction of organic matter from the B horizon of soil (19, 43), and it was considered advisable to investigate the use of oxine for the extraction of organic phosphorus because of the mild conditions under which it can be used. A two-phase system oxine-benzene: aqueous soil suspension similar to that described by Coffin (19) was used. By the chelation of iron and aluminum cations of insoluble phosphorus compounds the phosphorus containing anions would be expected to be released to the aqueous phase.

5 gram samples of each of the Greensboro and St. Bernard soils which had been converted to the ammonium form were placed in flasks with 50 ml. distilled water and 20 ml. of 2.5% (w/v) solution of oxine in benzene. The flasks were shaken for 805 hours and the pH of the aqueous phase was maintained at 6.5 ± 0.1. 10 samples of the aqueous phase were collected periodically during the run, and distilled water was added to the flask to maintain the soil/water ratio. The oxine-benzene solution was also changed periodically to ensure an excess of oxine at all times. These prolonged extractions released 355 p.p.m. and 210 p.p.m. of total phosphorus from the Greensboro and St. Bernard soils respectively. This amounts to 63% and 15.3% respectively of the total phosphorus in these soils. Because of difficulties that were

being encountered at that time with turbidity in the Dickman and Bray (22) method for determining inorganic phosphorus, it was impossible to follow the extraction of organic phosphorus during this experiment. However at the time the extraction was concluded it was found, by using the Martin and Doty (44) procedure for inorganic phosphorus, that the organic phosphorus in the final extracts amounted to 67% and 52% of the total phosphorus in the extracts from the Greensboro and St. Bernard soils respectively.

The release of total phosphorus with time is shown in Fig. 3 and it will be noted that although the St. Bernard soil released more total phosphorus initially than the Greensboro soil, after about ten hours the release from the Greensboro soil was continuously superior to the release from the St. Bernard soil. By withdrawing 10 ml. samples from the flasks and replacing them with distilled water, the amount of phosphorus in solution would be steadily depleted if phosphorus were not released from the soil. Phosphorus was however released from both soils, although in different amounts, and the phosphorus content of the aqueous solutions, especially after the tenth analysis (65 hours of extraction), remained fairly constant for each soil as shown in Fig. 4. This observation suggested an equilibrium state for each soil-water system, therefore the effect on extraction of widening the soil/water ratio was investigated.

One gram of Greensboro soil was placed in each of three suitably sized flasks and different amounts of distilled water, namely 15 ml., 50 ml. and 100 ml., were added to each flask along with 20 ml. of oxine-benzene solution. The aqueous phase was adjusted and maintained at pH 6.5 for a 24 hour extraction period. The St. Bernard soil was

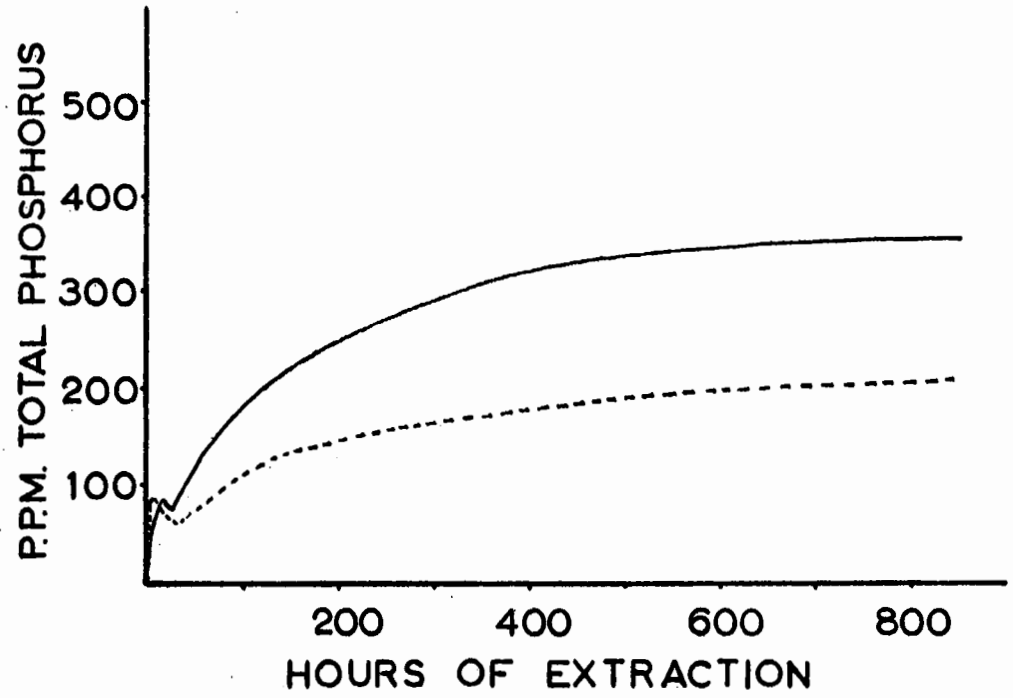


Fig. 3. Release of total phosphorus from soils during a prolonged oxine extraction at pH 6.5.

Solid line - Greensboro soil.

Broken line - St. Bernard soil.

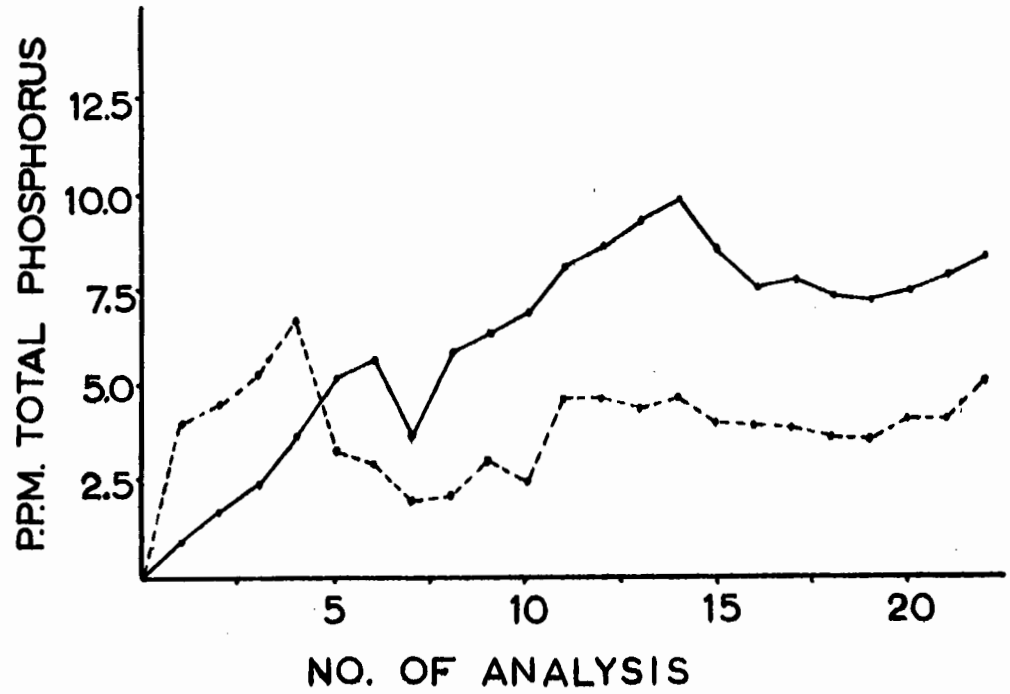


Fig. 4. Total phosphorus content of aqueous solutions during prolonged oxine extraction.

Solid line - Greensboro soil.

Broken line - St. Bernard soil.

treated similarly. The results of these experiments are given in Table 8.

It would appear that the organic phosphorus release is not influenced to any extent by varying the soil/water ratio although the St. Bernard shows a small increase from 6.9 to 9.2 percent as the ratio was changed from 1:15 to 1:100. However the release of inorganic phosphorus from both the Greensboro and St. Bernard soils was more than doubled and tripled respectively as the soil/water ratio was changed from 1:15 to 1:100.

The influence of pH level in the aqueous phase and of the presence of oxine on the release of organic phosphorus was investigated next. Five grams of the Greensboro soil were placed in each of four flasks. To each flask were added 50 ml. of distilled water, and the pH of the aqueous suspension was adjusted to 6.5 in two of the flasks and 9.2 in the two remaining flasks. 20 ml. of oxine-benzene solution were added to one of the flasks in each of the two pH series. The flasks were shaken for five hours and the pH checked periodically and if necessary adjusted to the required value. The St. Bernard soil was treated similarly. The results obtained are shown in Table 9.

Both soils released more organic phosphorus at the higher pH extraction. The Greensboro soil gave about a six-fold increase at pH 9.2 but the St. Bernard soil only a two-fold increase. Similarly, the use of oxine-benzene was responsible for an increased organic phosphorus release by both soils and again the Greensboro soil gave about a six-fold increase while the St. Bernard soil gave only a two-fold increase. It is apparent that there was a marked positive interaction between pH and oxine-benzene on the Greensboro soil in that the

TABLE 8

Phosphorus released by the Greensboro and St. Bernard soils at pH 6.5 at different soil/water ratios during a 24-hour extraction period.

Soil/Water Ratio	GREENSBORO SOIL					ST. BERNARD SOIL				
	Inorganic Phosphorus		Organic Phosphorus		Organic Phosphorus as % of Total P in Solution	Inorganic Phosphorus		Organic Phosphorus		Organic Phosphorus as % of Total P in Solution
	p.p.m.	%	p.p.m.	%		p.p.m.	%	p.p.m.	%	
1:15	15	7.1	96	27	86	52	6.2	36	6.9	41
1:50	29	13.7	88	25	75	130	15.5	44	8.4	25
1:100	35	16.6	93	26	73	178	21.2	48	9.2	21

TABLE 9

Phosphorus released from the Greensboro and St. Bernard soils at pH 6.5 and 9.2 with and without oxine-benzene during a 5 hour extraction period.

pH	SOIL	WATER ONLY					WATER + OXINE-BENZENE				
		Inorganic phosphorus		Organic phosphorus		% Organic P in solution	Inorganic phosphorus		Organic phosphorus		% Organic P in solution
		p.p.m.	%	p.p.m.	%		p.p.m.	%	p.p.m.	%	
6.5	GREENSBORO	0.6	0.3	4.5	1.3	88	4.4	2.1	24.0	6.8	85
	ST. BERNARD	28.0	3.3	19.0	3.6	40	45.0	5.4	26.0	5.0	37
9.2	GREENSBORO	3.4	1.6	27.0	7.6	89	12.0	5.7	166.0	47.0	93
	ST. BERNARD	24.0	2.9	33.0	6.3	58	37.0	4.4	77.0	14.8	68

release of organic phosphorus was about thirty-seven times greater at pH 9.2 with oxine-benzene than it was at pH 6.5 with a water extraction only. The St. Bernard soil showed a similar trend but the increased release of organic phosphorus at the higher pH with oxine-benzene was not nearly as pronounced as in the Greensboro soil.

For the Greensboro soil its inorganic phosphorus release showed a similar pattern to its organic phosphorus release in that both increasing the pH and the use of oxine increased the inorganic phosphorus release. For the St. Bernard soil, however, the inorganic phosphorus release showed a different pattern than the organic phosphorus release. Inorganic phosphorus release was increased by the use of oxine but it was decreased by increasing the pH. This fact suggests that the high calcium content of the St. Bernard soil plays a major role in the release of inorganic phosphorus, inorganic calcium phosphates being more insoluble at pH 9.2 than at pH 6.5 (38).

The oxine extractions at pH 9.2 looked rather promising for removing a high percentage of the organic phosphorus from the Greensboro soil, and it was decided to subject a sample of this soil to successive 24-hour oxine extractions. For this purpose 2.5 gm. of soil, 62.5 ml. of distilled water and 20 ml. of oxine-benzene were added to a 125 ml. Erlenmeyer flask. Four such extractions were performed simultaneously for a total of six 24-hour periods. At the end of each 24-hour period the contents of the Erlenmeyer flasks were placed in separatory funnels where the soil and water were separated from the benzene phase. Following centrifugation of the aqueous phase the soil was returned to the Erlenmeyer flask which contained distilled water and newly prepared oxine-benzene solution. The aqueous and benzene extracts were then

TABLE 10

Phosphorus, iron and aluminum released from the Greensboro soil during six 24-hour extractions with oxine at pH 9.2.

EXTRACT NO.	AQUEOUS PHASE					BENZENE PHASE		
	Inorganic Phosphorus		Organic Phosphorus		% Organic Phosphorus in solution	Total Phosphorus	Iron	Aluminum
	p.p.m.	%	p.p.m.	%		p.p.m.	p.p.m.	p.p.m.
1	61.8	29.3	217.2	61.5	78	3.0	2026	1692
2	14.8	7.0	41.2	11.7	74	7.6	1828	780
3	10.6	5.0	10.7	3.0	50	6.2	1302	493
4	6.1	2.9	4.5	1.3	42	2.1	425	197
5	3.5	1.7	3.3	0.9	49	0.9	190	76
6	3.2	1.5	1.4	0.4	30	1.8	310	99
Total	100.0	47.4	278.3	78.8		21.6	6081	3337

analysed and the data are shown in Table 10.

By these extractions a total of 78.8 percent of the total organic phosphorus estimated to be present in the soil was released. It is also interesting to note that 78 percent of the organic phosphorus released was found in the first 24-hour extraction. After the first extraction the amount of both inorganic and organic phosphorus in the aqueous phase dropped rapidly. In the benzene phase total phosphorus only was determined and the amount present was highest in the second and third extractions. It is possible that a large part of this total phosphorus in the benzene phase is organic in nature since Coffin (19) reported as much as two thirds of the organic matter released from the B horizon of a soil by such an extraction to be preferentially soluble in the benzene phase. However in the case of the Greensboro soil, if all the phosphorus in the benzene phase were organic it would amount to only 7.8 percent of the organic phosphorus released to the aqueous phase.

The iron and aluminum released by this soil decreased progressively until the sixth extraction when both constituents increased to an amount somewhat higher than in the fifth extraction. The release of aluminum decreased more rapidly with the number of extractions than did the release of iron.

In an attempt to increase the release of organic phosphorus, particularly from the St. Bernard soil, the 'normal' soils were pre-treated with 0.5 N hydrochloric acid for varying lengths of time on a rotary shaker, washed with distilled water to remove chloride, and then extracted with oxine-benzene solution at pH 9.2 for 18 hours. The phosphorus released by each soil is given in Table 11.

TABLE 11

Phosphorus released from the Greensboro and St. Bernard Soils by a 0.5 N hydrochloric acid pretreatment and by a subsequent 18 -hour oxine extraction.

