CHEMICAL COMPOSITION AND BIOLOGICAL BEHAVIOUR OF SOIL ORGANIC PHOSPHORUS

by

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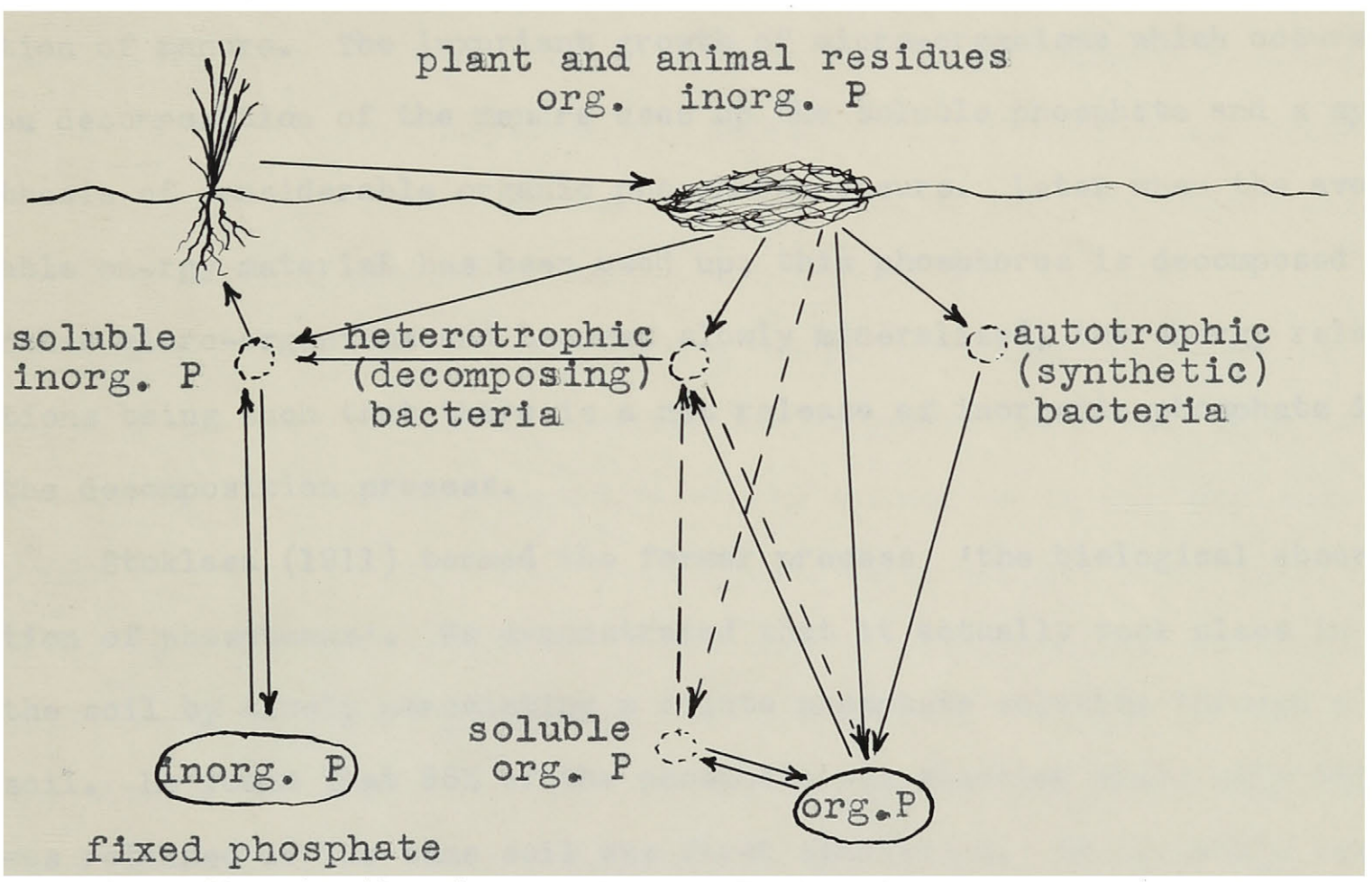
INTRODUCTION AND HISTORICAL

Phosphorus deficiency is one of the major agricultural problems. Although most of our Canadian soils are well supplied with total phosphorus, only a very small proportion of this is available to plants. From 30 - 85% of the phosphorus in our surface soils is present in an organic form (Odynski, 1936, Wrenshall and Dyer, 1939). Since our soils are relatively very poor in available phosphorus, it appears that the large store of organic phosphorus is unavailable for plant use.

The evidence of culture solution experiments (Schreiner and Skinner, 1912, Schulow, 1913, Weissflog and Mengdehl, 1933, Pierre and Parker, 1927) indicates that phosphorus is assimilated principally as the mono-phosphate ion ($H_2PO_4^-$). Therefore, the soil organic phosphorus probably will have to be hydrolyzed into inorganic phosphate before it can be assimilated by plant roots. Pierre and Parker (1927) found that corn and soybeans could not use the organic phosphorus of soil extracts. On the other hand, Wrenshall and McKibbin (1937) concluded from a study of the utilization of phosphorus by plants in pot tests, that some phosphorus must have been supplied from the organic phosphorus of the soil through decomposition. Stoklasa (1911) believed that a soil was fertile with respect to phosphate if it had a large store of organic phosphorus.

We may state the question thus: Does the large quantity of organic phosphorus in our soils represent simply an accumulation of the end-products of the decomposition of organic material added to the soil, thus being inactive and of little use for the phosphorus nutrition of plants; or does it still constitute an active part of the phosphorus cycle in our soils, being a store, as it were, of organic phosphorus continuously being synthesized by micro-organisms or repleted from plant residues, and simultaneously being decomposed and mineralized by other micro-organisms, thus providing a source of phosphate readily

available for plant assimilation? Before this question can be answered, a great deal has to be learned about the nature and properties of the organic phosphorus in soils. Undoubtedly, phosphorus in the soil takes part to some extent in a dynamic biological cycle, as do nitrogen, carbon and sulphur. Just as the availability of nitrogen in the soil is greatly influenced by soil conditions (pH, moisture etc.) so also it is probable that the transformations of phosphorus are likewise affected and thus it may be possible to change the conditions in our soils so that organic phosphorus may be more readily mineralized. The phosphorus cycle in soil may be summarized as in the following diagram.



According to the above scheme, plant and animal residues reaching the soil contain both organic and inorganic phosphorus compounds. A rapid growth of micro-organisms ensues immediately and a part of the inorganic phosphate is converted into organic phosphorus of the microbial protoplasm. Part of the organic phosphorus is decomposed by other

bacteria and some of this reaches the soil solution while the rest is again built into the microbial cells. When the micro-organisms die, this organic phosphorus, now part of the soil organic phosphorus, is attacked by other decomposing micro-organisms and the same process continues.

Thus the soil organic phosphorus originates from two sources, the non-decomposed organic phosphorus of plant and animal residues and that synthesized by the soil micro-organisms. There is therefore a continuous simultaneous synthesis and decomposition of organic phosphorus compounds in the soil. Tottingham and Hoffman (1913) have shown that this synthesis of organic phosphorus occurs during the decomposition of manure. The luxuriant growth of micro-organisms which occurs on decomposition of the manure uses up the soluble phosphate and a synthesis of considerable organic phosphorus occurs. Later when the available energy material has been used up, this phosphorus is decomposed by other micro-organisms and becomes slowly mineralized, the energy relations being such that there is a net release of inorganic phosphate in the decomposition process.

Stoklasa (1911) termed the former process 'the biological absorption of phosphorus'. He demonstrated that it actually took place in the soil by slowly percolating a dilute phosphate solution through a soil. He found that 98% of the phosphate was absorbed while only 65% was retained if the same soil was first sterilized. He is mainly responsible for the idea of a phosphorus cycle in the soil.

It is thus evident that micro-organisms play a very important role in the transformations of phosphorus in the soil.

The extent of accumulation of organic phosphorus in some of our infertile soils makes us wonder if the biological absorption of phos-

phorus is as important from the standpoint of improving soil fertility as Stoklasa and later Schreiner (1923) considered it to be.

Some of the properties of the organic phosphorus compounds liable to occur in soil will now be considered.

There are five classes of organic phosphorus compounds which could conceivably occur in soils.

1. Phospholipids. It has repeatedly been shown that only a small part of the organic phosphorus of soil is present as phospholipids (Stoklasa 1911). Wrenshall and McKibbin (1937) found 0.31 per cent of the soil organic phosphorus in this fraction.

Further, lecithin is very readily decomposed by micro-organisms, (Stoklasa, 1911, Plimmer 1913, Dox 1911) and is assimilated by plants in culture solution (Schreiner and Skinner 1912, Weissflog and Mengdehl 1933). Auten (1923) showed that lecithin was 60 per cent decomposed in two months in sand cultures.

2. Hexose Phosphate and related compounds. These compounds are very soluble and very labile to chemical and enzymatic agencies. (Dox 1911, Plimmer 1913) Thus they would be easily decomposed in the soil and would be almost as available as inorganic phosphate for plant nutrition. Mengdehl and Weissflog (1933) showed that glycerophosphate and hexose diphosphate were readily absorbed by corn plants. Hilbert et al (1938) and Spencer and Stewart (1934) have shown that glycerophosphates and such compounds were readily mineralized in the soil.