SOIL	ACID PRETREATMENTS					OXINE EXTRACTION				BOTH EXTRACTIONS			
	Duration of Acid Pretreatment	Inorganic Phosphorus		Organic Phosphorus		Inorganic Phosphorus		Organic Phosphorus		Inorganic Phosphorus		Organic Phosphorus	
		p.p.m.	%	p.p.m.	%	p.p.m.	%	p.p.m.	%	p.p.m.	%	p.p.m.	%
GREENSBORO	0 min.	- -	--	--	--	40.1	19.0	190.2	53.9	40.1	19.0	190.2	53.9
	15 "	24.8	11.8	29.6	8.4	45.6	21.6	207.9	58.9	70.4	33.4	237.5	67.3
	30 "	24.8	11.8	24.9	7.1	46.6	22.0	209.8	59.4	71.4	33.8	234.7	66.5
	60 "	26.8	12.7	21.3	6.0	45.6	21.6	218.6	61.9	72.4	34.3	239.9	67.9
	120 "	33.1	15.7	18.2	5.2	49.4	23.4	245.1	69.4	82.5	39.1	263.3	74.6
ST.BERNARD	0 Min.	--	--	--	--	29.6	3.5	33.0	6.3	29.6	3.5	33.0	6.3
	15 "	406.1	48.3	45.1	8.6	123.0	14.6	354.0	67.8	529.1	62.9	399.1	76.4
	30 "	413.6	49.2	37.6	7.2	133.8	15.9	356.7	68.3	547.4	65.1	394.3	75.5
	60 "	421.0	50.0	22.0	4.2	147.9	17.6	381.6	73.1	568.9	67.6	403.6	77.3
	120 "	413.6	49.2	13.2	2.5	155.1	18.4	437.4	83.8	568.7	67.6	450.6	86.3

The inorganic phosphorus released by the Greensboro soil to the acid pretreatment increased slightly with time, but no apparent effect was carried over to the subsequent oxine extraction where practically the same amount of inorganic phosphorus was extracted with or without acid pretreatment. A notable contrast was found for the St. Bernard soil, however, where as a result of acid pretreatment four to five times as much inorganic phosphorus was released to the oxine extraction.

The percentages of total organic phosphorus released by acid pretreatment for varying lengths of time, and by a subsequent 18-hour oxine extraction of the Greensboro soil are shown graphically in Fig. 5. This figure shows that the amount of organic phosphorus extracted from the Greensboro soil by the acid pretreatment decreased with time of contact and that the subsequent release of organic phosphorus in the oxine extraction increased with increased time of acid pretreatment. The increase in organic phosphorus released following a 2-hour acid pretreatment amounts to about 15 percent of the total organic phosphorus in the soil. The percentages of total organic phosphorus released by acid pretreatment for varying lengths of time, and by a subsequent 18-hour oxine extraction of the St. Bernard soil are shown graphically in Fig. 6. This figure shows that the response of the St. Bernard soil to the acid pretreatment was very similar to that of the Greensboro soil in that the organic phosphorus decreased with the time of acid pretreatment. However, in the case of the St. Bernard soil, the release of organic phosphorus to the oxine extraction following acid pretreatment was very pronounced. Thus, the 15 minute acid pretreated soil released over ten times as much organic phosphorus as soil which received no acid pretreatment. The increase in organic phosphorus released following

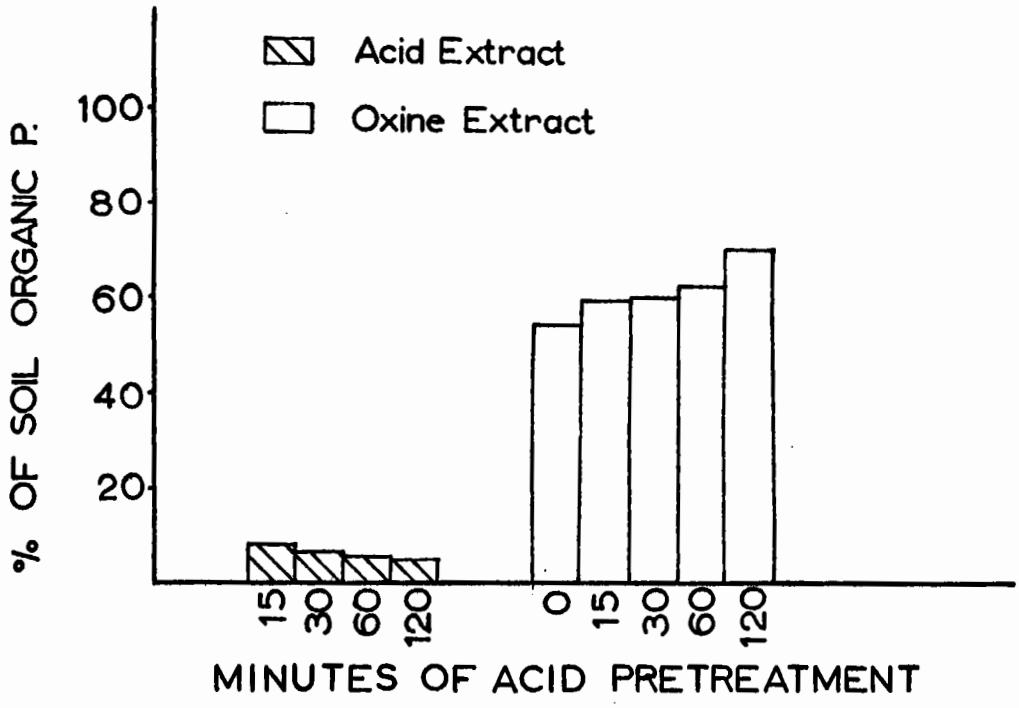


Fig. 5. Effect of pretreatment with acid on release of organic phosphorus from Greensboro soil.

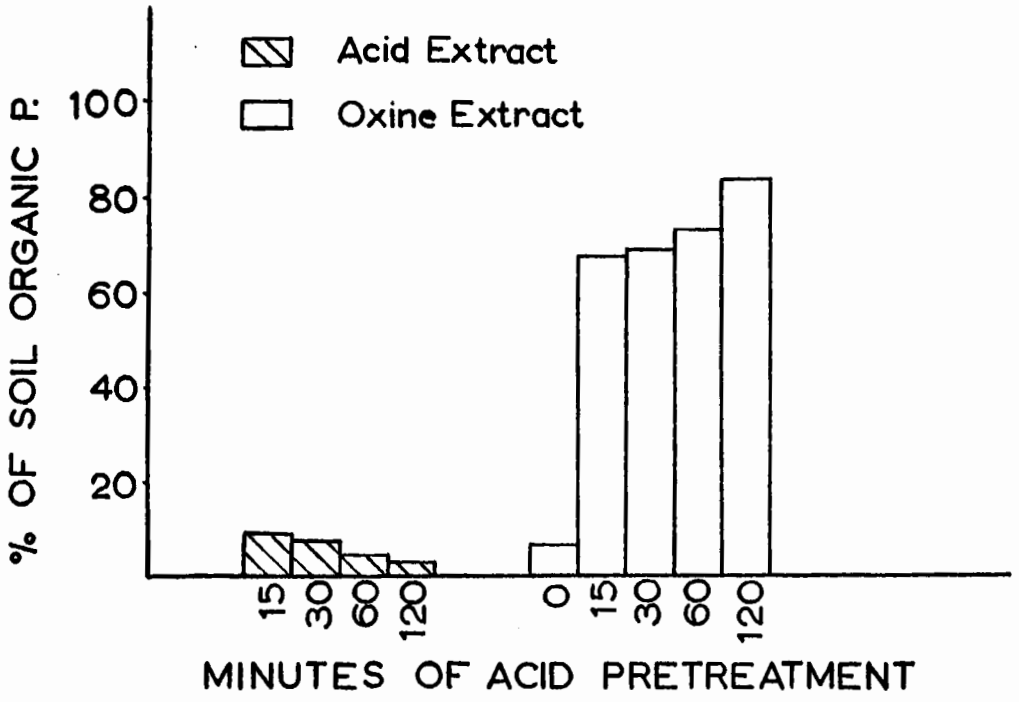


Fig. 6. Effect of pretreatment with acid on release of organic phosphorus from St. Bernard soil.

a 2-hour acid pretreatment amounts to about 76 percent of the total soil organic phosphorus. The total amount of organic phosphorus released by both the acid and oxine extractions amounted to 75 percent and 86 percent of the total organic phosphorus estimated to be present in the Greensboro and St. Bernard soils, respectively.

b. Extraction of soil organic phosphorus with sodium hydroxide.

Both the Greensboro and St. Bernard soils were extracted with sodium hydroxide in order to obtain a large amount of organic phosphorus by an alkaline extraction for hypobromite oxidation and subsequent analysis for phytic acid.

250 grams of each 'normal' soil were leached with 2 liters of 0.5 N hydrochloric acid and 2 liters of distilled water to remove chlorides. The soil was then heated on a steam bath for four hours with 1.0 N sodium hydroxide. The soil and sodium hydroxide extract were separated by centrifugation and the soil residue was washed with 1 liter of distilled water to remove soluble phosphorus compounds. The results of these extractions are given in Table 12.

The removal of 71 percent and 89 percent of the total organic phosphorus in the Greensboro and St. Bernard soils respectively by the combined acid-alkali procedure compares very closely with the values obtained by oxine extraction at pH 9.2 following an acid pretreatment. However the higher values obtained for the percent inorganic phosphorus removed by the alkali extraction suggests that hydrolysis of organic phosphorus compounds has resulted. This is to be expected in view of the results of recent studies on the hydrolysis of soil organic phosphorus compounds by alkaline extractants (45, 60).

TABLE 12

Phosphorus released by the Greensboro and St. Bernard soils by acidic and alkaline extractions.

Extraction agents	GREENSBORO SOIL					ST. BERNARD SOIL				
	Inorganic phosphorus		Organic phosphorus		% Organic in solution	Inorganic phosphorus		Organic phosphorus		% Organic in solution
	p.p.m.	%	p.p.m.	%		p.p.m.	%	p.p.m.	%	
0.5 N HCl	42.7	20.3	79.7	22.6	65.1	506.8	60.3	6.0	1.1	1.2
DISTILLED WATER	0.7	0.3	17.6	5.0	96.2	14.8	1.7	3.6	0.7	19.6
1.0 N NaOH	117.6	55.7	141.8	40.2	54.7	247.2	29.4	409.6	78.5	62.4
DISTILLED WATER	8.3	3.9	11.7	3.3	58.5	32.0	3.8	45.6	8.7	58.8
Total	169.3	80.2	250.8	71.1		800.8	95.2	464.8	89.0	

The amounts of organic phosphorus extracted from the two soils by the oxine procedure, the sodium hydroxide procedure, and the ammonium fluoride procedure are given in Table 13. For the latter procedure values obtained by Hamilton (35) are also included.

TABLE 13

Soil organic phosphorus extracted by different methods

EXTRACTANT	GREENSBORO SOIL		ST. BERNARD SOIL	
	Organic Phosphorus p.p.m.	% of Total Organic Phosphorus	Organic Phosphorus p.p.m.	% of Total Organic Phosphorus
Oxine (See Page 49)	263.3	74.6	450.6	86.3
NaOH (See Page 53)	250.8	71.1	464.8	89.0
NH_4F (BOSWALL)	183	51.9	150	28.8
(HAMILTON)	163	46.2	139	26.6

The proportion of the total organic phosphorus released into the aqueous phase by oxine-benzene extraction at pH 9.2 following a mild acid pretreatment approaches that obtained by older more drastic extraction procedures. The oxine-benzene extracts have the further advantage of a very low content of cations, and no extraneous anions are introduced which might interfere with subsequent attempts to identify the compounds extracted. Hydrolysis of soil organic phosphorus compounds such as inositol phosphates might occur to a small extent even under the mild (pH 9.2) conditions of the oxine extraction procedure. Desjobert et al. (21) found that hydrolysis of inositol phosphates falls to zero in weakly alkaline solutions, but that the pH above which they are stable depends on the number of phosphate groups present, ranging from 7.5 for the monophosphate to more than 11 for the hexaphosphate.

4. Fractionation of Inositol Phosphates and of Soil Organic Phosphorus

The organic phosphorus extracted from soils has been shown to be chiefly inositol phosphate and nucleic-acid-like materials (3, 8, 9, 26, 80, 82, 83). Improvements in the techniques of identifying extracted compounds have enabled workers to show that the amounts of these types of compounds present in soils are in reality much less than was originally believed. Adams et al. (1) using chromatographic techniques were unable to show any appreciable amounts of nucleic acids in extracts of two soils containing 327 p.p.m. and 575 p.p.m. of organic phosphorus. Smith and Clark (67), using a similar technique, showed that two thirds or more of the organic phosphorus containing material from soil that heretofore has been considered as phytin, is admixed material that chemically behaves as phytin but that chromatographically is not inositol hexaphosphate. Nevertheless the inositol hexaphosphate content of the organic phosphorus extracted from the soils they investigated ranged from 7% to 34%. Quebec soils have been shown to contain inositol phosphate by the isolation of a compound that contained phosphorus and iron in the same ratio as did ferric phytate similarly prepared (26, 80). It was therefore considered desirable to use the newer chromatographic techniques to separate any inositol phosphates that might be present in the oxine-benzene extracts of the Greensboro and St. Bernard soils.

Two chromatographic methods for studying soil inositol phosphates which were considered for use in this study were those of Smith and Clark (67, 68) and Anderson (3). The latter employs paper partition chromatography and although it offers a method which should be rapid and convenient, it has the obvious disadvantage of yielding only minute

quantities of separated compounds. The anion exchange column chromatographic method of Smith and Clark, on the other hand, offers the possibility of obtaining the larger amounts of separated compounds which are necessary for identification purposes, such as determining the inositol to phosphate ratio. This method has the disadvantage of being extremely slow, and with the use of only one automatic fraction collector it took up to five days to run a single sample. However the method was found to give reproducible results and, with some modifications, it was accepted for use in the present study.

a. Fractionation of 'phytic acid' by stepwise elution and gradient elution.

Phytic acid (Nutritional Biochemicals Ltd.), also known as inositol hexaphosphate, was obtained for use as a reference for the anion exchange chromatography of inositol phosphates. Although phytic acid is reported in the literature to be an amorphous powder (34), the material used in this case was a rather viscous or syrupy solution. A working solution of phytic acid was prepared by diluting 1 ml. of phytic acid to 100 ml. with distilled water. This solution was found to contain 260 p.p.m. inorganic orthophosphate and 1140 p.p.m. organic phosphorus.