3. Phosphoproteins. The phosphorus of casein is very readily split off by alkaline hydrolysis (Plimmer and Bayliss 1906, Rimington and Kay 1926). Dox (1911) showed that casein phosphorus is very readily hydrolyzed by micro-organisms, and Kelly (1915) and Neubauer (1933) found that this compound was rapidly decomposed in the soil.

4. Nucleic Acid and Nucleotides. Compounds of this type are known to be present in the soil (Shorey 1913, Bottomly 1919, Wrenshall and McKibbin 1937).

Nucleic acids are known to be susceptible to acid and alkaline hydrolysis. The purine nucleotides are completely decomposed by two or three hours boiling with 5% H_2SO_4, while boiling under pressure for several hours with 25% H_2SO_4 is used to decompose the pyrimidine nucleotides (Levene and Jorpes 1930). The nucleotides are readily decomposed by enzymes, which are present in the soil micro-organisms (Dox 1911, McFayden 1934), and it is difficult to see how these compounds could accumulate in the soil. Various workers (Stoklasa 1911 , Schreiner and Skinner 1912, Weissflog and Mengdehl 1933) have shown that nucleic acid is readily absorbed in culture solutions. Auten (1923) showed that nucleic acid is 80 to 85 per cent decomposed in sand cultures in one to three months, and Neubauer (1933) found about 70 per cent assimilation by rye seedlings in sand or mixed soil and sand cultures.

Potter and Snyder (1918) determined the rate of hydrolysis of the organic phosphorus in soil by 5 per cent H_2SO_4 at 100° C and found it different from nucleic acid, and more similar to the pyrimidine nucleotides or to phytin.

The pyrimidine nucleotide theory was further advanced by Potter and Benton (1916) and Schollenberger (1918). Auten (1923), on the other hand, concluded that soil organic phosphorus was not present in any appreciable amount in the form of lecithin, nucleic acid, phytin, or pyrimidine dinucleotides, and he postulated the presence of Ca, Mg, or other salts of an 'organic amphoteric complex' in which phosphate becomes 'buried' in the soil humus organic matter complex.

Thus the presence or absence of any large quantity of nucleic acid or its derivatives in soil is still debatable, although small amounts of nucleotide material have been isolated.

Recently Gulland and Jackson (1938) have observed that nucleic acid is only 75 per cent dephosphorylated by enzymes. Thus there is an enzyme-stable residue of unknown composition which contains 25 per cent of the phosphorus. This does not fit in with our present day conception of the structure of nucleic acid, but nevertheless, this is what is observed. Gulland and Jackson (1939) claimed it to be of an adenine uracil nature, although close inspection of their published data does not confirm this conclusion.

It is quite possible that this residual compound occurs in soil. The soil micro-organisms build up large amounts of inorganic phosphate to nucleoproteins in their bodies and this is later broken down enzymatically by other micro-organisms, part being released as inorganic phosphate, and part being again synthesized to nucleoproteins (Stoklasa 1911). If only 75 per cent of the nucleoprotein phosphorus can be decomposed, obviously there will be an accumulation of resistant organic phosphorus in the soil.

5. Phytin. This compound had not been detected in soil up to the time of the present investigation.

Phytin is known to be quite stable. It is very resistant to alkaline hydrolysis although not so stable to acids, however being still relatively resistant. (Plimmer 1913)

Phytin, like the nucleotides, is very readily decomposed by micro-organisms. It is easily hydrolyzed by the enzyme phytase occurring abundantly in the soil micro-organisms. (Dox and Golden 1911, Kawahara 1929, Shimoda 1927)

Phytin is readily assimilated by plants from culture solution, (Weissflog and Mengdehl 1933, Schreiner and Skinner 1912) although less readily than nucleic acid.

In sand cultures, Auten (1923) finds 65 per cent hydrolysis at two months. Whiting and Heck (1926) and Heck and Whiting (1927) found that phytin added to sand cultures was utilized by oats and clover.

Since completion of this work it has come to our attention that Neubauer (1933) found that the addition of soil or permutite to the sand cultures rendered phytin stable and valueless for plant assimilation. This will be discussed later.

Potter and Snyder (1918) found that phytin added to the soil could not be extracted by 1% HCl. There seemed to be no explanation for this behavior.

Although it has been known at least since 1914 that phytin forms an iron salt which is very insoluble in acid solution, the significance of this in connection with the occurrence of phytin in the soil (in fact, also in plants) has not been realized. It seems very probable that in soils, especially acid soils, phytin would be precipitated as ferric phytate and hence would accumulate. Thus the fixation of phytin observed by Potter and Snyder is readily explained.

The above discussion of the properties of the various organic phosphorus compounds shows that it is very difficult to account for organic phosphorus accumulation in soils.

Nucleic acid and derivatives were considered as the most likely compounds liable to occur in soil. Otherwise one could only assume the existence of some hypothetical combination of phosphorus with humus or lignin-humus, and this was tantamount to saying that we did not know how organic phosphorus actually existed in the soil. After the publication of Gulland and Jackson's results on nucleic acid and serious consideration of the presence of phytin, it became very much easier to explain the accumulation of soil organic phosphorus.

EXPERIMENTAL AND DISCUSSION

A. Extraction and Separation of Organic Phosphorus from Soil

1. Introduction and Historical

There must be some explanation why our infertile, phosphorus deficient, Quebec podsol soils contain such a large proportion of organic phosphorus (P) which apparently is not available for assimilation by plants.

In studying this question it was deemed desirable to obtain information from several aspects. Firstly, a study of the decomposition of various organic phosphorus compounds when added to the soil, and a comparison of the rates of decomposition of these with organic P preparations obtained from the soil itself; secondly, an investigation of the chemical properties and identity of the organic P compound or compounds in the soil. To these ends, it was necessary to obtain a quantity of the soil organic P. If this preparation is to be representative of the soil organic P it must be relatively pure and represent a fairly large proportion of the total soil organic P.

Previous to 1910 very little work had been done on the actual isolation of organic P from soil. It was known (Stewart 1910) that dilute alkalies extracted a large proportion of the soil organic P along with other organic matter, and that the amount of organic P dissolved was increased when the soil was leached previously with dilute acid (Mulder 1844). On precipitation of humic acid by acidifying the alkaline extract, most of the organic P remained in solution but as early as 1889 Eggertz noted that a portion remained with the precipitate. Later, Dumont (1904) showed that the acid used influenced the partition. Smoeger (1893), Aso (1904) and Stoklasa (1911), all demonstrated the presence of small quantities of lecithin in the soil by ether extraction. These authors believed that most of the soil organic P was of nuclein nature.

Shorey (1913) isolated material from the soil which he considered to be nucleic acid. This was followed by Bottomley's work in 1917. He obtained a preparation which he considered to be an adenine-uracil dinucleotide. The method of isolation showed that this material was closely related to that obtained by Shorey.

Potter and Snyder (1916) showed that nucleic acid added to soil was completely recoverable on NaOH extraction.

Schollenberger (1918) made a thorough study of the extraction of organic P by NH_3. The soil was first preleached with HCl (about 1%) and then extracted with 4% NH_4OH for several hours. Clay was removed by filtering through a layer of soil on a Buchner funnel.

Auten (1923) concluded that the soil organic P does not exist to any extent as either nucleic acid, phytin, lecithin, purine or pyrimidine nucleotides.

The work of Shorey and of Bottomley has been confirmed by Wrenshall and McKibbin (1937) who were able to obtain a product containing about 65% of the organic P extractable by NH_3 from a muck soil. They demonstrated the presence of adenylic and uridylic acids in the preparation.

In Shorey's method for the isolation of nucleic acid the soil was extracted with NaOH and the humic acid was then precipitated by acidifying. Sodium acetate was added to the filtrate along with 3 or 4 volumes of alcohol . The precipitate was washed with alcohol and on hydrolysis yielded H_3PO_4, pentose, adenine, hypoxanthine and cytosine.

Bottomley (1919), on the other hand, found that $NaHCO_3$ dissolved the organic P from a raw peat while leaving most of the troublesome humic acid undissolved. The $NaHCO_3$ extract was neutralyzed, concentrated in vacuo and poured into four volumes of alcohol containing sodium acetate and HCl. The flocculent precipitate was considered to be an adenine-uracil-dinucleotide

on the basis of isolation of adenine and uracil from the hydrolyzed material.

Wrenshall and McKibbin (1937) used a method similar to that of Shorey, but modified the procedure in several details. The soil was first extracted with dilute HCl in order to effect the maximum solution of organic P by alkali. This treatment removes Ca and other salts and renders the humic acid and other organic compounds more soluble (Schollenberger 1918, Auten 1923).

NH_4OH was used instead of NaOH, since the excess could readily be removed by evaporation. (Schollenberger showed that NH_4OH was just as efficient as NaOH). The humic acid was reprecipitated to recover the organic P carried down in this fraction (Potter and Snyder 1916). A higher final concentration of alcohol--80%--was found necessary to completely precipitate organic P from solution.

In beginning the further investigation of this problem it was evident immediately that there were no satisfactory methods for the determination of phosphate in colored soil extracts, or for the determination of the total organic phosphorus of soils.

The ceruleomolybdate method for the colorimetric determination of phosphorus was improved and with the use of a photoelectric colorimeter was applied to the determination of phosphate in soil extracts (colored or otherwise) (Dyer and Wrenshall 1938, Smith, Dyer, Wrenshall and Delong 1939).

This method was satisfactorily applied to the determination of organic phosphorus in soil extracts and total organic phosphorus in soils (Wrenshall and Dyer 1939).

These methods have proved indispensible in carrying out the experimental work recorded in this thesis and in a great many cases results could not have been obtained without them.

2. Separation of Organic Phosphorus from Soil

In this experiment the method used was essentially that described by Wrenshall and McKibbin (1937).

One kilogram of air-dry St. Clothilde muck soil was extracted with 2,000 ml. of 1 N HCl by shaking for several hours. After filtering on a Buchner funnel and washing, the soil was extracted with 8,000 ml. of cold 4 per cent NH_4OH for 24 hours. The mixture was then filtered through glass wool, as it was found to be virtually impossible to filter the solution through filter paper. The residue was washed two to three times with hot water in order to peptize as much organic material as possible. In order to remove excess ammonia the filtrate was evaporated overnight in a vacuum still at a temperature not exceeding 40° C. The liquid was then acidified with 2 N HCl (about 450 ml.) until the humic acid was precipitated. This was filtered off on a fast filter paper and washed carefully with warm water (slightly acidified with HCl). Excess washing was avoided since peptization takes place very readily.

In preliminary experiments acetic acid was used to neutralize the ammonia extract but the yield of nucleotide was then found to be very low so that the procedure was abandoned.

A reprecipitation of the humic acid was not carried out since the increase in yield would not compensate for the extra work entailed in evaporating the large volumes of liquid obtained. The filtrate was then evaporated at 35° to 40° C. in a 22 litre vacuum still to a small volume (1450 ml.). A 5 per cent excess of HCl was then added and the liquid was poured into alcohol such that the final concentration of alcohol was 80 per cent. The whitish gelatinous precipitate was filtered off on hardened filter paper and dried in a vacuum oven at a temperature below 50° C.

The yield obtained was 0.9183 gms. and the phosphorus content was

2.78 per cent P (≈ 4.6% total soil organic P). Another extraction using the same soil and procedure as above gave the following results: yield 1.7170 gms., P content 2.15 per cent P (≈ 6.7% total soil organic P).

It is evident that the method gives highly unsatisfactory results. An experiment was therefore carried out in which the distribution of organic P in each fraction was determined.

500 g. muck soil (ground in a Wiley mill) was moistened with 500 ml. water and treated with 1,000 ml. N HCl. It was shaken for several hours and filtered on a Buchner funnel. 2,500 ml. of acid extract (a) were obtained.

The residue was treated with 5 litres of 4 per cent NH_4OH and shaken intermittently for 2 days. It was then filtered off on glass wool filters, and washed once with hot water. The filtrate (b) amounted to 5,000 ml. The residue was re-extracted with 2.5 litres of 4 per cent NH_4OH, filtered as before, and washed well with warm water. The volume of the filtrate (c) was 5,500 ml.

After taking samples for analysis, the filtrates (b) and (c) were combined and the excess NH_3 was evaporated off in a vacuum still at low temperature. The liquid was then acidified with HCl until the humic acid was precipitated (pH 2.4). The precipitate was filtered off and washed with hot water (acidulated with HCl). The filtrate (d) amounted to 12,080 ml.

This was then evaporated in a large vacuum still at a temperature below 40° C. The final volume (e) was 500 ml. To this solution a 5 per cent excess of HCl (conc.) was added and it was poured into 95 per cent alcohol, (2,667 ml.) such that the final concentration of alcohol was 80 per cent. After standing overnight the precipitated nucleotide (1) was filtered off and dried in a vacuum desiccator, (11.468 g.). The filtrate (f) (3,200 ml.) was neutralized with NH_4OH and the precipitate (3) obtained was filtered off, (18.332 g.) The filtrate (g), 3, 350 ml., was

found to contain no phosphorus, organic or inorganic.

In order to determine if any appreciable amount of organic phosphorus was carried down with the humic acid precipitate above, this was dissolved in a minimum amount of ammonia (120 ml. conc. NH_3) and diluted to about 7 litres with water. After standing for a day the excess ammonia was evaporated off in the vacuum still, and the liquid was acidified with HCl until the humic acid was again precipitated. The precipitate was filtered off and washed with hot water. The filtrate (d^1), (10,800 ml.) was evaporated in the vacuum still to 425 ml. (e^1). The nucleotide was then precipitated from this solution by alcohol as described above. The nucleotide precipitate (2) amounted to 0.7831 g. The filtrate (F_2^1) (2850 ml.) was neutralized with ammonia as above and the precipitate (4) (5.994 g.) was filtered off.

The results are shown diagrammatically below.

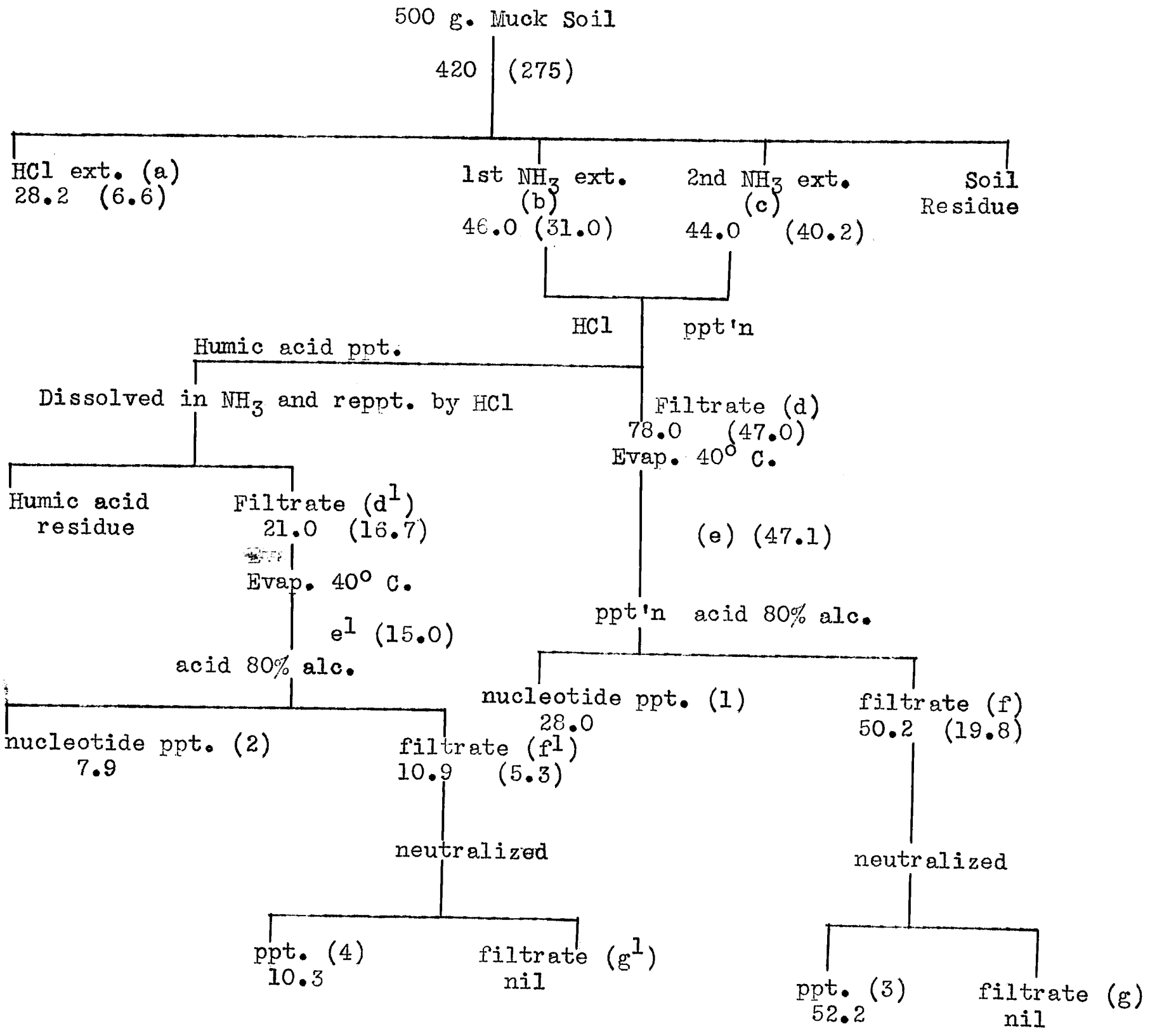
The diagram shows that only 25.9% of the soil organic P was extracted by two ammonia extractions, and that 66% of this remained in the filtrate after humic acid precipitation. Again only 60% of this was precipitated by alcohol so that the nucleotide precipitate contained only 10.2% of the soil organic P.

By reprecipitation of the humic acid a further 2.9% was obtained, bringing the phosphorus contained in the nucleotide preparation to 13.1% (35.9 mg. P). Further these nucleotide preparations were very impure. Nucleotide ppt. (1) contained 1.01% P.

Thus reprecipitation of humic acid does not give a sufficient increase in yield to compensate for the extra work entailed.

The data also show that evaporation of the extract in vacuo at a temperature of approximately 40° C. does not result in any measurable hydrolyses of organic P.

The figures not in parenthesis represent Total Phosphorus (mg.); those in parenthesis, Organic Phosphorus (mg. P).