5 ml. of 1% phytic acid solution were applied to the top of the De-Acidite column with a pipette and stepwise elution was effected by beginning with 0.85 N hydrochloric acid. After 500 ml. passed into the column the 1.4 N acid was used. 10 ml. fractions of the eluate were collected with the aid of a Shandon automatic fraction collector and these were analysed for inorganic and total phosphorus. The results are shown in Fig. 7. It is apparent that the phytic acid solution

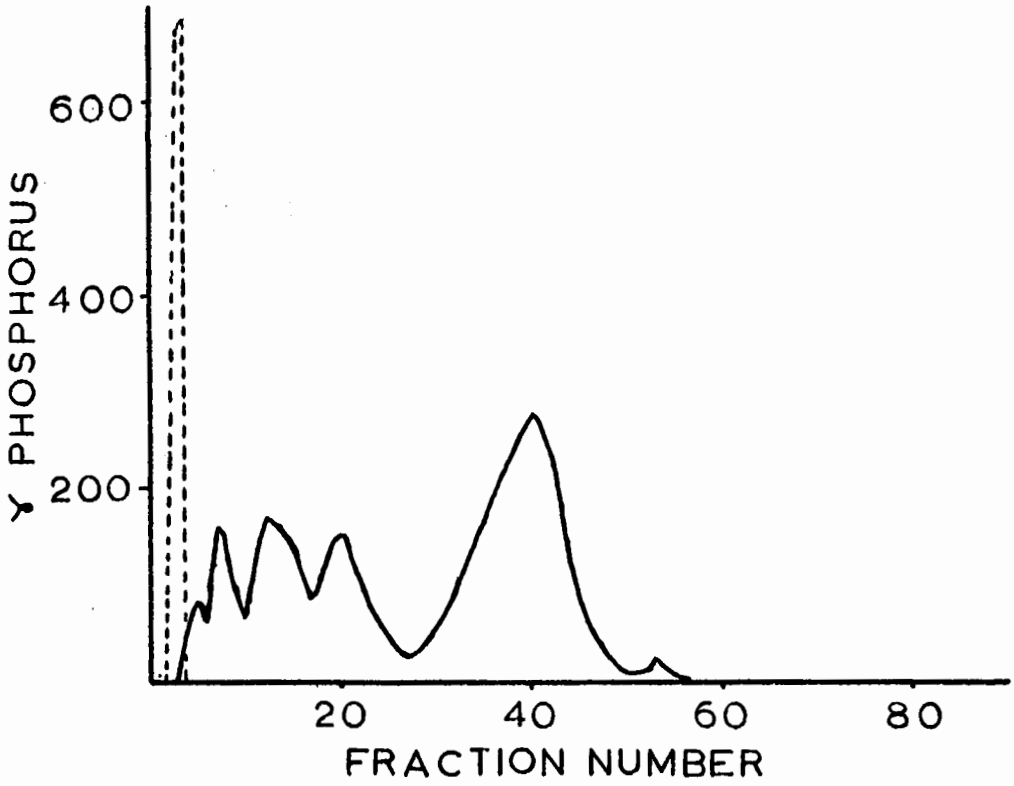


Fig. 7. Elution curve of 5 ml. 1% phytic acid solution (stepwise)

Solid line - organic phosphorus.

Broken line - inorganic phosphorus.

contained organic phosphorus in addition to inositol hexaphosphate. This appears as several poorly resolved peaks which leave the column previous to the largest peak which was considered at the time and later confirmed by its inositol/phosphate ratio to be inositol hexaphosphate. It should be noted too, that fraction 52 which was the first eluate from the 1.4 N hydrochloric acid, gives what might be termed a spurious peak. This was particularly noticeable when soil extracts were chromatographed by stepwise increasing the strength of the eluent.

In the attempt to obtain a better separation of the organic phosphorus compounds and to reduce or eliminate the suspected spurious peaks it was considered advisable to begin elution with a more dilute solution of hydrochloric acid and to increase its strength gradually throughout the run by employing the technique of gradient elution (32). Accordingly 1 ml. of 1% phytic acid solution was added to the De-Acidite column, and gradient elution, type A, as outlined in the section on Experimental Methods, was employed throughout the run.

The separation of the various compounds into distinct peaks by gradient elution is illustrated in Fig. 8. The resolution of the organic phosphorus peaks is very clear and six separate organic phosphorus peaks are evident. Aliquots of 1 ml. each of the 1% phytic acid solution were separated on the column by the above procedure on three different occasions and although comparable amounts of organic phosphorus did not occur in the same eluate fractions in each run (compare Fig. 8 and Fig. 9), the percentage of the total organic phosphorus that occurred in comparable peaks was essentially the same. Slight changes in the mesh of the resin, the length of the resin column, and strength of the

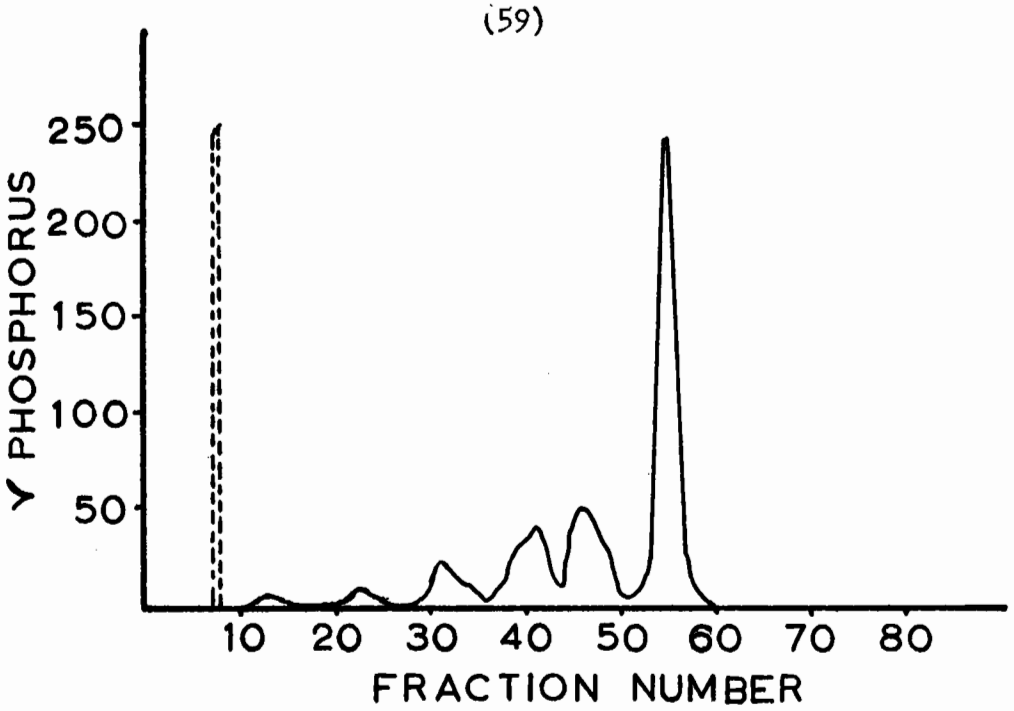


Fig. 8. Elution curve of 1 ml. 1% phytic acid solution (gradient, type A).

Solid line - organic phosphorus.

Broken line - inorganic phosphorus.

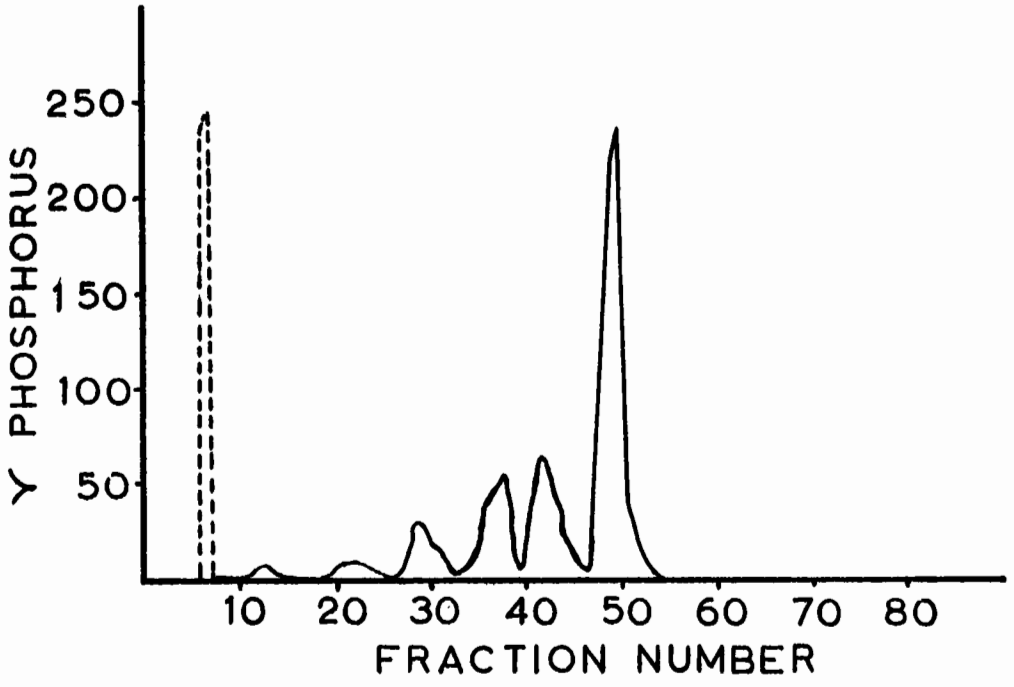


Fig. 9. Elution curve of 1 ml. 1% phytic acid solution (gradient, type A).

Solid line - organic phosphorus.

Broken line - inorganic phosphorus.

eluting solutions can account for an organic phosphorus peak occurring one or two fractions early or later in any particular run. It should also be noted that the organic phosphorus curves obtained by gradient elution contained no spurious peaks such as were found at fractions 52 and 53 in Fig. 7 which were apparently due to the sudden increase in the strength of the eluting solution. Examination of the elution curves of Smith and Clark (67) suggests that spurious peaks were present.

De-Acidite resin in the sulphate form swells considerably if distilled water or dilute hydrochloric acid is passed through it. This is an unfortunate characteristic because the resin (60 - 80 mesh) swells to such an extent when using 0.01 N hydrochloric acid in gradient elution, that the flow of solution through the resin is almost completely stopped. This was found to be particularly serious when soil extracts were added to the De-Acidite column. The initial flow was good but by the time the 5th fraction was collected the rate of flow became extremely slow requiring several days to collect the next 25 fractions. When the strength of eluting solution approached 0.5 N the rate of flow gradually increased and at about the 35th fraction the stopcock was partially closed to maintain the rate of flow at 20 to 30 ml. per hour. Because of this difficulty it was decided to begin gradient elution with 0.85 N hydrochloric acid (gradient elution, type B) instead of 0.01 N hydrochloric acid (gradient elution, type A). This procedure was used to chromatograph 0.25 ml. of 1% phytic acid solution which would provide approximately the same amount of inositol hexaphosphate as did dilute soil extracts that were being studied at that time. The results are shown in Fig. 10. This procedure does not separate the first five compounds into separate peaks as does type A gradient elution.

However at this point in the study of organic phosphorus in soil extracts, emphasis was being placed on the separation of inositol hexaphosphate and a distinct organic phosphate peak which left the column after inositol hexaphosphate. For this reason it was considered advisable to use type B gradient elution in order to obtain a satisfactory rate of flow of solution through the column.

In order to determine the inositol/phosphate ratio of the compounds occurring in the organic phosphate peaks from the chromatographic separations of the phytic acid solution, it was necessary to obtain a larger quantity of the first three or four peaks. To obtain these fractions it was considered that it would be more fruitful to hydrolyze partially the phytic acid solution rather than make several chromatographic separations and combine comparable fractions. Accordingly 3.0 ml. of 1% phytic acid solution and 3 ml. of 2 N hydrochloric acid were placed in a sealed test tube and heated at 123° C for two hours. Analysis of the orthophosphate content of the partially hydrolyzed sample showed that approximately 50% of the organic phosphorus was hydrolyzed. The sample was then chromatographed using gradient elution, type A. The results are shown in Fig. 11. It was gratifying to find that the six peaks found in Fig. 8 again were present in appreciable amounts. This procedure gave good yields of the first three peaks but the 4th and 5th peaks were present in smaller amounts than in the previously chromatographed unhydrolyzed samples. Since the inositol hexaphosphate peak was almost completely eliminated and the first three peaks have been enhanced, the first peak by a factor of 10 or more, it is to be assumed that the first five organic phosphate peaks are esters of a lower degree of phosphorylation than inositol hexaphosphate.

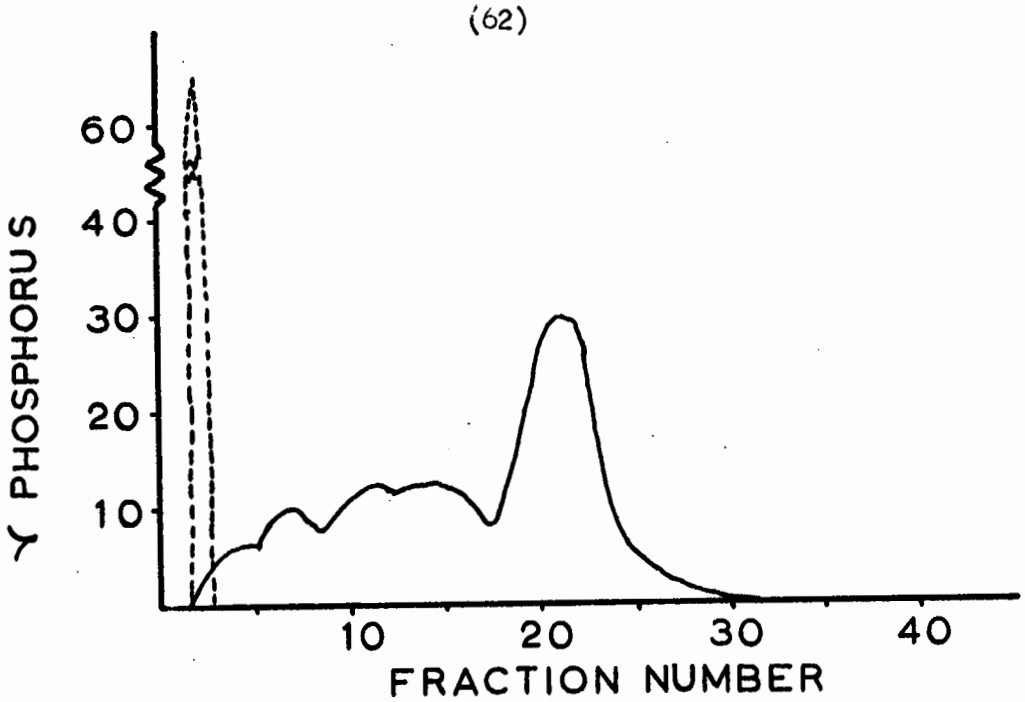


Fig. 10. Elution curve of 0.25 ml. 1% phytic acid solution (gradient, type B).

Solid line - organic phosphorus.

Broken line - inorganic phosphorus.