In this experiment about 35-40% of the organic P present remained in solution in acid, 80% alcohol. Neutralization of the alcoholic solution resulted in the precipitation of all organic and inorganic phosphorus, but the precipitate was very much contaminated with both organic and inorganic material. An acid solution is used to prevent the humic acid, Fe, Al, and inorganic phosphate from being precipitated along with the nucleotide substances. The yield of organic P in this experiment is also very unsatisfactory.

3. Proposed HCl Extraction Method

It was observed that the strong acid used in the determination of total organic phosphorus in soils by the method of Wrenshall and Dyer (1939), dissolved considerable organic phosphorus from the soil. Accordingly, this was investigated further in efforts to improve on the ammonia extraction method.

In a preliminary experiment, a little soil was extracted with about twice its volume of 6 N HCl and filtered. Alcohol was added to the filtrate to 80 per cent concentration and the precipitate obtained was filtered off and dried under a vacuum. It resembled the product obtained by ammonia extraction and contained about 0.9 per cent P. The method was then applied to a larger sample.

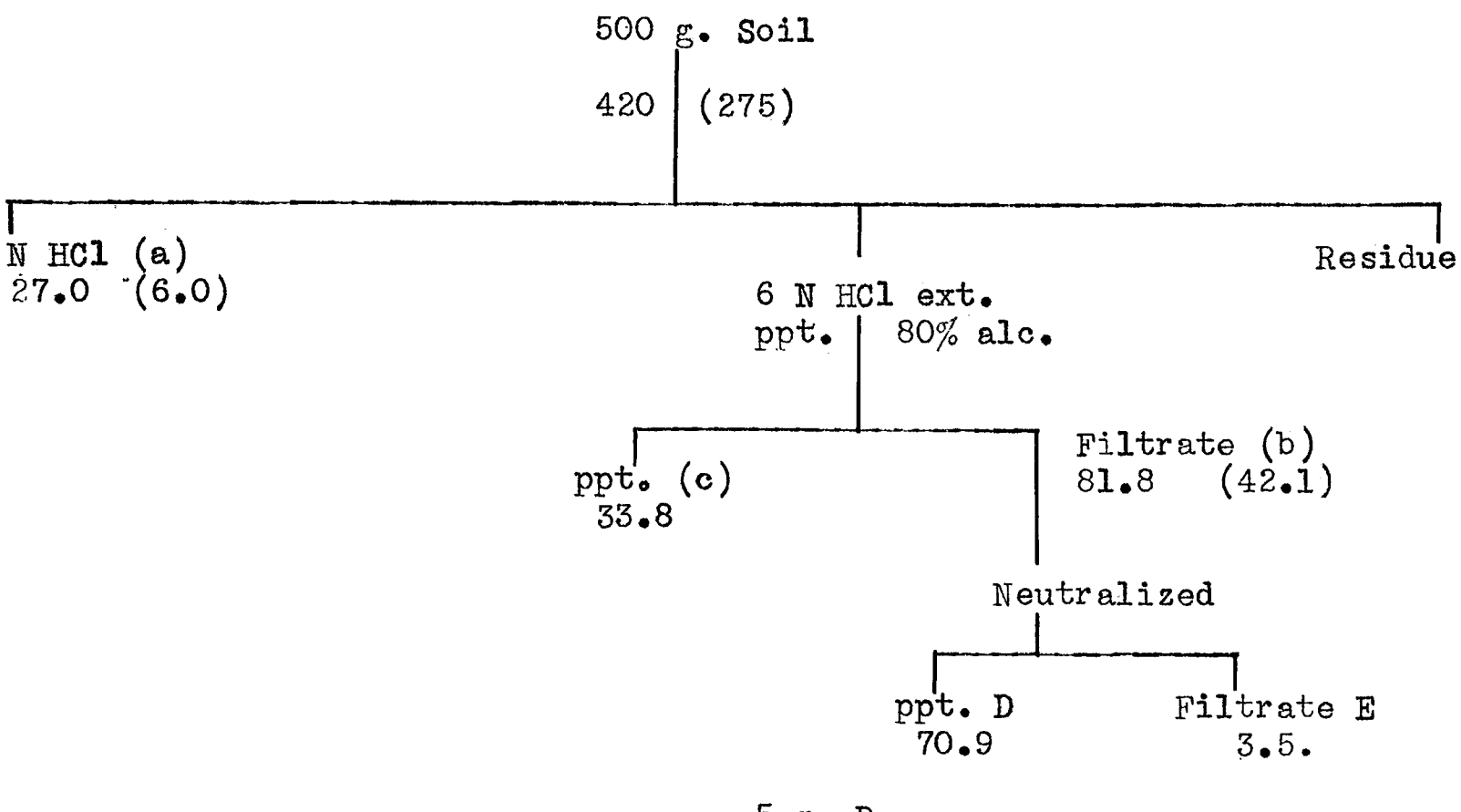
500 g. of the muck soil was extracted with 1,500 ml. 1 N HCl, as above. (Acid extract (a) 1,000 ml.)

To the residue was added 500 ml. 12 N HCl (equiv. to 1,600 ml. 6 N HCl in the soil). After shaking occasionally for a period of 24 hours, the liquid was filtered off, first through glass wool, and finally on filter paper in a Buchner funnel, washing a little with N HCl. The filtrate (1,880 ml.) was made to 80 per cent alcohol and after standing a few hours the precipitate was filtered off and dried in a vacuum desiccator. (Nucleotide precipitate (C) = 5.400 g., 0.625 per cent P, 33.8 mg. P). Analysis showed that the filtrate (b), 12,030 ml. still contained organic phosphorus to the extent of 50 per cent of its total phosphorus.

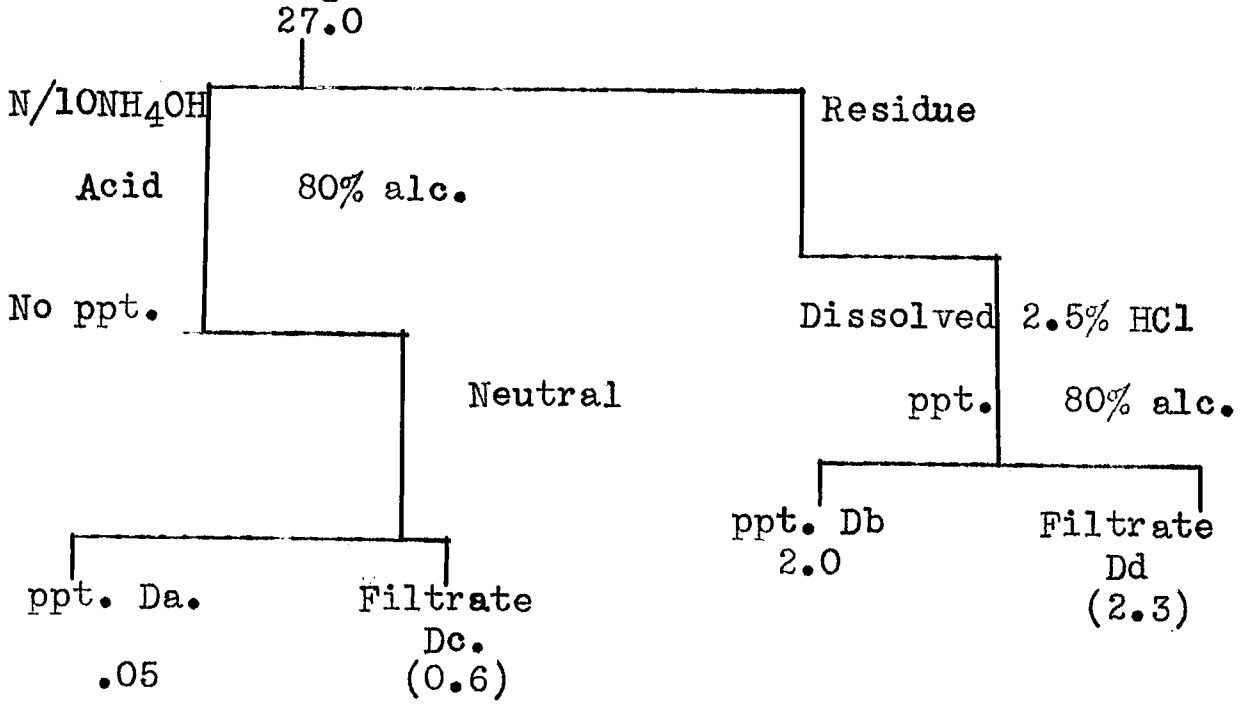
Accordingly the filtrate (b) was neutralized with ammonia and the heavy precipitate obtained filtered off and dried in a vacuum desiccator (Precipitate D, 13.296 g., 0.55 per cent P, 74.3 mg. P). The filtrate E (11,800 ml.) contained only traces of phosphorus, 3.55 mg. total P.

This precipitate (D) was grossly contaminated with Fe and Al. hydroxides, phosphates and organic material. In order to determine if any separation of organic phosphorus could be made, 5 g. of (D) was treated with 10 ml. NH_4OH and 90 ml. water. The brownish red suspension was filtered. The filtrate was acidified with an excess of 2.5 per cent of its volume of concentrated HCl and treated with alcohol (final conc. 80 per cent). No precipitation occurred, but on neutralizing a nucleotide-like precipitate was formed. (Da, 0.107 g., containing 0.05 mg. P).