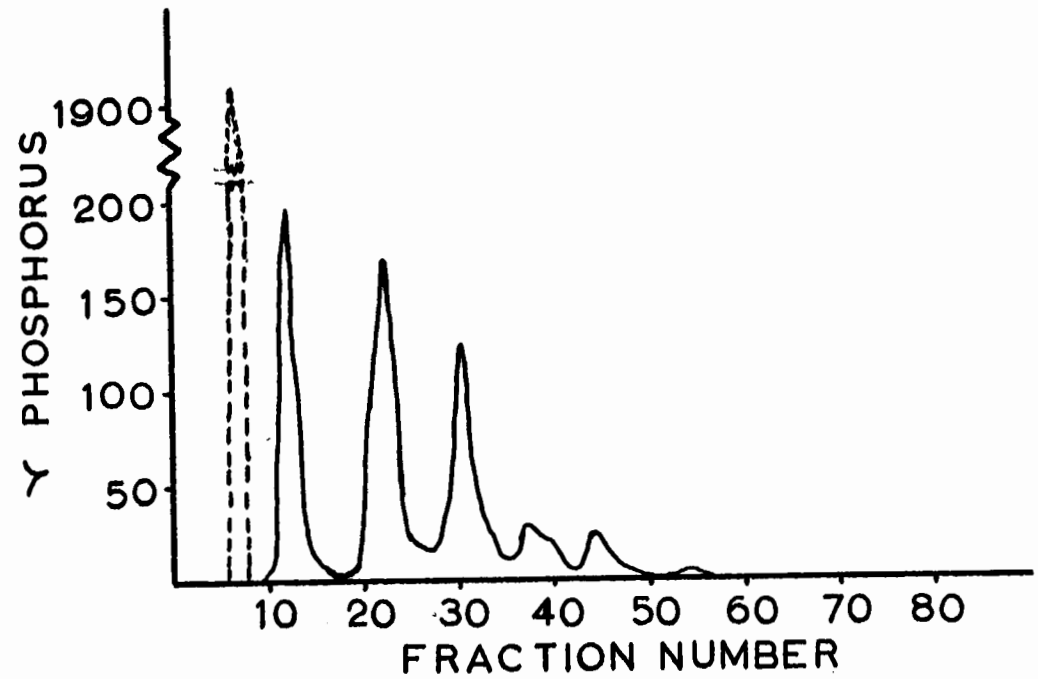


Fig. 11. Elution curve of 3 ml. 1% phytic acid solution, partially hydrolyzed (gradient, type A).

Solid line - organic phosphorus.

Broken line - inorganic phosphorus.

The distribution of the total organic phosphorus among the various peaks and interpeak regions is given in Table 14. In the case of poor resolution such as is found in Figs. 7 and 10 it is obvious that only an approximation of the phosphorus belonging to any one peak can be obtained. However the approximations agree quite well with the values for phosphorus in peaks which are well defined at the base. The quantity of organic phosphorus applied to the resin has some influence on the separation of the organic phosphorus peaks, but the concentration of the hydrochloric acid eluting solution has the greatest influence, as can be seen from Figs 7 and 10 where the concentration of the eluting solutions was greater than that used for Figs. 8, 9, and 11. The quantity of organic phosphorus subjected to chromatographic separation ranged from 290 γ to 5380 γ in the various runs. The fact that the phosphorus present in the inositol hexaphosphate peak ranges from 53.4% to 56.4% of the total organic phosphorus in the sample, indicated that the chromatographic separation of this peak was satisfactorily reproducible and the method was suitable for separating similar compounds from soil extracts.

b. Fractionation of organic phosphorus from soil by application of an oxine extract to the resin.

(1) 'Raw' oxine extract.

Throughout this investigation emphasis was placed on the use of reagents which would provide mild conditions for the extraction of soil organic phosphorus compounds and their subsequent separation. Since the mild conditions of the oxine-benzene extraction were effective in removing substantial amounts of organic phosphorus from the Greensboro and St. Bernard soils, it was desirable that separation of the extracted

TABLE 14

Organic phosphorus found in each peak obtained from the anion exchange chromatography of a 1% phytic acid solution.

SAMPLE	5 ML. 1% PHYTIC ACID		1 ML. 1% PHYTIC ACID		1 ML. 1% PHYTIC ACID	
Eluting Agent Hydrochloric Acid	500 Ml. 0.85 N, 250 Ml. 1.4 N. 250 Ml. 3.0N		0.01 N and 3.0 N Gradient Elution		0.01 N and 3.0 N Gradient Elution	
Peaks	γ Organic P*	%	γ Organic P*	%	γ Organic P*	%
1	)		10.40	0.9	7.53	0.7
	) 176.2	3.3				
2	)		29.82	2.7	27.89	2.6
3	469.0	8.7	87.21	7.8	90.66	8.4
4	837.0	15.6	154.80	13.8	156.03	14.4
5	915.5	17.0	188.88	16.8	182.62	16.9
6	2981.1	55.4	633.06	56.4	584.49	54.1
Interpeak	———	———	17.89	1.6	32.05	2.9
Total	5378.8	100.0	1122.06	100.0	1081.27	100.0
Ortho-P	1264.3		258.38		260.25	
Ortho-P as % of Total P	19.0		18.7		19.4	

(continued on next page)

* Not more than 3 digits significant.

TABLE 14 (continued)

Organic phosphorus found in each peak obtained from the anion exchange chromatography of a 1% phytic acid solution.

SAMPLE	1 ML. 1% PHYTIC ACID		0.25 ML. 1% PHYTIC ACID		3 ML. 1% PHYTIC ACID (PARTIALLY HYDROLYSED)	
Eluting Agent Hydrochloric Acid	0.01 N and 3.0 N Gradient Elution		0.85 N and 3.0 N Gradient Elution		0.01 N and 3.0 N Gradient Elution	
Peaks	γ Organic P*	%	γ Organic P*	%	γ Organic P*	%
1	9.32	0.8	)		383.7	27.4
2	28.75	2.6	) 41.49	14.4	481.0	34.4
3	95.96	8.7	)		313.9	22.4
4	162.65	14.7	36.79	12.7	110.9	7.9
5	197.95	17.9	55.41	19.2	56.6	4.1
6	588.31	53.4	155.22	53.7	11.0	0.8
Interpeak	20.96	1.9	—		41.3	3.0
Total	1103.90	100.0	288.91	100.0	1398.4	100.0
Ortho-P	266.98		70.74		1978.8	
Ortho-P as % of Total P	19.5		19.5		58.6	

* Not more than 3 digits significant.

compounds be effected without resorting to harsh methods such as the alkaline hypobromite oxidation procedure used by previous workers (8, 67, 80) for preparing the 'phytic fractions'. Consequently several attempts were made to fractionate the organic phosphorus of "raw" oxine extracts of soil on De-Acidite anion exchange columns.

These attempts were not successful because as soon as the soil extract was applied to the resin, it was impossible to maintain a flow of eluting solution by either the stepwise or gradient elution procedures. This was apparently due to two factors. 1) A large amount of the organic matter in the extract appeared to precipitate within the resin column, and 2) the extract caused the resin to swell considerably. On one occasion a strong hydrochloric acid eluting solution was successfully passed through the column but no fractionation of the organic phosphorus resulted. It is therefore concluded that application to the soil extracts obtained from these two soils by the oxine method, of the chromatographic procedure (gradient elution, type A) previously found to provide relatively clear-cut separation of organic phosphorus fractions from a commercial 'phytic acid' solution, failed to yield satisfactory results.

(II) Acidified oxine extract.

Separation of the organic phosphorus from other organic matter of the oxine extract by acidification with hydrochloric acid produced a relatively light colored supernatant solution. This solution was evaporated to dryness in a vacuum desiccator over solid sodium hydroxide to remove hydrochloric acid. The resulting residue when taken up in 0.01 N sulphuric acid could be applied to the resin without the serious complications noted for non-acidified oxine extracts, although some

swelling of the resin occurred. Fig. 12 shows the peaks of organic phosphorus obtained from a Greensboro soil extract together with the peaks obtained from a 1% commercial phytic acid solution which was chromatographed under identical conditions using gradient elution, type B. The general shape of the soil organic phosphorus elution curve in Fig. 12 is characteristic of the shape of other soil organic phosphorus elution curves obtained in this study. Four peaks can usually be found and they are identified as peaks A, B, C and D. The inorganic phosphorus of the extracts was always eluted abruptly in the first and second fractions that contained Peak A, but the elution of inorganic phosphorus is not shown in any of the figures. Fig. 12 shows that peak C passed through the De-Acidite resin at the same rate as inositol hexaphosphate.

The same procedure applied to the organic phosphorus extracted from the St. Bernard soil failed to produce organic phosphorus peaks. Certain differences can be noted in the oxine extracts of the two soils. The oxine extract of the St. Bernard soil contained more organic phosphorus than did the extract from the Greensboro soil, and furthermore the amount of organic phosphorus remaining in solution on acidification amounted to about 50% of that extracted from the St. Bernard soil, while it was only about 30% of that extracted from the Greensboro soil. This resulted in more residue on evaporating to dryness and much of the residue was insoluble in 0.01 N sulphuric acid. The phosphorus that went into solution was about 90% inorganic, and the remaining 10% (organic phosphorus) when eluted from the anion exchange column appeared at the same time as the inorganic phosphorus and then gradually decreased.

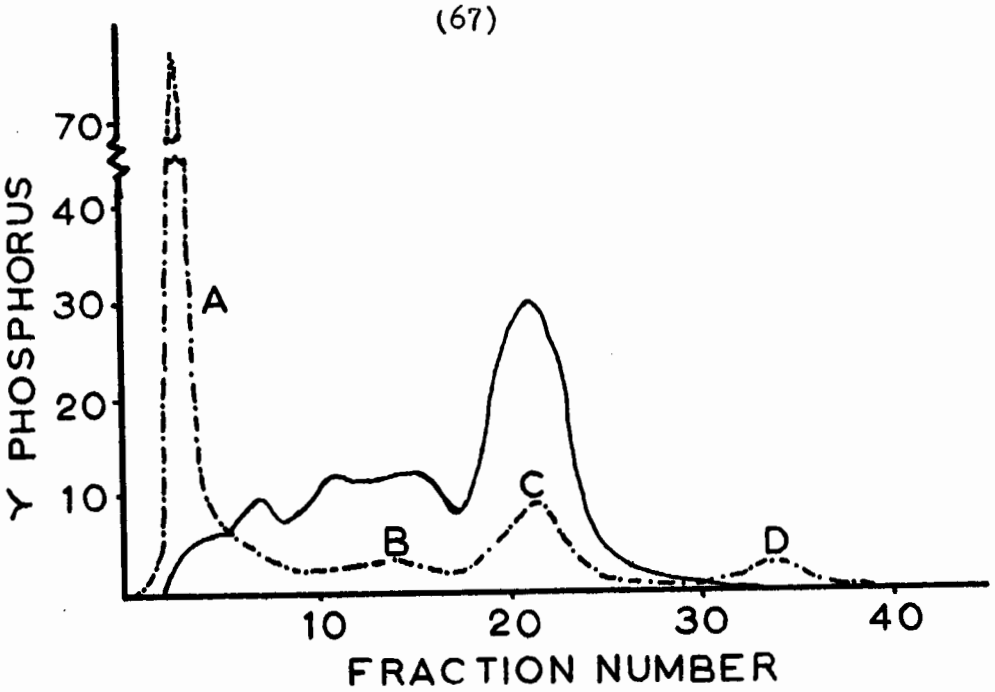


Fig. 12. Organic phosphorus elution curves (gradient, type B).

Solid line - 0.25 ml. 1% phytic acid.

Broken line - Greensboro oxine extract, acidified.

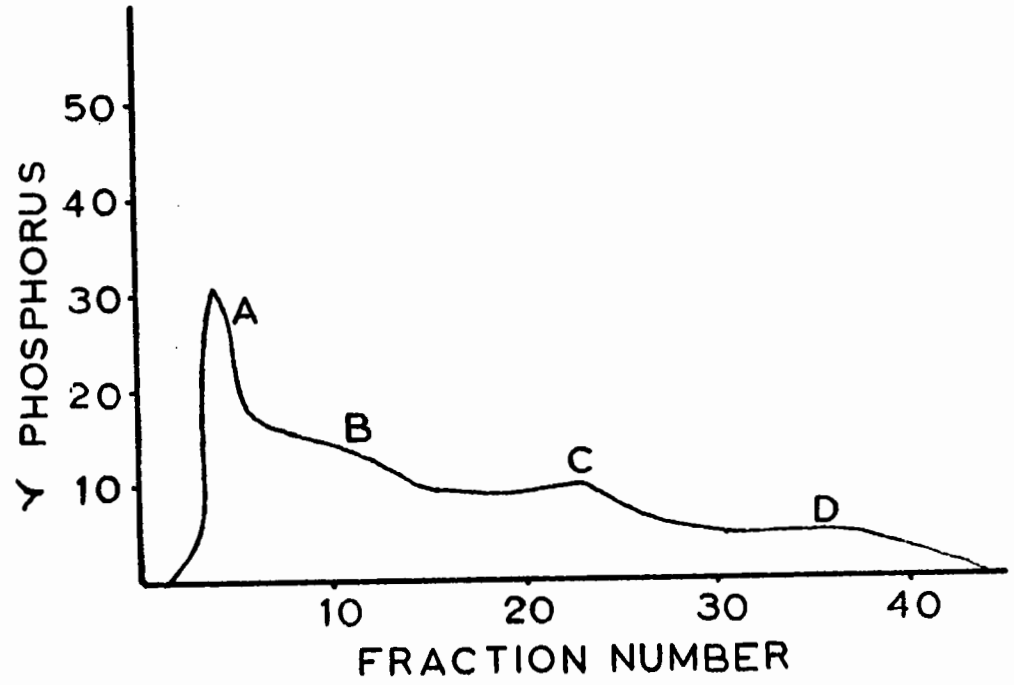


Fig. 13. Organic phosphorus elution curve of oxine extract of St. Bernard soil.

The residue insoluble in 0.01 N sulphuric acid was treated with a dilute ammonium hydroxide solution. This dissolved much of the residue and the solution so obtained was chromatographed by gradient elution, type B. The results given in Fig. 13 show that there was a small increase in the amount of organic phosphorus eluted where peak C would be expected but only a plateau where peak D would be expected. This procedure of chromatographing (gradient elution, type B) oxine extracts which were partially purified by removal of organic matter by acidification also yielded less satisfactory results than reported by previous workers for organic phosphorus solutions obtained from soils by more drastic treatment, e.g., treatment with sodium hydroxide solutions of high alkalinity. The unsatisfactory results obtained with oxine extracts may be attributable to differences in the character of the non-phosphorus containing organic matter extracted by oxine as compared to that extracted by use of sodium hydroxide solutions. For example, the milder oxine extraction may have liberated non-phosphorus-containing organic compounds of higher molecular weight and/or organic phosphorus complexes of higher molecular weight and more complex constitution. Hence it was decided to have recourse to more drastic treatment of the oxine extracts in an attempt to improve the isolation of organic phosphorus compounds from them.

(III) Hypobromite treatment of oxine and sodium hydroxide extracts of soil.

Sodium hypobromite has been used for preparing the 'phytic fraction' of soils and its use on extracts of the St. Bernard soil appeared justified at this time. Since the organic phosphorus of the Greensboro soil extracts could be resolved by chromatography into distinct peaks

when mild extraction and clarification procedures were used, it was considered desirable to chromatograph a Greensboro oxine-benzene extract which had been treated with sodium hypobromite in order to determine whether the organic phosphorus in the peaks could withstand such a treatment. Accordingly an oxine-benzene extract of the Greensboro soil was given the sodium hypobromite treatment outlined in the section on Experimental Methods. An aliquot of the treated sample was chromatographed using gradient elution, type B, and the results are shown in Fig. 14.

The prominence of peaks A, B, C and D indicates the feasibility of treating soil extracts with sodium hypobromite before subjecting them to chromatographic fractionation. The distribution among these peaks, of the organic phosphorus in oxine extracts with and without sodium hypobromite treatment, is given in Table 15.

TABLE 15

Chromatographic distribution of organic phosphorus in oxine extracts of Greensboro soil with and without sodium hypobromite treatment.

Type of Extract	Amount of Organic Phosphorus chromatographed	Amount of Organic Phosphorus in peaks (% of Organic Phosphorus chromatographed)			
	γ	A	B	C	D
Oxine	208	62	9	20	5.9
Oxine + NaOBr.	440	27	18	44	11.1

The successful recovery of the organic phosphorus comprising peaks C and D prompted an alkaline extraction of both soils in order to obtain rapidly large amounts of the organic phosphorus compounds

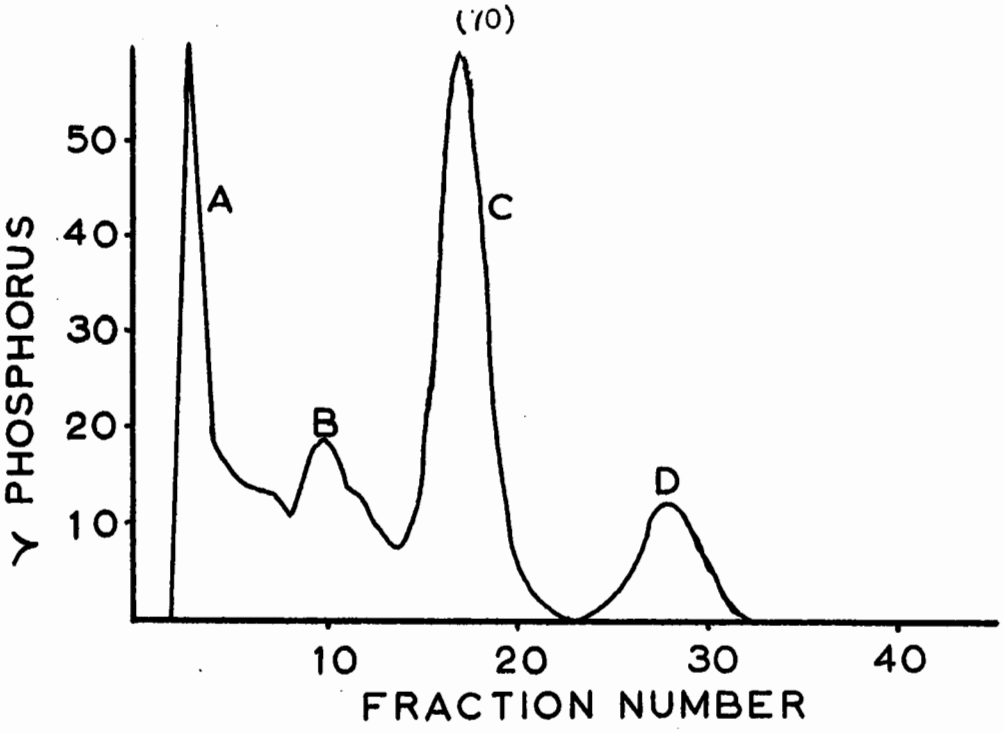


Fig. 14. Organic phosphorus elution curve of oxine extract of Greensboro soil, sodium hypobromite treated.

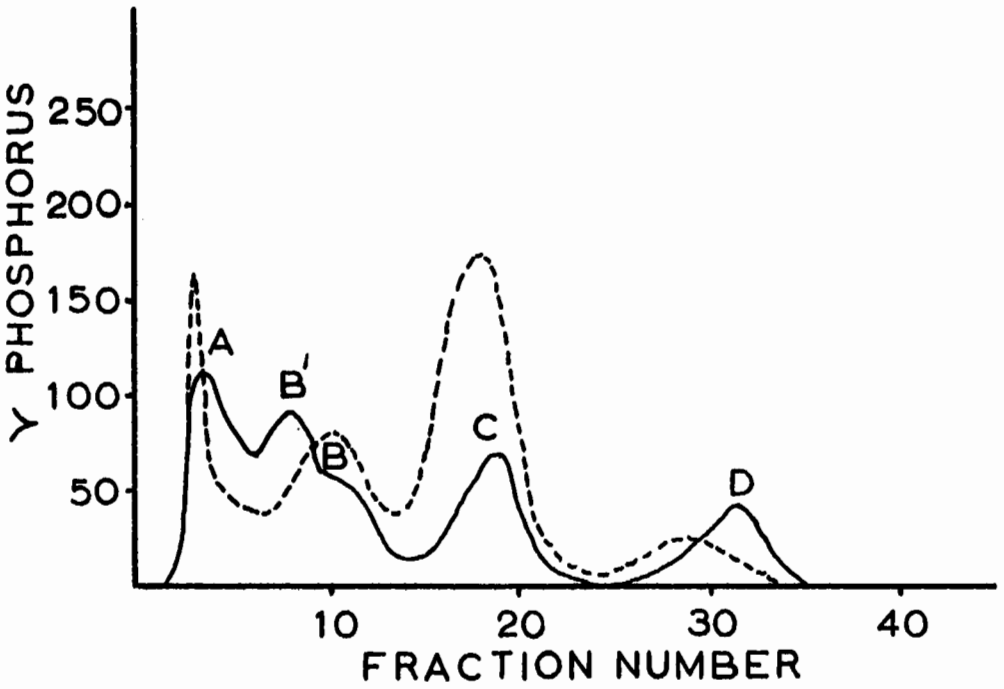


Fig. 15. Organic phosphorus elution curves of sodium hydroxide soil extracts, sodium hypobromite treated.

Solid line - St. Bernard soil.

Broken line - Greensboro soil.

so that some studies of their composition and properties could be undertaken. The results of these extractions were discussed previously and are shown in Table 12. These alkaline extracts were treated with sodium hypobromite and aliquots were chromatographed using gradient elution, type B. The elution curves for both soils are shown in Fig. 15.

The organic phosphorus elution curves of Fig. 15 have very similar characteristics. Peak A represents the first large amount of organic phosphorus eluted from the column and it occurs simultaneously with the elution of the inorganic phosphorus. The inorganic phosphorus always left the column abruptly and was found in not more than two or three fractions. With gradient elution, type B, the inorganic phosphorus was usually found in the third to fifth fractions. Peak B is noted in both the elution curves but it is not consistent in its shape as compared to the other peaks. It sometimes has the appearance of two peaks which are not well separated and these are identified as B and B'. Peak C as was previously noted in Fig. 12 coincides with the inositol hexaphosphate peak of the phytic acid curve. Peak D is noted in the elution curves of the soil extracts but not in the phytic acid elution curves.

(IV) Results of rechromatographing peaks C and D.

The organic phosphorus in each of peaks C and D was rechromatographed separately following evaporation to dryness in a vacuum desiccator over sodium hydroxide to remove hydrochloric acid. The data given in Table 16 includes the inorganic phosphorus that resulted.

TABLE 16

Redistribution of phosphorus when peaks C and D rechromatographed.

	Phosphorus Content			
	Organic		Inorganic	Total
	Peak C	Peak D		
	γ	γ	γ	γ
Original Peak C	85	--	--	85
Peak C Rechromatographed	70.1	--	3.1	80.2
Original Peak D		21		21
Peak D Rechromatographed	5.2	9.7	1.2	19.7

The repeat chromatographing shows that neither compound was found entirely in the region of the chromatograph that they occupied originally. Both have apparently broken down somewhat on the repeat treatment but only a small amount of inorganic phosphorus was eluted, indicating that only a small amount of hydrolysis took place. The organic phosphorus in Peak C when rechromatographed did not appear as peak D, but the organic phosphorus in peak D on rechromatographing appeared as both peak C and peak D. This behavior of peak C and D suggests that peak C contains little organic phosphorus other than inositol hexaphosphate, and that peak D, although more strongly adsorbed by the resin, also contains a considerable amount of inositol hexaphosphate. An organic phosphorus elution peak corresponding to peak D has been reported by the Iowa workers (16, 67), and they consider it is an isomer of inositol hexaphosphate but they have not presented any evidence that it contains organic phosphorus that can be eluted from the resin column with peak C. The data obtained from the rechromatographing of peaks C

and D suggests that a major component of both peaks can be eluted from the anion exchange column at the same time as inositol hexaphosphate.

(V) Hydrochloric acid extract of Greensboro soil.

The hydrochloric acid extract obtained from the acid pretreatment of the Greensboro soil contained 20% of the estimated organic phosphorus content of the soil. The organic phosphorus in the extract, with and without sodium hypobromite treatment, was chromatographed. The portion of the extract that was not sodium hypobromite treated was passed through a column of Dowex-50 in the hydrogen form and the effluent was evaporated to dryness in a vacuum desiccator over solid sodium hydroxide to remove hydrochloric acid. The residue was taken up in 0.01 N sulphuric acid for application to the De-Acidite column. The distribution among the four peaks, of the organic phosphorus in the hydrochloric acid extracts, with and without sodium hypobromite treatment is given in Table 17.

TABLE 17

Chromatographic distribution of organic phosphorus in hydrochloric acid extracts of Greensboro soil with and without sodium hypobromite treatment.

	Amount of Organic Phosphorus Chromatographed	Amount of Organic Phosphorus in peaks (% of Organic Phosphorus Chromatographed)			
Type of Extract	γ	A	B	C	D
0.5 N HCl	284	70	7	16	6.1
0.5 N HCl + NaOBr.	1010	6	20	60	13.9

It is seen that the organic phosphorus contained in the hydrochloric acid soil extract was successfully fractionated without any treatment to remove organic matter, and furthermore when the hydrochloric acid

extract was treated with sodium hypobromite the percentage of the chromatographed organic phosphorus which appeared in peak C was higher than that obtained when either oxine or sodium hydroxide extracts were so treated. Although hydrochloric acid is not a very effective extractant for organic phosphorus, its use in organic phosphorus fractionation studies merits further consideration because of the clean separation into distinct peaks, of the organic phosphorus extracted from soil.

(VI) General conclusions.

Consideration of the results of attempts to fractionate soil organic phosphorus of the various solutions chromatographed leads one to believe that the relatively inefficient separations observed are due primarily to inefficient separation of extraneous organic matter from the organic phosphorus prior to application to the column. No fractionation of organic phosphorus was obtained when attempts to chromatograph 'raw' oxine extracts were made. Fractionation of organic phosphorus of oxine extracts was obtained however when the extracts were acidified to remove organic matter and treated with sodium hypobromite. Both of these treatments caused a large loss of organic phosphorus as is shown in Table 18, but that which remained could be separated into distinct peaks.

It was noted that the use of sodium hypobromite on soil extracts altered the distribution of the organic phosphorus among the peaks so that a relatively larger percentage of the organic phosphorus chromatographed appeared in peaks B, C, and D and a smaller percentage in Peak A. This is illustrated in Fig. 16 which shows the elution curves of a hydrochloric acid extract chromatographed before and after sodium hypobromite treatment. This altered distribution could be brought

TABLE 18

Chromatographic distribution of organic phosphorus in various soil extracts.

Soil	Type of Extract	Treatment of Extract	% of Soil Organic P Extracted	Organic Phosphorus in Extract γ/ml	% Organic Phosphorus Lost	Organic Phosphorus Applied to resin γ	% of Organic Phosphorus Chromatographed			
							A	B	C	D
G	oxine	acidified	59	10.4	70	208	62	9	20	59
G	oxine	NaOBr	70	19.0	91	440	27	18	44	11.1
G	NaOH	NaOBr	40	35.4	88	1732	19	26	46	8.3
St. B.	NaOH	NaOBr	78	102	63	1197	31	34	21	13
St. B.	NaOH	NaOBr	78	102	63	4660	24	35	24	14
G	HCl	H-Dowex-50	23	19.9	--	284	70	7	16	6.1
G	HCl	NaOBr	23	19.9	93	1010	6	20	60	13.9

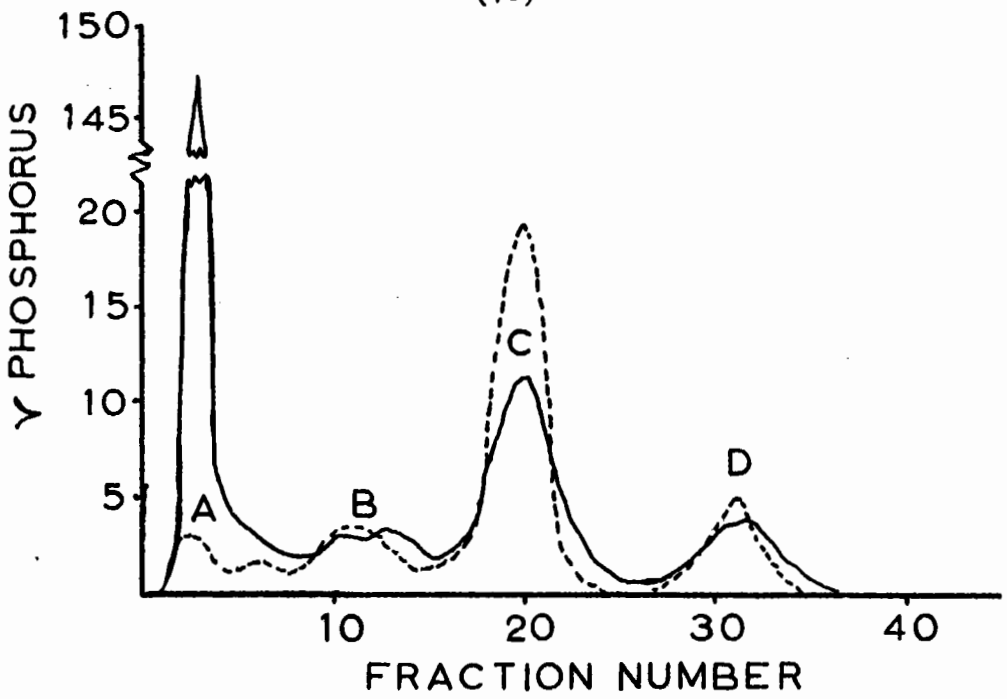


Fig. 16. Organic phosphorus elution curves of hydrochloric acid extracts of Greensboro soil.

Solid line - no hypobromite treatment.

Broken line - sodium hypobromite treated (P = γ x 10).