The residue from the above was taken up in 5 ml. 6 N HCl and water to make 100 ml. The dark brownish-black solution was treated with alcohol (final conc. 80 per cent alc.) and the precipitate filtered off (Db, 0.875 g. containing 2.00 mg. P). The operations are shown diagrammatically below:



5 g. D



The attempted separation of organic phosphorus from ppt. D did not prove very successful, only 2.0 mg. of P being obtained in a very impure state.

This procedure is much simpler than the ammonia extraction method, but the low yield of organic phosphorus obtained, 33.8 mg. P≒ 12.2% total organic P, was very disappointing (about the same as Expt. A).

Extraction of a mineral soil was next carried out to determine if better results could thus be obtained.

500 g. of Macdonald College soil was treated with 1,000 ml. N HCl, as before. The residue was treated with 1,000 ml. 12 N HCl and 900 ml. H_2O (to make about 2,000 ml. 6 N HCl). After shaking occasionally and standing overnight, the extract was filtered off (C_2, 2,085 ml.). 95 per cent alcohol was added until the final concentration was 80 per cent and the precipitate was filtered off. (C_4, yellowish ppt., 0.6433 g., 0.7 per cent P).

Since it was thought that this solution was too acid to allow the precipitation of nucleotides, NH_4OH was added, but not enough to bring about the precipitation of iron hydroxide. At a pH of 2.8, a light yellow precipitate was obtained, filtered off and dried as above. (C6, 2.193 g. (6.3 per cent P)≏138.6 mg. P). It is evident from the diagram that the phosphorus of this precipitate was mainly inorganic.

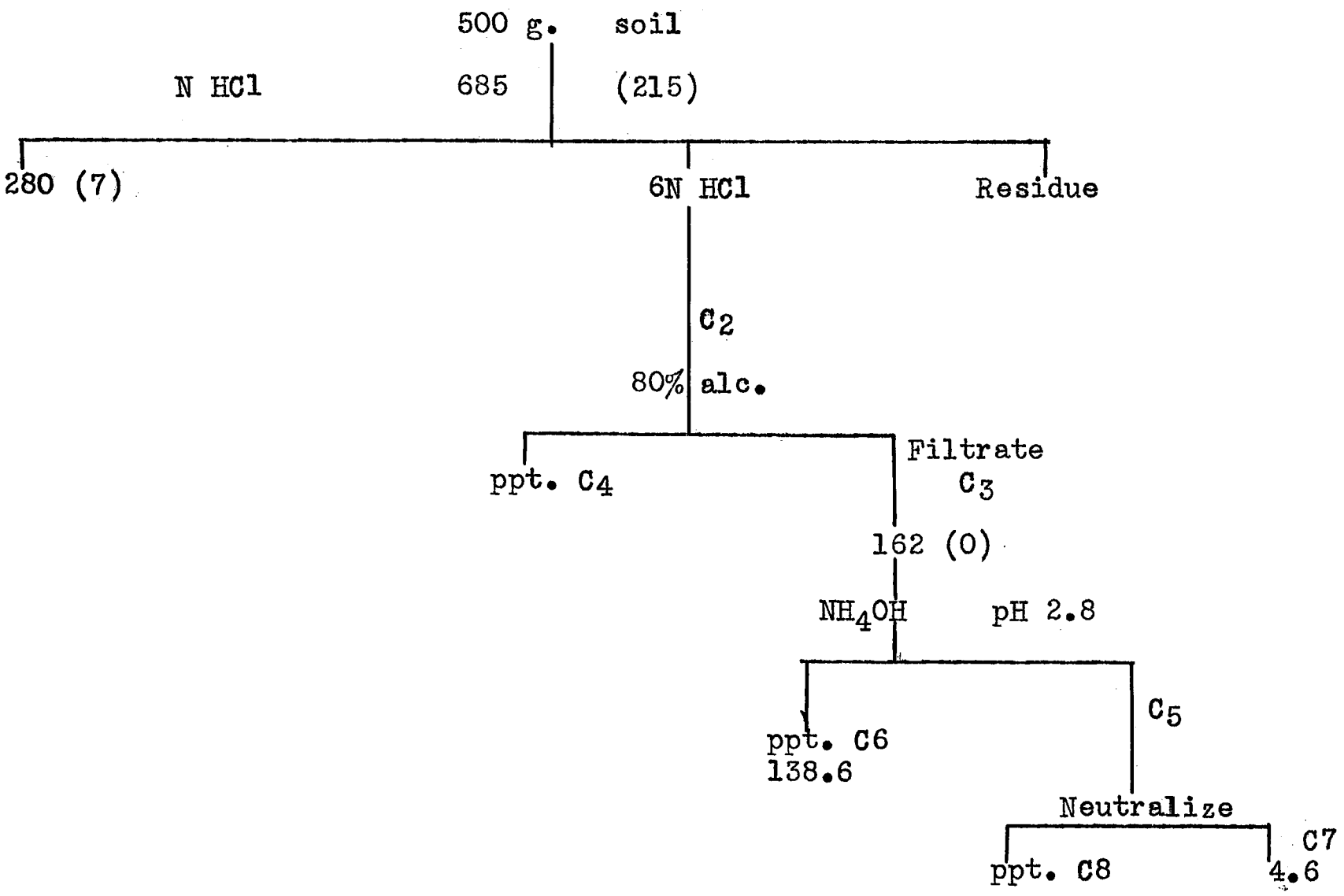
On completely neutralizing the filtrate another precipitate C8, was obtained, and only 4.6 mg. P remained in the filtrate. This is represented diagrammatically below. (see p. 18)

4. Comparison of Efficiency of Various Extracting Solutions

The above experiments led to a study of the efficiency of various methods and extracting solutions for organic P since it was obvious that the inefficiency of the methods used up to the present would not allow the isolation of sufficient nucleotide material to permit continued investigation of its properties.

In this experiment, NH_4OH and Na_2CO_3 were compared as extractants for organic P and were used both with and without preliminary leaching of the soil with HCl.

A St. Clothilde muck soil ground in a Wiley mill to pass a 20-mesh sieve was used.



Here an almost negligible amount of organic nucleotide was obtained.

In the tests where acid preleaching was carried out 20 g. of soil were moistened with about 25 ml. of water and 40 ml. N HCl were added. After standing several hours with occasional shaking, the soil mass was filtered off, washed with about 25 ml. N HCl and finally washed thoroughly with cold water.

For the alkaline extracts, the soil (20 g.) was shaken up with 200 ml. of solution, and after standing several hours with occasional shaking was finally allowed to stand overnight. The extracts were filtered on a Buchner funnel and washed well with warm water.

The treatments were as follows:

1. 20 g. soil / 200 ml. 1% Na_2CO_3
2. 20 g. soil / 200 ml. 4% NH_4OH
3. 20 g. soil / 200 ml. 4% NH_4OH (hot)
4. 20 g. soil, preleached with N HCl / 200 ml. 1% Na_2CO_3
5. 20 g. soil, preleached with N HCl / 200 ml. 4% NH_4OH
5. (a) Residue from No. 5, re-extracted with 200 ml. 4% NH_4OH
6. 20 g. soil, preleached with N/10 HCl / 200 ml. 4% NH_4OH
7. 20 g. soil, preleached with N HCl / 200 ml. 4% NH_4OH (hot)
8. 20 g. soil, preleached with N HCl / 200 ml. 4% NH_4OH (hot) / $Ca(NO_3)_2$ (added before filtering)

In Nos. 3, 7 and 8, the soil and ammonia solution were placed in a closed bottle and heated in a steam bath for several hours, with occasional shaking. The mixture was filtered after standing at room temperature overnight.

The ammonia extract in No. 8 was treated with a saturated $Ca(NO_3)_2$ solution before filtering. This precipitated most of the humic acid material and rendered the filtration process much easier.

No appreciable loss of organic phosphorus was occasioned by the acid extraction, since, as may be seen from Table I, only a small quantity of organic phosphorus is thus dissolved.

TABLE I. PHOSPHORUS IN ACID EXTRACTS

Treatment	Total P	Inorganic P	Organic P	Org. P % Total P of Soil ★
	mg. P per 100 g. soil			
3	6.138	5.038	1.100	1.30
4	6.489	5.187	1.302	1.56
5	2.944	2.576	0.368	0.35

★ Total P of soil = 0.084%

Organic phosphorus, total solids and ash were determined on the alkaline extract and the data are summarized in Table II.

TABLE II DISTRIBUTION OF PHOSPHORUS AND ORGANIC MATTER IN ALKALINE EXTRACTS OF MUCK SOIL

Treatment	mgms. P per 100 g. soil			Org. P as % Total soil Org. P* (A)	% Soil			A/B
	Direct P	Total P	Organic P		Total Solids	Ash	Organic Matter (B)	
1. Na_2CO_3	3.32	3.45	0.13	2.18	5.53	5.16	-	-
2. NH_4OH	2.29	10.00	7.71	14.0	6.95	1.18	5.77	1.34
Washings	2.83	6.85	4.03	7.33	3.32	0.59	2.73	1.48
Total			11.74	21.3				
3. NH_4OH (hot)	5.60	37.65	32.05	58.4	22.89	1.84	21.04	1.52
4. HCl , Na_2CO_3	5.66	14.84	9.18	16.7	7.62	4.65	-	-
5. HCl, NH_4OH	4.0	31.88	27.9	50.7	18.67	1.17	17.50	1.59
5.(a) 2nd NH_4OH	1.04	11.8	10.8	19.7				
Total			38.7	70.5				
6. N/10 HCl, NH_4OH	6.00	26.40	20.4	37.8	15.63	2.00	13.63	1.50
7. HCl, NH_4OH (hot)	5.0	62.0	57.0	103.8	40.66	1.06	39.26	1.52
8. HCl, NH_4OH(hot)$Ca(NO_3)_2$	1.05	21.1	20.1	36.4	35.40	4.71	30.69	1.18

* Total soil organic P = 55.0 mg. P per 100 g. soil

The data show that sodium carbonate which was used by Bottomley (1919) is a very inefficient extractant for the soil organic phosphorus. Cold ammonia is a little more effective while hot ammonia extracts a fairly large proportion of the soil organic phosphorus (58.4 per cent).