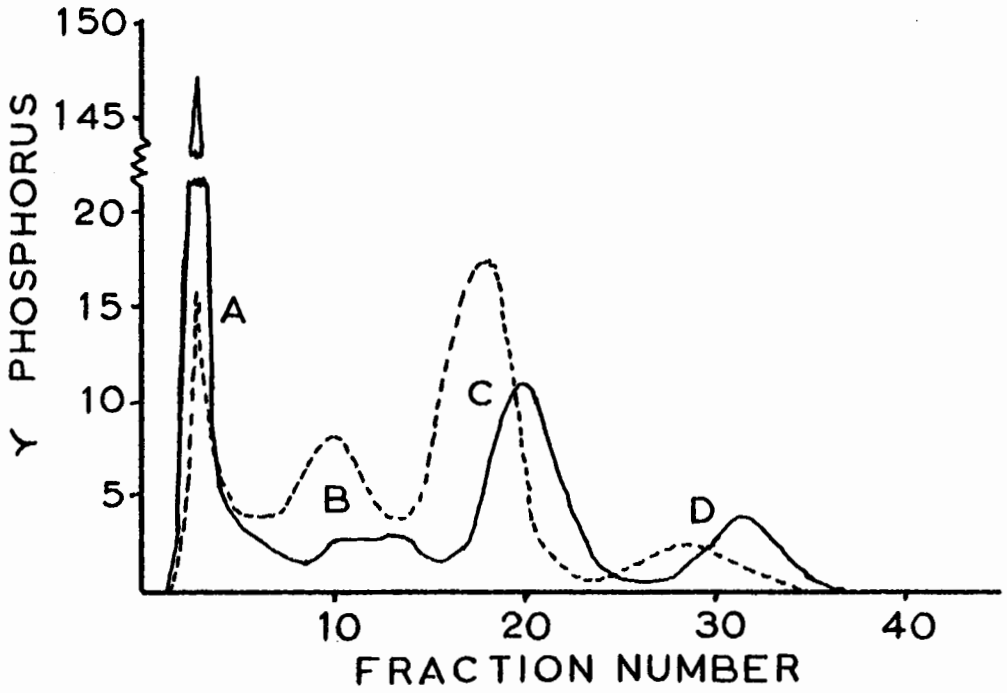


Fig. 17. Organic phosphorus elution curves.

Solid line - Greensboro HCl extract.

Broken line - Greensboro NaOH, hypobromite treated

(P = γ x 10).

about by either a redistribution of the organic phosphorus among peaks or by the fact that the additional loss of organic phosphorus that was noted with sodium hypobromite treatment was more pronounced in the organic phosphorus contributing to peak A than in the other peaks. Of these two possibilities the latter is the more likely in view of the fact that Smith and Clark (67) found that there was almost complete loss of the mono-, di-, and triphosphates of inositol when they were subjected to the sodium hypobromite procedure which includes a calcium precipitation, and Bower (9) showed that calcium does not precipitate the nucleic-acid-like organic phosphorus of soils. Therefore if any of these lower phosphates of inositol or nucleic-acid-like organic phosphorus are present in soil extracts they could not be expected to be recovered when the sodium hypobromite procedure is used.

As compared to sodium hydroxide and oxine, hydrochloric acid is not an effective agent for the removal of soil organic matter. Nevertheless it removed about 20% of the organic phosphorus from the Greensboro soil. The organic phosphorus in this extract, which would not have as high an organic matter content as sodium hydroxide and oxine extracts, was fractionated into well defined peaks without any treatment to remove organic matter. The separation of the organic phosphorus from the hydrochloric acid extract which did not receive a sodium hypobromite treatment is given in Fig. 17 along with the separation of organic phosphorus from a sodium hydroxide extract which was treated with sodium hypobromite. The relative sharpness of the peaks of organic phosphorus from the untreated hydrochloric acid extract as compared to those of the sodium hypobromite treated sodium hydroxide extract is further evidence for the interference of extraneous organic

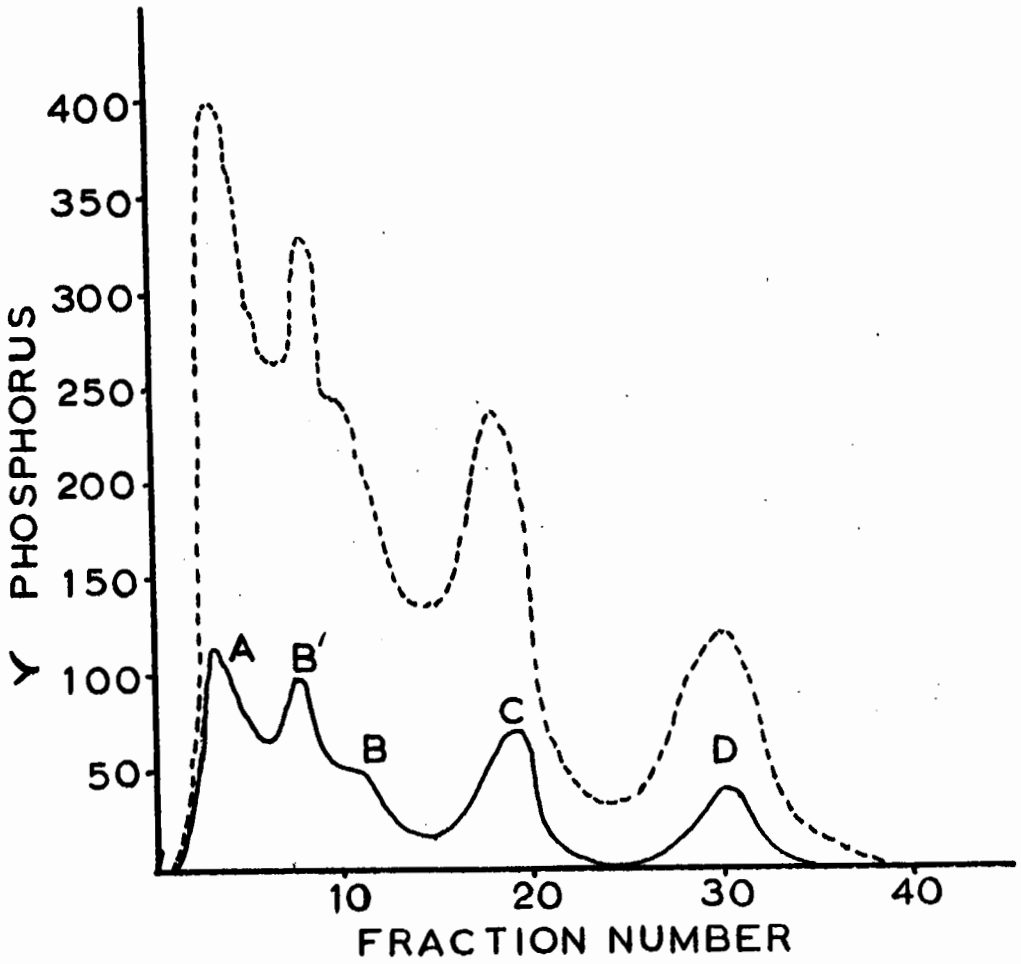


Fig. 18. Organic phosphorus elution curves for two amounts of a sodium hypobromite treated sodium hydroxide extract of the St. Bernard soil.

matter, in sodium hydroxide and oxine extracts, in the fractionation of organic phosphorus. Two different amounts of organic phosphorus of a hypobromite treated sodium hydroxide extract of the St. Bernard soil were chromatographed. The calculated distribution among the peaks of the organic phosphorus chromatographed was not influenced very much by the amount of organic phosphorus applied to the column (see Table 18). However, examination of Fig. 18 shows that although comparable peaks occur in the same region a clean separation of peaks was not obtained when the larger amount of organic phosphorus was chromatographed.

The results presented above show that even the best methods of previous workers in the study of soil organic phosphorus are inefficient for the extraction of soil organic phosphorus and its subsequent fractionation. Further progress in the fractionation of organic phosphorus extracted from soils requires either a more efficient procedure for the separation of extraneous organic matter from the extracted organic phosphorus, or the development of an extraction procedure which removes less extraneous organic matter, or both.

5. Estimation of Inositol Content and Inositol/Phosphorus Ratios.

An estimation of the inositol and the inositol/phosphorus ratios of organic phosphorus fractions contained in elution peaks corresponding to the hexaphosphate of inositol and its supposed isomer was made by employing a microbiological assay for inositol. A commercial phytic acid preparation was separated into six distinct peaks by anion exchange chromatography and an estimation of the inositol/phosphorus ratios of the organic phosphorus in these peaks was made before the method was used for the estimation of inositol in organic phosphorus fractions of elution peaks from soil.

a. Chromatographic fractions from commercial phytic acid.

The microbiological assay of inositol with a strain of 'Schizosaccharomyces pombe' as outlined by Norris and Darbre (48) had just been published at the time this work was being done. Because it was considered capable of a high order of sensitivity and precision it was selected as a suitable method for this investigation. Since the method when employed to estimate the inositol in dilute solution from chromatographic separations would result in a high sodium chloride content in the neutralized hydrolyzed fractions, some preliminary assays were made on inositol solutions to which sodium chloride was added, to establish the suitability of the method for this work. These assays are reported in the section on Experimental Methods.

Commercial phytic acid was separated by chromatography into six organic phosphorus peaks (Fig. 9 and Table 14). It was presumed that the organic phosphorus compounds in these peaks would be the mono-, di-, tri-, tetra-, penta-, and hexaphosphate esters of inositol. On this presumption assays were made on hydrolyzed aliquots of these organic phosphorus compounds and the volumes were finally adjusted so that the solutions would contain 1 γ of inositol per milliliter if the presumptions were correct. Hydrolysis was carried out by heating in normal hydrochloric acid in sealed tubes for 48 hours at 123° C. The results of the assay are given in Table 19. The presumed monophosphate of inositol was not present in sufficient quantity to perform an assay.

The compounds which were presumed to be the penta- and hexaphosphates of inositol gave inositol/phosphorus ratios which were very close to the theoretical ratios. For the lower esters the agreement with the expected values was not good and a progressive decrease in

the recovery of inositol was evident. The question remained as to whether these organic phosphorus peaks did in fact contain individual esters of inositol. If it was presumed they did then it was only reasonable to give consideration to the possibility that inositol was being destroyed, probably in the hydrolysis process, or that something inhibited the growth of the yeast.

TABLE 19

Inositol found in organic phosphorus compounds separated from commercial phytic acid.

Presumed Inositol Phosphate	Theoretical Inositol/P ratio	Ratio found	% of Theoretical Inositol recovered*
hexa-	0.97	0.99	102 ± 3
penta-	1.16	1.22	105 ± 4
tetra-	1.45	1.28	88 ± 3
tri-	1.94	1.67	86 ± 3
di-	2.90	2.02	70

* Values without estimates of the fiducial limits were shown by statistical analysis to be invalid. However the mean value obtained is given to show the trend toward progressively poorer values.

A chromatographic separation of partially hydrolyzed phytic acid was made (Fig. 11) and it was decided to hydrolyze several aliquots of solutions containing the organic phosphorus compounds for different lengths of time in sealed tubes at 123°C to determine when hydrolysis was complete. The data are shown in Fig. 19. Aliquots of all six organic phosphorus peaks were included in the experiment and since

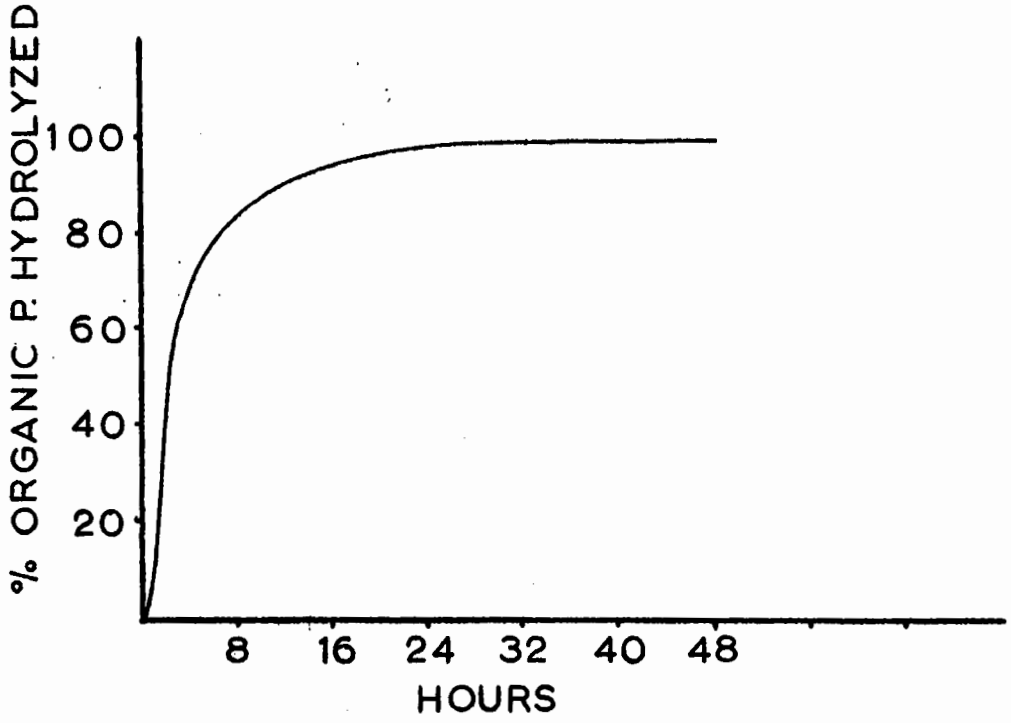


Fig. 19. Rate of hydrolysis of inositol phosphates in 1 N hydrochloric acid at 123°C.

there was close agreement between them the average is plotted in Fig. 19. It can be concluded that hydrolysis is complete in about 24 hours under these conditions.

Aliquots of these organic phosphorus compounds were hydrolyzed at 123° C in normal hydrochloric acid for 24 hours and microbiological assays were again run. The data are presented in Table 20. Again except for the penta- and hexaphosphates of inositol, the data are low and equally as disappointing as those presented in Table 19, so that destruction of the inositol by heating for too long during hydrolysis was ruled out.

TABLE 20

Inositol found in organic phosphorus compounds separated from commercial phytic acid. Aliquots hydrolyzed for 24 hours at 123°C.

Presumed Inositol Phosphate	Theoretical Inositol/P ratio	Inositol/P ratio found	% of Theoretical Inositol found
hexa-	0.97	0.98	101 ± 3
penta-	1.16	1.12	97 ± 3
tetra-	1.45	1.20	83 ± 4
tri-	1.94	1.49	77
di-	2.90	1.89	65
mono-	5.81	2.73	47

The possibility that some material being leached from the resin column was inhibiting the growth of the organism had to be considered. Accordingly a gradient elution of the De-Acidite column to which no organic phosphorus had been added was performed and 10 ml. eluates were collected. Qualitative tests for sulphuric acid showed that it was present in fractions 1 to 31 with the highest concentration in

fractions 8 to 20. It was also noted that when the resin swelled and the eluting solution ran slowly colored material was transferred from the resin to the eluates. The appropriate fractions of these eluates which contained no organic phosphorus were used for controls or standards for the presumed lower esters of inositol by adding inositol and putting them through the hydrolysis process for 48 hours. Assays were performed as before. Again the standard solutions promoted excellent growth of the organism while the presumed inositol phosphates did not. The values for the inositol/phosphorus ratios are given in Table 21.