Preleaching with HCl is a very effective in increasing the amount of organic phosphorus extracted, N HCl being more effective in this respect than N/10 HCl. A second extraction with ammonia removes a considerable proportion of organic phosphorus, as also does washing the residue with hot water. Preleaching the soil with N HCl followed by treatment with hot ammonia completely extracts the organic phosphorus from this soil. This is an important finding since it shows that the organic phosphorus compounds were not hydrolyzed by this treatment.

Treatment of the ammonia extract with $Ca(NO_3)_2$ reduced considerably the amount of organic phosphorus extracted. In the light of the final isolation of phytin it seems likely that calcium phytate which is almost insoluble in alkaline solution would be precipitated under these conditions. The actual organic matter in solution is only 22% lower than that of the hot ammonia extract without $Ca(NO_3)_2$ treatment, while the organic phosphorus content is 67% lower. This would indicate a specific precipitation of organic phosphorus, thus confirming the above reasoning.

Williams (1937) and Dean (1938) found that NaOH extracted only a part of the soil organic phosphorus unless Ca was first removed, and this effect was more marked in neutral or calcareous soils. This now seems to be explained by a precipitation of Ca phytate as phytin is brought into solution by the alkali.

For this soil there is a fairly constant ration between the amount of organic matter and organic phosphorus dissolved. This will be discussed later.

Extraction of the soil by hot NH_4OH (4 per cent) preceded by leaching with HCl (1 N) is the best extraction method.

5. Separation of Organic Phosphorus from Soil by the Modified Ammonia Extraction Method

Previously cold ammonia has been used in the extraction but hot ammonia was now used for the separation of the nucleotide from the soil since it was shown that two extractions with cold ammonia gave only 70 per cent extraction while hot ammonia completely extracted the organic phosphorus. The soil and ammonia mixture was heated in a steam bath for from 5-8 hours with occasional shaking and was then allowed to cool overnight. The mass was then filtered through cloth and washed with hot water. This procedure gave very satisfactory results giving extractions of 75-90 per cent in most of the large scale experiments.

1000 g. Macdonald College soil was extracted with 2000 ml. 1 N HCl, and the residue was filtered off and washed as in the regular procedure. 10 litres of 4 per cent NH_3 was added to the residue and the mixture was treated as described above. The excess ammonia in the extract was evaporated off under reduced pressure. The humic acid was precipitated by acidifying with 250 ml. N HNO_3 and the precipitate was filtered off and washed with hot water. The ammonia extract contained 260 mg. organic phosphorus while the humic acid filtrate contained only 27 mg., i.e., only 10.4 per cent of the organic phosphorus remains in the humic acid filtrate. This shows very markedly that most of the organic phosphorus remains in the humic acid precipitate and also why we have been unsuccessful in isolating any appreciable quantity of organic phosphorus material from this soil.

To determine the effect of reprecipitation the humic acid precipitate was dissolved in 200 ml. conc. NH_3 and 10 litres of hot water, and then allowed to stand overnight. The excess NH_3 was then evaporated off under reduced pressure, and the liquid was acidified with 1 N HNO_3.

The humic acid precipitate was filtered off and washed as above. The filtrate contained only 8 mg. organic P (4.1 per cent of organic P of NH_3 extract). To determine the effect of the volume of solution in which the humic acid is precipitated, the humic acid was dissolved in four times the volume of solution used above. The organic P in the filtrate after reprecipitation of humic acid was still only 16 mg. P. Analysis showed that the humic acid contained the calculated amount of organic P. Thus reprecipitation of the humic acid did not result in the recovery of any significant amount of organic P.

Apart from the fact that this soil is different from the one used above, the low recovery of organic P in the humic acid filtrate (10.4%) may be due in part to the more complete extraction of organic P compounds from the soil. (60.5% of the total soil organic P was extracted as compared with 30% or less in former experiments.) The more difficultly extractable organic P compounds may be more subject to precipitation in the humic acid fraction.

However since these results differ from thos/obtained with the muck soil, it was decided to study the organic P partition between the humic acid precipitate and filtrate in the extracts of various soils.

6. Partition of Organic Phosphorus Between Humic Acid Precipitate and Filtrate in Various Soils

The following soils were used in this experiment:

Soil 1. St. Chlothilde muck (55 mg. organic P per 100 g. soil)

2. Eastern Townships podsol (38.6 mg. organic P per 100 g. soil)

3. Macdonald College loam (43 mg. organic P per 100 g. soil)

4. Eastern Townships podsol.

5. " " "

6. Podsol A_1 Horizon (Halliday) (137 mg. organic P per 100 g. soil)

7. " " " (Moist) (110 mg. organic P. per 100 g. soil)

8. Cultivated Podsol.

The procedure, which imitated that of the large scale experiments for the preparation of the ammonia extracts and humic acid filtrates of the soil was as follows: 20 g. of each soil contained in a 500 ml. Erlenmeyer flask was extracted with 40 ml. 1 N HCl by shaking occasionally during several hours. The soil was then filtered off and washed with water. It was extracted with 200 ml. 4 per cent NH_3 solution by shaking and heating on a steam bath for several hours. After standing overnight, the mixture was filtered through cloth and washed with hot water. The excess NH_3 was evaporated off under reduced pressure and the supernatant liquid was decanted from the clay precipitate which settled after standing. The liquid was made to 400 ml. and 25 ml. samples were taken for the determination of total and inorganic phosphorus. The remainder was acidified with 1 N HNO_3. After standing overnight the humic acid precipitate was filtered off and washed with hot water. The filtrates were made to a volume of 500 ml. and analyzed for total and inorganic phosphorus. The results are given in Table III.

The acid preleaching of the soil extracts only a very small proportion of the total organic P, varying from 2-5 per cent.

Extraction with hot ammonia has removed a very high proportion of the organic P contained in all these soils - podsols, 90, 91, 92 per cent; muck, 77 per cent; calcareous loam, 78 per cent.

Recently Smith (1939) has shown that a small part of the organic P in these soils is quite labile and is easily decomposed by boiling ammonia, although it is stable in cold solutions. The data from this experiment show that the loss due to this cause does not amount to more than 5-20 per cent and is probably less than this.

The results show definitely that the amount of organic P contained in the humic acid filtrate is very small. In the podsol soils only 8-14.8 per cent of the organic P in the NH_3 extract remains in the filtrate,

TABLE III. ORGANIC PHOSPHORUS DISTRIBUTION IN ACID AND AMMONIA EXTRACTS IN THE HUMIC ACID FILTRATE OF VARIOUS SOILS.

SOIL		ACID EXTRACT Mg. P per 100 g. soil			% Total Soil Org.P	AMMONIA EXTRACT Mg. P per 100 g. soil			%Total Soil Org.P	HUMIC ACID FILTRATE Mg. P per 100 g. soil			% Org.P NH_3 Extract
		Inorg.P	Total P	Org.P		Inorg.P	Total P	Org.P		Inorg.P	Total P	Org.P	
St. Chlotilde Muck	1	5.51	6.99	1.48	2.7	2.70	44.8	42.1	77.0	3.68	15.5	12.8	30.4
Podsol	2	1.52	3.71	2.19	5.6	5.86	40.8	34.9	90.5	1.58	4.98	3.6	10.3
Macdonald College	3	37.9	38.8	0.9	2.1	25.6	59.0	33.4	78.0	18.4	21.2	2.8	8.4
Podsol	4	1.74	3.65	1.91	-	6.86	52.0	45.1	-	3.20	6.91	6.7	14.8
Podsol	5	3.54	5.41	1.87	-	8.58	47.4	39.0	-	4.65	8.04	3.4	8.7
Halliday A_1 Podsol	6	17.9	21.0	3.1	2.3	9.62	134.0	124.4	91.0	7.83	20.4	12.6	10.1
Halliday A_1 (Moist)	7	16.1	18.8	2.7	2.4	7.98	109.0	101.0	92.0	6.88	17.5	10.6	10.5
Podsol	8	1.90	4.17	2.27	-	8.10	46.8	38.7	-	2.34	5.37	3.0	7.8

while this figure is increased to 30.4 per cent in the case of muck soil. These data explain why it has not been possible to isolate any appreciable quantity of organic P from mineral soils and why better results were obtained previously with the muck soil. In the former experiment with muck soil (p. 11) 60% of the NH_3 soluble organic P remained in the filtrate. However, only a 25% extraction of organic P was obtained in the former experiment whereas in the present case the extraction was 80%. Apparently, the more difficultly extractable organic compounds are also more liable to precipitation with the humic acid fraction. This may indicate the existence of two classes of organic P compounds.

The podsols (and calcareous loam) all show about the same distribution of organic P between precipitate and filtrate (7.8-14.8 per cent in the filtrate) and differ markedly from the muck soil. This difference is probably not due to organic matter alone since the Halliday soil contained almost as much as the muck. There are two possible explanations. First, the organic P in the muck soil may be of different identity from that in the podsols. We know nothing about this. Second, there may be some specific substance in the humic acid precipitate which combines with the organic phosphorus and the proportion of this material may vary in the different soil types. There are several substances which might act in this manner. Undoubtedly, the Fe, Al, and clay content of mineral soil extracts is considerably higher than the muck soil extracts. In this connection it is known that ammonia exerts a considerable solvent action on Fe phosphates (Fraps 1911, Schollenberger 1918).

In this experiment HNO_3 was used in the precipitation of humic acid and it is possible that the use of other acids might give a different partition. However, the results of Schollenberger (1918) indicate that there is very little difference in the effect of the ordinary mineral acids.

It was decided to determine the partition with acetic acid and also to determine whether the addition of compounds containing α-OH groups such as oxalic acid, would inactivate the Fe (or Ca) or other possible interfering substances and allow a larger proportion of organic P to pass into the humic acid filtrate.

Humic acid filtrates of soils 1, 2 and 7 were prepared in the same way as in the previous experiment, except that 1 N acetic acid was used instead of HNO_3 for the precipitation of humic acid. The analyses of the extracts are given in Tables IV, (a) and (b).

TABLE IV (a). ORGANIC PHOSPHORUS IN AMMONIA EXTRACTS AND ACETIC ACID FILTRATES.

SOIL	AMMONIA EXTRACT mg.P per 100 g. soil			ACETIC ACID FILTRATE mg. P per 100 g. soil			(A) ORG. P. OF FILTRATES % Org. P of HN_3 Ext.
	Inorg. P	Total P	Org. P	Inorg. P	Total P	Org. P	
Muck, 1	1.73	60.8	59.1	3.47	42.0	39.5	66.8
Podsol, 2	7.83	48.0	40.2	2.83	10.0	7.2	17.9
Halliday A_1,7	10.2	115.8	105.6	9.50	91.2	81.7	77.5

TABLE IV (b). TOTAL SOLIDS, ASH AND ORGANIC MATTER IN AMMONIA EXTRACTS AND ACETIC ACID FILTRATES.

SOIL	AMMONIA EXTRACT g. per 50 ml. ext.				ACID FILTRATE g. per 50 ml. ext.				(B) y/x x 100	B/A RATIO
	T.S.	Ash	Org.	Org.% Soil(x)	T.S.	Ash	Org.	Org.% Soil(y)		
1	0.5454	0.0202	0.5252	33.09	0.2439	0.0080	0.2359	24.77	74.9	1.12
2	.1337	.0439	.0898	5.39	.0214	.0024	.0190	1.90	35.2	1.96
7	.1778	.0478	.1300	7.80	.1058	.0240	.0818	8.18	105.0	1.35

Table IV (a) shows that a good recovery of organic phosphorus was obtained in the humic acid filtrates from soils 1 and 7, while the recovery was very low in the case of soil 2. On the other hand, Table IV (b) shows that very little organic matter was removed from the extracts of soils 1 and 7 by the acetic acid precipitation and that a greater amount was removed from the extract of soil 2. Thus the precipitation of organic phosphorus parallels that of the organic matter. In Table IV (a) and (b) (A) shows the organic P remaining in the humic filtrate as per cent of the organic P in the ammonia extract while (B) shows the percentage of NH_3 soluble organic matter remaining in the filtrate. The ratio B/A then gives the relative amounts of organic P and organic matter remaining in solution in the humic acid filtrate. The figures show that relatively more organic phosphorus than organic matter was removed from solution by the acetic acid treatment, thus showing that CH_3COOH partition is a disadvantage rather than an advantage.

The CH_3COOH filtrate was treated with 1 N HNO_3 until the remainder of the organic matter was precipitated. After filtering and washing, organic P was determined in the filtrate. Table V shows that acetic acid acidification followed by HNO_3 precipitation gives results very little different from the use of HNO_3 alone.

7. Proposed Oxalate Method

As mentioned above the effect of oxalate ion on the partition of organic P was now investigated.

Ammonia extracts of soils 1, 2 and 7 were prepared as above. To 100 ml. ($\simeq$ 3.3 g. soil) of each extract was added 0.5 g. ammonium oxalate ($\simeq$ about 4.3 per cent Fe_2O_3 in the soil). The solutions were then acidified with N HNO_3 and the humic acid was filtered off and washed as usual. The distribution of P was then determined in the filtrate. The results are reported in Table VI.

TABLE V. PHOSPHORUS IN THE FILTRATE AFTER HNO_3 PRECIPITATION OF ACETIC ACID FILTRATES.

Soil	MG. P PER 100 G. SOIL			Org. P, % Org. P of NH_3 Ext.
	Inorg. P	Total P	Org. P	
1	6.1	19.3	13.2	22.4
2	- ✱	-	-	-
7	11.3	27.4	16.1	15.3

✱ Soil 2 gave no further precipitate.

TABLE VI. ORGANIC PHOSPHORUS IN HUMIC ACID FILTRATE IN THE PRESENCE OF OXALATE.

SOIL	MG. P PER 100 G. SOIL			ORG. P, % OF ORG. P OF NH_3 EXT.	INCREASE IN RECOVERY OVER HNO_3 ALONE ✱
	Total P	Inorg. P	Org. P		
1	34.3	12.7	21.6	36.6	6.2 (20%)
2	24.5	13.1	11.4	28.4	18.1 (175%)
7	70.1	15.3	54.8	52.0	41.9 (400%)

✱ Table III

TABLE VII. EFFECT OF OXALATE ON THE PARTITION OF ORGANIC PHOSPHORUS.

TREATMENT	MG. P PER 100 G. SOIL			ORG. P, % OF ORG. P OF NH_3 EXT.
	Total P	Inorg. P	Org. P	
1	38.0	20.1	17.9	19.2
2	77.5	20.6	56.9	61.0
3	72.0	22.0	50.0	53.7
4	72.5	23.3	49.2	52.9
5	63.5	20.9	42.6	45.7

Since oxalate ion interferes with the ceruleomolybdate reaction, (Dyer and Wrenshall, 1938) it was necessary to determine the corrections for the incomplete recovery of inorganic phosphate (usually 80-100 per cent.)

The effect of the oxalate addition is very marked. The recovery of the organic P in the humic acid filtrate is increased in each of the three soils; for the muck from 30.4 to 36.6 per cent, for the podsol, from 10.3 to 28.4 per cent; and for the Halliday podsol A_1 layer from 10.6 to 52.0 per cent. The filtrates contained no more organic matter than would be present with HNO_3 alone so that the use of oxalate is a very definite advantage.

Reprecipitation of the humic acid from soil No. 7 by HNO_3 after solution in dilute NH_3, yielded only a very small further amount of organic P in the filtrate (1.42 per cent). It seems therefore that a fairly definite fraction is precipitated in the presence of oxalate.

The optimum concentration of oxalate was determined next. An ammonia extract of the Halliday soil (40 g.) was prepared in the usual way. The extract (1000 ml.) contained 93.2 mg. organic P per 100 g. soil. 50 ml. aliquots ($\simeq$ 2 g. soil) were diluted to 100 ml. and treated as follows:

Treatment 1. Acidified with N HNO_3, excess of amount required to precipitate humic acid.

" 2. 0.3 g. $COO(NH_4)_2$, just acidified sufficiently to precipitate humic acid.

" 3. 0.3 g. $COO(NH_4)_2$, acidified in excess of amount required to precipitate humic acid.

" 4. 1.0 g. $COO(NH_4)_2$, acidified in excess of amount required to precipitate humic acid.

" 5. 0.1 g. $COO(NH_4)_2$, acidified in excess of amount required to precipitate humic acid.

After standing a few hours the humic acid was filtered off and washed with hot water (acidified with HNO_3). Organic P was determined in the filtrates.

The results, presented in Table VII, indicate that, (1) the addition of only enough HNO_3 for complete precipitation gives the best results but it was observed that the rate of filtration was much slower than when excess acid was used, (2) the addition of 0.15 g. ammonium oxalate to 50ml. of extract (≙ 1. g. soil) seems to give the optimum concentration of oxalate. The use of less oxalate results in a decreased recovery of organic P and more only complicates the inorganic phosphate determination.

Since oxalic acid is so effective in increasing the solubility of the organic phosphorus it would seem quite probable that Fe, or possibly Ca, was the cause of the precipitation of the organic phosphorus.

With podsol soils, therefore, the use of oxalate should allow the isolation of about three to five times as much organic P as with the former method. At the same time the amount of impurities remain approximately the same so that the final product should be much purer.