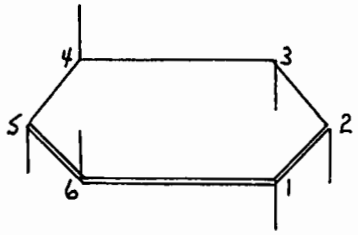
TABLE 21

Inositol found in organic phosphorus compounds separated from commercial phytic acid. Aliquots hydrolyzed for 48 hours at 123°C. Inositol in simulated eluates used as standard.

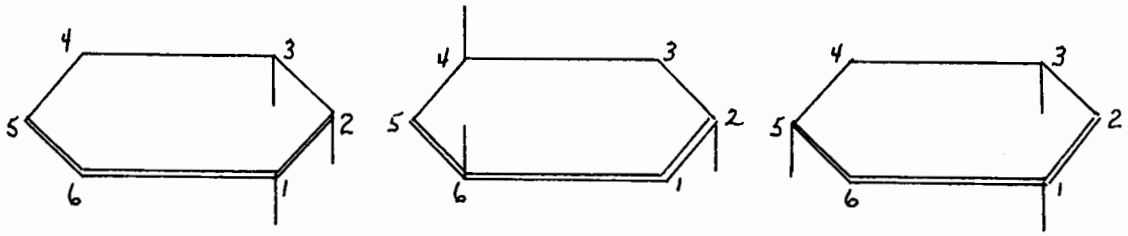
Presumed Inositol Phosphate	Theoretical Inositol/P ratio	Inositol/P ratio found	% of Theoretical Inositol found
tetra-	1.45	1.16	80
tri-	1.94	1.38	71
di-	1.90	1.94	67
mono-	5.81	1.88	49

At this point, study of the chromatographed organic phosphorus compounds from commercial phytic acid was discontinued. Nevertheless some comments on the results are in order. It is apparent from the inositol/phosphate ratios that we are dealing with inositol phosphates of a lower degree of phosphorylation than inositol hexaphosphate, and any one of these lower phosphates of inositol could assume several

configurations. Meso-inositol hexaphosphate which has the maximum number of phosphate groups attached to the inositol, has a phosphate group at each carbon atom of the cyclohexane ring and only one configuration will exist,



However, if three phosphate groups are removed several isomeric inositol triphosphates could result, and three possible configurations are listed here.



Similarly, different configurations could exist for the other lower inositol phosphates. It is known that isomeric meso-inositol monophosphates (inositol 1-phosphate and inositol 2-phosphate) move at different rates on paper chromatographs (55), and it is possible that such a phenomenon occurs in anion exchange chromatography. Thus if one configuration of a triphosphate should have a resin affinity equal to one configuration of a tetraphosphate, mixed esters of inositol could occur in any one peak of organic phosphorus. Suffice it to say that the first four organic phosphorus peaks arising from commercial phytic acid have not been adequately identified.

b. Chromatographic fractions from soil extracts.

The chromatographic separation of organic phosphorus compounds from soil extracts gave four fractions which have been identified throughout as peaks A, B, C, and D. Peaks corresponding to C and D were isolated by Smith and Clark (67), who identified the former as inositol hexaphosphate because it was eluted from the De-Acidite simultaneously with known inositol hexaphosphate. Caldwell and Black (16) followed up this work and determined the inositol/phosphorus ratio of the compound in peak C. The value was 0.90 ± 0.49 and they had no clear evidence that the compound was not meso-inositol hexaphosphate whose inositol/phosphorus ratio is 0.97. They also found that although positive chemical tests for inositol were obtained for the compound in peak D, the hydrolysate of a solution from peak D would not cause growth of Saccharomyces carlsbergensis or of a mutant of Neurospora crassa, both of which require meso-inositol. They therefore suggested that it was an isomer of inositol hexaphosphate.

Preliminary assays on hydrolyzed aliquots of the two peaks C and D, separated from a Greensboro oxine extract which was acidified to remove extraneous organic matter, indicated that the inositol values were low. For peak C it was lower than that for inositol hexaphosphate and also lower than the value reported by Caldwell et al. (16) for a corresponding peak from soil. For peak D the Schizosaccharomyces pombe showed a very slight growth response.

In order to obtain dependable assay values for inositol by this method, the concentration of inositol in both the test and standard solutions should be about equal. Consequently the volumes of the test

solutions from hydrolyzed chromatographic fractions of the sodium hypobromite treated extracts of the two soils were adjusted to attain this equality for the assay.

Assays for inositol were performed on the materials in peaks C and D obtained from sodium hydroxide extracts of both the Greensboro and St. Bernard soils, and on the material in peak B from the Greensboro soil. The soil extracts were treated with sodium hypobromite before being chromatographed (see Table 18). The inositol/phosphorus ratios obtained for the materials in peaks B, C, and D are given in Table 22.

TABLE 22

Inositol/phosphorus ratios of materials in organic phosphorus fractions separated from extracts of the Greensboro and St. Bernard soils.

Soil	Peak	Fraction No. (Fig. 15)	Inositol/Phosphorus ratio	Fiducial limits
Greensboro	B	10	0.49	--
Greensboro	C	19	0.82	0.79-0.85
Greensboro	D	29	0.14	--
St. Bernard	C	19	0.83	0.80-0.86
St. Bernard	D	31	0.10	--

A statistical check on the assay data indicated that the assays were of questionable value for the B and D peaks, consequently fiducial limits are not given. There is no doubt however that the meso-inositol content was low in both peaks. If fraction 10 of the Greensboro soil contains a lower phosphate of meso-inositol such as the tri- or tetraphosphate an inositol/phosphorus ratio of 1.45 - 1.94 would exist. Even with the low values obtained for the third and

fourth peaks from commercial phytic acid (the presumed tri- and tetraphosphates) the ratios found were 1.16 - 1.67. The inositol/phosphorus ratio of 0.49 which was obtained for the material in peak B from the Greensboro soil indicates that inositol phosphates are present, but the low value suggests that other organic phosphorus compounds are also present. The rather incomplete chromatographic separation of the soil organic phosphorus in the peak B region, supports the possibility of the presence of organic phosphorus impurities.

The response of 'Schizosaccharomyces pombe' to the material in peak D from both the Greensboro and St. Bernard soils was very low and the inositol/phosphorus ratios obtained were 0.14 and 0.10 respectively. Caldwell et al. (16) reported a negative response of 'S. carlsbergensis' and 'N. crassa' to the material in a peak corresponding to peak D, but found that the material gave positive chemical tests for inositol. Because naturally occurring inositols, other than meso-inositol, have practically no growth promoting effect on assay organisms, they suggested the presence of an isomer of inositol hexaphosphate in the peak corresponding to peak D. The present study has shown that the material in peak D on rechromatographing produces an organic phosphorus peak C, which suggests that peak D is an artifact. Therefore further study of the material in peak D is necessary in order to understand the significance of its occurrence in the chromatographic fractionation of organic phosphorus in soil extracts.

The inositol/phosphorus ratios of 0.82 ± .03 and 0.83 ± .03 for

the peak C material from the Greensboro and St. Bernard soils, respectively, are considerably lower than those obtained by Caldwell et al. (16). Furthermore these values are significantly different from the inositol/phosphorus ratio of 0.97 for meso-inositol hexaphosphate. The fact that the organic phosphorus peak C,obtained from soil extracts,coincides with the inositol hexaphosphate peak of commercial phytic acid indicates that inositol hexaphosphate is present in the soil extracts. However the low inositol/phosphorus ratios found for the materials in the C peaks suggest that while inositol hexaphosphate is the major organic phosphate present, the procedures used to isolate it failed to remove completely other organic phosphates. Furthermore the failure to obtain any inositol/phosphorus ratios for fractions isolated from soil which are in agreement with known inositol phosphates, is additional evidence for the inadequacy of the methods used in this and previous studies for separating soil organic phosphorus into fractions containing pure compounds.

SUMMARY

Throughout this investigation of soil organic phosphorus, emphasis was placed on using reliable methods that would give accurate estimates of the phosphorus contents of solutions. Two separate studies on methods for determining phosphorus in solution were made. One concerned a study of methods for the estimation of inorganic phosphorus in ammonium fluoride soil extracts, and the other a study of the estimation of total phosphorus in solution by the Martin and Doty procedure.

Because previous work by Hamilton (35) at Macdonald College showed that the Martin and Doty method (44) and the Truog and Meyer method (74) gave values for the inorganic phosphorus contents of ammonium fluoride soil extracts which were not in agreement, a study of methods for estimating inorganic phosphorus in solutions containing fluoride was made. This study showed that the boric acid modification of Kurtz (39) for the removal of fluoride interference was very effective when used in the Dickman and Bray method (22), but was only partially effective when used in the Truog and Meyer method. Therefore the discrepancies noted by Hamilton in the inorganic phosphorus contents of ammonium fluoride soil extracts by the two methods, can be attributed to fluoride interference in the Truog and Meyer procedure. Because of this interference the Truog and Meyer procedure is not a suitable method for estimating the inorganic phosphorus contents of fluoride-containing solutions.

The Martin and Doty procedure was used to estimate the total phosphorus contents of solutions. When it was found that the estimate of total phosphorus was sometimes lower than the estimation of inorganic phosphorus, it was necessary to find the reason for this anomaly.

Since total phosphorus was determined on solutions digested with mixed acids (HNO_3, H_2SO_4, $HClO_4$), interference from these ions was suspected, after loss of phosphorus by volatilization was ruled out. It was found that both sulphate and nitrate ions interfere in concentrations above 70,000 p.p.m. and 20,000 p.p.m. respectively, but perchlorate ions have no undersirable effects. It was also shown that the interference of sulphate is caused by the fact that the isobutanol-benzene extraction of molybdophosphoric acid from the aqueous phase is incomplete when a high sulphate concentration is present. Because of the interference of sulphate and nitrate ions in the determination of total phosphorus by the Martin and Doty procedure, perchloric acid was used to digest organic phosphorus-containing solutions.

A mild procedure was developed for the extraction of organic phosphorus from soil. The procedure, employing oxine in a water-benzene system, was applied to two unimproved pasture soils which differed widely in total phosphorus, ratio of organic to inorganic phosphorus, pH, calcium content, and clay content. Although both soils showed individuality in their release of organic phosphorus to the extraction procedure, it was found that the oxine extraction was more effective at pH 9.2 than at pH 6.5. It was also found that an acid pretreatment of the soils increased the release of organic phosphorus to a subsequent oxine extraction. The total release of organic phosphorus to both the acid and oxine treatments amounted to 76% and 85% of the organic phosphorus estimated to be present in the Greensboro and St. Bernard soils respectively. This agrees very closely to the extraction effected by a more conventional hot sodium hydroxide procedure by which 71% and 89% of the organic phosphorus of these two soils were extracted.

Fractionation of the organic phosphorus extracted from the Greensboro and St. Bernard soils was carried out by employing an anion exchange chromatographic system. The organic phosphorus was eluted from the resin column with hydrochloric acid which was progressively increased in strength by a gradient elution procedure. Gradient elution was used in preference to stepwise elution because the latter produced spurious organic phosphorus peaks. The organic phosphorus in hydrochloric acid extracts of the Greensboro soil could be separated into well defined chromatographic fractions without using any treatment to remove organic matter, while oxine and sodium hydroxide extracts had to be treated with sodium hypobromite or acidified to remove organic matter, before a chromatographic separation of their organic phosphorus could be obtained. However the chromatographic fractionation of organic phosphorus from treated oxine and sodium hydroxide extracts was not as sharp as that from hydrochloric acid extracts, indicating that the relatively inefficient chromatographic separations achieved, were due primarily to inefficient separation of extraneous organic matter from the organic phosphorus prior to application to the resin column.

The organic phosphorus elution curves of both soils contained four peaks which are identified as A, B, C and D. The first two peaks A and B, possibly because of the strong eluting agent used, were not as well separated as peaks C and D. Peak C coincided with an organic phosphorus peak produced by inositol hexaphosphate. Peak D which was eluted subsequently to the hexaphosphate was found, on rechromatographing, to contain organic phosphorus that could be eluted from the resin

column with peak C, indicating that peak D is an artifact. Because of the elution pattern of the soil organic phosphorus and the presence of a considerable amount of inositol in peaks B and C, the presence of inositol phosphates in the extracts of the Greensboro and St. Bernard soils is indicated. Furthermore since a large well defined soil organic phosphorus peak coincides with the inositol hexaphosphate peak, presumptive evidence is available to indicate that inositol hexaphosphate is the major inositol phosphate present. However the fact that the inositol/phosphorus ratio of the material in this soil organic phosphorus peak is significantly lower than that for inositol hexaphosphate indicates that the methods used to separate soil organic phosphates have failed to provide fractions which contain pure compounds.

CLAIMS OF CONTRIBUTIONS TO KNOWLEDGE

It was demonstrated that the Truog and Meyer procedure is not suitable for routine use in the determination of inorganic orthophosphate in fluoride-containing solutions. Boric acid can be used successfully to remove fluoride interference in the method only if the standard and unknown solutions contain equal amounts of fluoride.

In the estimation of the total phosphorus contents of dilute solutions by the Martin and Doty procedure it was found that serious interference can result from the sulphate ions introduced by the mixed sulphuric-nitric acid digestion procedure. This interference results from incomplete extraction of the molybdophosphoric acid by the isobutanol-benzene. However the use of perchloric acid for digesting the samples enables the Martin and Doty procedure to be used successfully.

A new mild extraction procedure using oxine in a benzene-water system adjusted to pH 9.2 was developed for the removal of organic phosphorus from soil. When this method was used on soils which received a dilute hydrochloric acid pretreatment the amount of organic phosphorus removed compared favorably with that removed by a hot sodium hydroxide extraction. To the writer's knowledge such an effective procedure carried out under mild conditions has not previously been reported.

The results obtained in the present study of attempts to chromatograph soil organic phosphorus, have demonstrated the need for the removal of extraneous organic matter from the organic phosphorus, prior to application of the latter to the resin column. Furthermore the results show that even the best methods used by previous workers are inefficient. Further progress in the fractionation of organic

phosphorus extracted from soils requires either a more efficient procedure for separation of extraneous organic matter or development of an extraction procedure which removes less extraneous organic matter, or both.

Elution procedures previously used for the chromatography of soil organic phosphorus involved stepwise increases in the strength of the eluting agent. It has been demonstrated in the present study that gradient elution is an effective elution procedure which eliminates the spurious organic phosphorus peaks noted in stepwise elution.

It was found that the organic phosphorus contained in hydrochloric acid soil extracts not treated to remove extraneous organic matter, could be separated by anion exchange chromatography into relatively well defined peaks, one of which (peak C) coincided with that of inositol hexaphosphate. Although previous workers have recognized that soil organic phosphorus displays appreciable solubility in mineral acids, to the writer's knowledge this is the first time that fractionation of the organic phosphorus so extracted has been reported.

Anion exchange chromatography of soil organic phosphorus, revealed a more strongly adsorbed organic phosphorus (peak D) than inositol hexaphosphate. This is in agreement with studies reported in the literature. However the present study has demonstrated for the first time that an organic phosphorus peak having an elution pattern corresponding to inositol hexaphosphate can be derived from peak D, thus indicating that peak D is an artifact.

BIBLIOGRAPHY

1. Adams, A. P., W. V. Bartholomew, and Francis E. Clark.
   Measurement of nucleic acid components in soil.
   Soil Sci. Soc. Amer. Proc. 18: 40 - 46, 1954.

2. Anastassiadis, P. A., and R. H. Common.
   The use of an ion-exchange resin for tissue hydrolysis in the determination of hexosamine.
   J. Sci. Fd. Agr. 4: 229 - 231, 1955.

3. Anderson, G.
   The identification and estimation of soil inositol phosphates.
   J. Sci. Fd. Agr. 7: 437 - 444, 1956.

4. Association of Official Agricultural Chemists.
   Official Methods of Analysis. 8th ed.
   Washington, D. C., 1955. p. 10.

5. Auten, J. T.
   Organic phosphorus of soils.
   Soil Sci. 16: 281 - 294, 1923.

6. Berenblum, I., and E. Chain.
   An improved method for the colorimetric determination of phosphate.
   Biochem. J. 32: 295 - 298, 1938.

7. Black, C. A., and C. A. I. Goring.
   Organic phosphorus in soils.
   Soil and Fertilizer Phosphorus. Academic Press Inc., New York, N. Y., 1953. p. 123 - 152.

8. Bower, C. A.

Separation and identification of phytin and its derivitives from soils.

Soil Sci. 59: 277 - 285, 1945.

9. Bower, C. A.

Studies on the forms and availability of soil organic phosphorus.

Iowa Agr. Expt. Sta. Res. Bull. 362, 1949.

10. Bray, R. H., and L. T. Kurtz.

Determination of total, organic, and available forms of phosphorus in soils.

Soil Sci. 59: 39 - 45, 1945.

11. Bremner, J. M.

A review of recent work on soil organic matter.

Part 1.

J. Soil Sci. 2: 67 - 82, 1951.

12. Bremner, J. M.

A review of recent work on soil organic matter. Part II.

J. Soil Sci. 5: 214 - 232, 1954.

13. Bremner, J. M., and H. Lees.

Studies on soil organic matter: II. The extraction of organic matter by neutral reagents.

J. Agric. Sci. 39: 274 - 279, 1949.

14. Caldwell, A. G.

Inositol hexaphosphate in soils.

Iowa State College J. Sci. 30: 337, 1956.

15. Caldwell, A. G., and C. A. Black.

Inositol hexaphosphate. I. Quantitative determination in extracts of soils and manures.

Soil Sci. Soc. Amer. Proc. 22: 290 - 293, 1958.

16. Caldwell, A. G., and C. A. Black.

Inositol hexaphosphate. II. Synthesis by soil microorganisms.

Soil Sci. Soc. Amer. Proc. 22: 293 - 296, 1958.

17. Caldwell, A. G., and C. A. Black.

Inositol hexaphosphate. III. Content in soils.

Soil Sci. Soc. Amer. Proc. 22: 296 - 298, 1958.

18. Cann, D. B., and P. Lajoie.

Soil survey of Stanstead, Richmond, Sherbrook and Compton counties in the Province of Quebec.

Canada Department of Agriculture, 1942. p. 27.

19. Coffin, D. E.

Investigations on the release of organic matter from the B horizon of podsol soils.

M. Sc. Thesis. McGill University, 1956.

20. Dean, L. A.

An attempted fractionation of the soil phosphorus.

J. Agr. Sci. 28: 234 - 246, 1938.

21. Desjobert, A., and P. Fleurent.

Bull. Soc. Chim. Biol.,

Paris. 36: 475, 1954.

22. Dickman, S. R., and R. H. Bray.

Colorimetric determination of phosphate.

Ind. Eng. Chem. Anal. Ed., 12: 665 - 668, 1940.

23. Dickman, S. R., and E. E. DeTurk.

A method for the determination of the organic phosphorus of soils.

Soil Sci. 45: 29 - 37, 1938.

24. Dion, H. G., and P. J. G. Mann.

Three - valent manganese in soils.

J. Agr. Sci. 36: 239 - 245, 1946.

25. Dyer, W. J., and C. L. Wrenshall.

An improved method for the determination of phosphate by photoelectric colorimetry.

Can. J. Res. 16 B: 97 - 108, 1938.

26. Dyer, W. J., C. L. Wrenshall.

Organic phosphorus in soils: I. The extraction and separation of organic phosphorus compounds from soil.

Soil Sci. 51: 159 - 170, 1941.

27. Dyer, W. J., C. L. Wrenshall, and G. R. Smith.

The isolation of phytin from soil.

Science 91: 319 - 320, 1940.

28. Eid, M. T., C. A. Black, and O. Kempthorne.

Importance of soil organic and inorganic phosphorus to plant growth at low and high soil temperatures.

Soil Sci. 71: 361 - 370, 1951.

29. Fraps, G. S.

Effect of ignition on the solubility of soil phosphates.

J. Ind. Eng. Chem. 3: 335 - 339, 1911.

30. Ghani, M. O.

Fractionation of soil phosphorus. I. Method of extraction.

Indian J. Agr. Sci. 13: 29 - 45, 1943.

31. Goring, C. A., and W. V. Bartholomew.

Microbial products and soil organic matter: II. The effect of clay on the decomposition and separation of the phosphorus compounds in microorganisms.

Soil Sci. Soc. Amer. Proc. 14: 152 - 156, 1950.

32. Grande, J. A., and John Beukenkamp.

Gradient elution chromatography of phosphates.

Anal. Chem. 28: 1497 - 1498, 1957.

33. Grandeau, L.

Recherches sur le rôle des matières organiques du sol dans les phénomènes de la nutrition des végétaux.

Compt. rend. 74: 988 - 991, 1872.

34. Hackh, I. W. D.

Hackh's Chemical Dictionary, 2nd. ed.

P. Blakiston's Son & Co. Inc.

Philadelphia, Pa., 1937. p. 722.

35. Hamilton, H. A.

Experiments on the extractability of soil phosphorus.

M. Sc. Thesis, McGill University, 1955.

36. Heintze, S. G., and P. J. G. Mann.

Soluble complexes of manganic manganese.

J. Agric. Sci. 37: 23 - 27, 1947.

37. Houlihan, J. E., and P. E. L. Farina.

The thiocyanate determination of iron with hydrogen peroxide.

Analyst 78: 559, 1953.

38. Jackman, R. H., and C. A. Black.

Solubility of iron, aluminum, calcium, and magnesium inositol phosphates at different pH values.

Soil Sci. 72: 179 - 186, 1951.

39. Kurtz, L. T.

Elimination of fluoride interference in the molybdenum blue reaction.

Ind. Eng. Chem. Anal. Ed. 14: 855, 1942.

40. Lajoie, P., and R. Baril.

Soil survey of Montreal, Jesus, and Bizard Islands in the Province of Quebec.

Canada Department of Agriculture, 1954. p. 32.

41. Legg, J. O., and C. A. Black.

Determination of organic phosphorus in soils. II. Ignition method.

Soil Sci. Soc. Amer. Proc. 19: 139 - 143, 1955.

42. Lutwak, L., and J. Sacks.

The catalytic effect of molybdate on the hydrolysis of organic phosphates.

J. Biol. Chem. 200: 565, 1953.

43. Martin, A. E., and R. Reeve.

Extraction of organic matter from podsolic B horizons with organic reagents.

Chem. and Ind. 356, 1955.

44. Martin, J. B., and D. M. Doty.

Determination of inorganic phosphate. Modification of isobutyl alcohol procedure.

Anal. Chem. 21: 965 - 967, 1949.

45. Mehta, N. C., J. O. Legg, C. A. I. Goring, and C. A. Black.

Determination of organic phosphorus in soils:

I. Extraction method.

Soil Sci. Soc. Amer. Proc. 18: 443 - 449, 1954.

46. Mulder.

Ueber die Bestandtheile der Ackererde.

J. prakt. chem. 32: 321 - 344, 1844.

47. Nachod, F. C.

Ion Exchange

Academic Press Inc. New York, N. Y., 1949. p. 388.

48. Norris, F. W., and A. Darbre.

The microbiological assay of inositol with a strain of Schizosaccharomyces pombe .

Analyst 81: 394 - 400, 1956.

49. Parker, F. W., and J. F. Fudge.

Soil phosphorus studies: I. The colorimetric determination of organic and inorganic phosphorus in soil extracts and the soil solution.

Soil Sci. 24: 109 - 117, 1927.

50. Pearson, R. W.

Determination of organic phosphorus in soils.

Ind. Eng. Chem. Anal. Ed. 12: 198 - 200, 1940.

51. Pearson, R. W., and R. W. Simonson.

Organic phosphorus in seven Iowa soil profiles: distribution and amounts as compared to organic carbon and nitrogen.

Soil Sci. Soc. Amer. Proc. 4: 162 - 167, 1940.

52. Peech, M., L. T. Alexander, L. A. Dean, and J. F. Reed.

Methods of soil analysis for soil fertility investigations.

U. S. Dept. Agr. Circ. 757, 1947. p. 8

53. Pierre, W. H.

The phosphorus cycle and soil fertility.

J. Am. Soc. Agron. 40: 1 - 14, 1948.

54. Pierre, W. H., and N. W. Parker.

Soil phosphorus studies: II. The concentration of organic and inorganic phosphorus in the soil solution and soil extracts and the availability of the organic phosphorus to plants.

Soil Sci. 24: 119 - 128, 1927.

55. Pizer, F. L., and C. E. Ballou.

Studies on myo-inositol phosphates of natural origin.

J. Amer. Chem. Soc. 80: 915 - 921, 1959.

56. Potter, R. S., and T. H. Benton.

The organic phosphorus of soil.

Soil Sci. 2: 291 - 298, 1916.

57. Potter, R. S., and R. S. Snyder.

The organic phosphorus of soil.

Soil Sci. 6: 321 - 322, 1918.

58. Robertson, G.

The colorimetric determination of aluminum in silicate materials.

J. Sci. Fd. Agr. 1: 59 - 63, 1950.

59. Russel, E. W.

Soil Conditions and Plant Growth, 8th ed. Longmans, Green & Co., London, New York, Toronto, 1952. p. 12 - 18.

60. Saunders, W. M. H., and E. G. Williams.

Observations on the determination of total organic phosphorus in soils.

J. Soil Sci. 6: 254 - 267, 1955.

61. Schmidt, G.

The biochemistry of inorganic pyrophosphates and metaphosphates.

A Symposium on Phosphorus Metabolism. Vol. 1.

The Johns Hopkins Press, Baltimore, Md. 1951. p. 443 - 475.

62. Schmoeger, M.

Ueber den Phosphor im Moorboden

Ber. Deut. Chem. Ges. 26: 386 - 394. 1893.

63. Schollenberger, C. J.

Organic phosphorus of soil: Experimental work on methods for extraction and determination.

Soil Sci. 6: 365 - 395, 1918.

64. Scott, Wilfred W.

Standard Methods of Chemical Analysis. Vol. 1.

D. Van Nostrand Company, Inc. 1925. p. 533.

65. Sherman, M. S.

Colorimetric determination of phosphorus in soils.

Ind. Eng. Chem. Anal. Ed. 14: 182 - 185, 1942.

66. Shorey, E. C.

Some organic soil constituents.

U. S. D. A. Bur. Soils Bull. 88, 1913.

67. Smith, D. H., and F. E. Clark.

Anion-exchange chromatography of inositol phosphates from soil.

Soil Sci. 72: 353 - 360, 1951.

68. Smith, D. H., and F. E. Clark.

Chromatographic separations of inositol phosphorus compounds.

Soil Sci. Soc. Amer. Proc. 16: 170 - 172, 1952.

69. Smith, G. R., W. J. Dyer, C. L. Wrenshall, and W. A. DeLong.

Further observations on the determination of phosphate by photoelectric colorimetry.

Can. J. Res. 17B: 178 - 191, 1939.

70. Stewart, J.

Organic phosphorus in the soil.

Orig. Com. 8 Intern. Congr. Appl. Chem. 15: 273 - 300, 1912.

71. Stewart, R.

Quantitative relationships of carbon, phosphorus and nitrogen in soils.

Illinois Agr. Expt. Sta. Bull. 145, 1910.

72. Thompson, L. M., and C. A. Black.

The mineralization of organic phosphorus, nitrogen and carbon in Clarion and Webster soils.

Soil Sci. Soc. Amer. Proc. 14: 147 - 151, 1950.

73. Thompson, L. M., C. A. Black, and F. E. Clark.

Accumulation of microbial organic phosphorus in soil materials.

Soil Sci. Soc. Amer. Proc. 13: 242 - 245, 1949.

74. Truog, E., and A. H. Meyer.

Improvements in the Deniges colorimetric method for phosphorus and arsenic.

Ind. Eng. Chem. Anal. Ed. 1: 136 - 139, 1929.

75. Weil-Malherte, R., and R. H. Green.

The catalytic effect of molybdate on the hydrolysis of organic phosphate bonds.

Biochemical J. 49: 286 - 292, 1951.

76. Williams, C. H.

Studies on soil phosphorus. I. A method for the partial fractionation of soil phosphorus.

J. Agric. Sci. 40: 233 - 242, 1950.

77. Williams, C. H.

Studies on soil phosphorus. III. Phosphorus fractionation as a fertility index in South Australian soils.

J. Agric. Sci. 40: 257 - 262, 1950.

78. Wood, E. C.

The efficient planning of microbiological assays.

Analyst 78: 451 - 460, 1953.

79. Wrenshall, C. L., and W. J. Dyer.

A method for the determination of organic phosphorus in soils and soil extracts.

Can. J. of Res. 17B: 199 - 205, 1939.

80. Wrenshall, C. L., and W. J. Dyer.

Organic phosphorus in soils: II. The nature of the organic phosphorus compounds. A. Nucleic acid. B. Phytin.

Soil Sci. 51: 235 - 248, 1941.

81. Wrenshall, C. L., W. J. Dyer, and G. R. Smith.

Recent studies on the nature of soil organic phosphorus.

Sci. Agr. 20: 266 - 271, 1940.

82. Wrenshall, C. L., and R. R. McKibbin.

Pasture studies XII. The nature of the organic phosphorus in soils.

Can. J. Res. 15B: 475 - 479, 1937.

83. Yoshida, R. K.

Studies on organic phosphorus compounds in soil; isolation of inositol.

Soil Sci. 50: 81 - 89, 1940.