8. Separation of Organic Phosphorus by the Oxalate Method.

A quantity of organic P was then separated from larger quantities of soil by the above method. Expt. H. 500 g. of Halliday soil (20 mesh) was extracted with 1000 ml. 1 N HCl as in the regular procedure. 5000 ml. 4 per cent NH_3 solution were added to the soil and this was then heated in a steam bath overnight (75° C. approx.). The mixture was filtered through cloth and the excess NH_3 in the filtrate was evaporated off under reduced pressure. After standing overnight, or longer, the supernatant liquid was decanted from the deposited clayey precipitate. The extract, 18 litres, contained 100.8 mg. organic P per 100 g. soil. 75 g. ammonium oxalate were added and the extract was acidified with 1 N HNO_3 until the humic acid was completely coagulated. After standing several hours the precipitate was filtered off and washed well with hot water (slightly acidified with HNO_3). The filtrate and washings were evaporated in the vacuum

still to small volume (Temp. about 35 ° C.) and a small amount of precipitate was filtered off. The liquid, 1275 ml. contained 57.4 mg. org. P per 100 g. soil.

A 5 per cent excess of conc. HCl was added and the liquid poured into 95 per cent alcohol such that the final concentration of alcohol was 80 per cent. After standing overnight the precipitate was filtered off on hardened filter paper and finally dried in a vacuum desiccator over H_2SO_4. The precipitate, 6.139 g., contained 4.05 per cent P (equivalent to 50.7 mg. P per 100 g. soil).

In the determination of inorganic phosphate in the humic acid filtrate and alcoholic filtrate, corrections were made for the interference due to the presence of oxalate ion. By using large dilutions the recovery of phosphate was brought up to about 90 per cent, and the results were checked at different dilutions.

Approximately 92 per cent of the total soil organic P was extracted by the hot ammonia and of this 53.2 per cent remained in the humic acid filtrate. Only 3.4 mg. organic P remained in the alcohol filtrate, amounting to only 3.1 per cent of the total organic P of the soil.

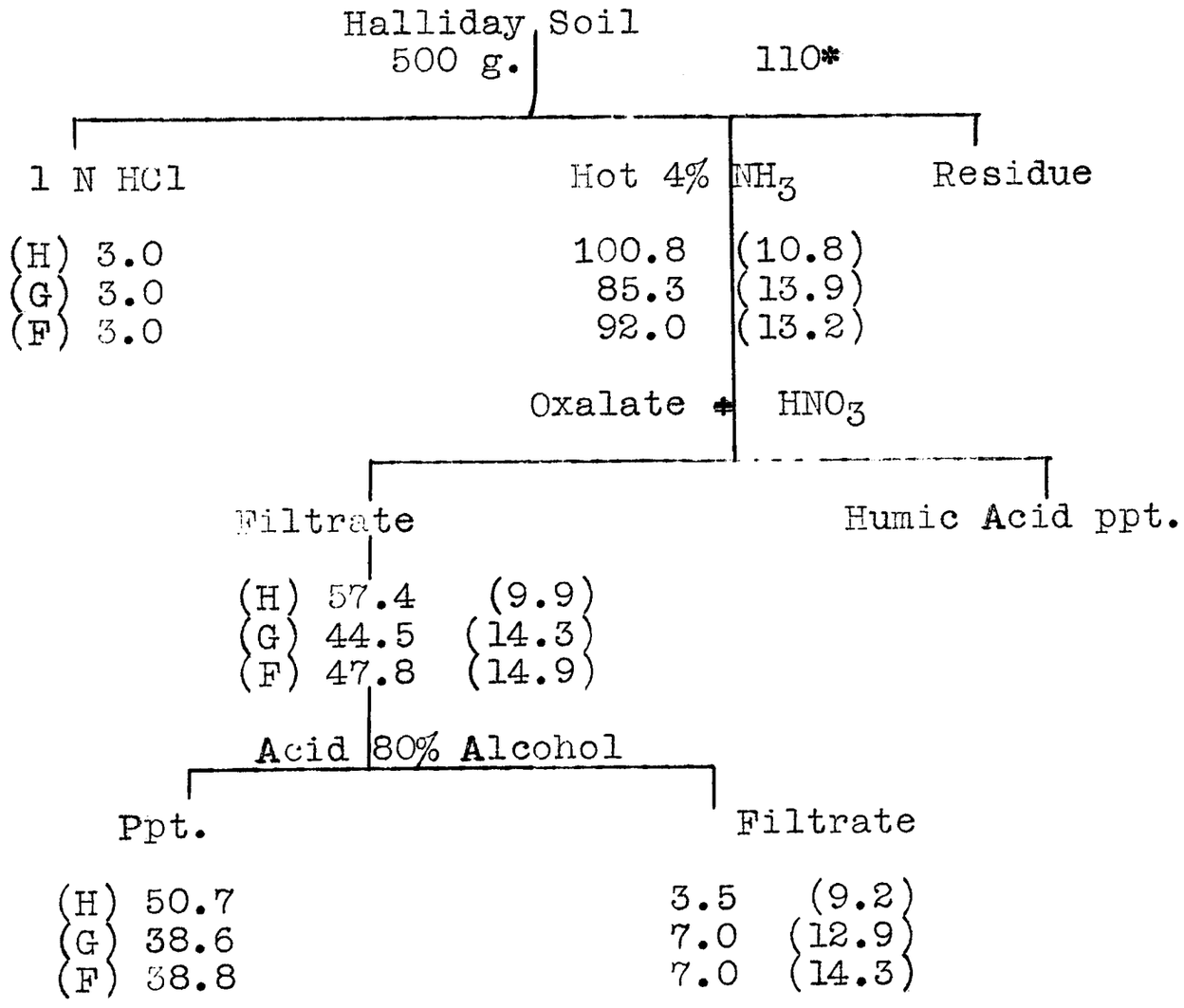
The precipitate contained a higher proportion of organic P than any obtained before in this work and represents a yield of 45 per cent of the soil organic P.

Expt. G and F were carried out in exactly the same way as the above.

The phosphorus distribution in the various fractions obtained in these three experiments is tabulated in Table VIII and in the accompanying diagram.

The diagram shows that the inorganic phosphate in the ammonia extract was practically all recovered in the alcoholic filtrate, and therefore that the nucleotide precipitate contained very little inorganic phosphate.

In experiments G and F the extraction of organic P by ammonia was not quite as complete as in the first expt. H, being 78 and 84 per cent as compared with 92 per cent.



*The figures show the phosphorus content in the various extracts expressed as mg. P per 100 g. soil. The figures in parenthesis represent inorganic phosphorus, the others organic phosphorus.

TABLE VIII. ORGANIC PHOSPHORUS DISTRIBUTION IN VARIOUS FRACTIONS IN THREE ISOLATION EXPERIMENTS.

EXPT.	ORG. P NH_3 EXTRACT		ORG. P HUMIC ACID FILTRATE		
	mg.P per 100 g. soil	% total soil org. P	mg.P per 100 g. soil	% Org. P NH_3 Ext.	% Soil Org.P
H	100.8	92.0	57.4	53.2	52.2
G	85.3	78.0	44.5	52.2	40.5
F	92.0	84.0	47.8	52.0	43.5

	ORG. P ALCOHOLIC FILTRATE			ORG. P NUCLEOTIDE PPT.				
	Mg.P per 100 g. soil	% org.P H.A. filtrate	% soil org. P	Weight	% P	mg. P per 100 g. soil	%org.P NHz ext.	% soil org.P
H	3.4	5.92	3.1	6.1389	4.05	50.7	50.3	46
G	7.0	15.7	6.3	5.7878	3.25	38.6	45.2	35
F	7.0	14.6	6.3	11.6527	1.62	38.8	42.2	35

The proportion of organic P contained in the humic acid filtrate is remarkably constant varying only from 52.0 per cent to 53.2 per cent of the organic P of the ammonia extract. It also agrees with the values obtained in the oxalate experiment for this same soil which ranged from 52.0--53.7. This constant ratio seems to indicate a very definite fractionation possibly of two distinct phosphorus compounds.

Precipitation with 80 per cent alcohol gave a satisfactory separation of the organic P from solution. It is recommended that the solution should stand with the alcohol overnight since in experiments G and F in which the solution was allowed to stand only an hour before filtration, less complete precipitation was obtained than in the experiment H, and on standing the filtrate of G and F deposited a further small amount of precipitate which, however, is not included in the analyses.

The nucleotide precipitate from experiment F contained a large amount of salt crystals (perhaps oxalic acid).

The organic P distribution in the various extracts has been calculated and is given in Table IX.

TABLE IX. DISTRIBUTION OF ORGANIC PHOSPHORUS IN VARIOUS SOIL FRACTIONS.

EXPT.	ORG. P - % TOTAL SOIL ORG. P						
	Acid Ext.	Soil Residue *	Alc. Filtrate	Humic Acid Ppt.	Isolated	Total	Error
H	3.0	6.2	3.1	39.8	46.0	98.1	-1.9
G	3.0	21.7	6.3	37.5	35.1	103.6	+3.6
F	3.0	15.0	6.3	40.5	35.3	100.1	+0.1

* Includes also the organic P decomposed by hot NH_3, if any.

This table shows that about 40 per cent of the soil organic phosphorus remained with the humic acid precipitate. From 35--46 per cent of the soil organic P was actually isolated. This compares very favourably with the yields of 5--15 per cent obtained with the former methods.

The degree of extraction of organic P from the soil is very satisfactory, only 6--20 per cent remaining in the soil residue, and this fraction includes also any organic P decomposed by hot NH_4OH (Smith (1939) having found indications that a small fraction was labile to ammonia at boiling temperature.)

This, varying only from 98--103, is convincing proof for the accuracy of our method of phosphate determination and its applicability to these soil extracts. This experiment included inorganic and total phosphate determinations on acid and alkaline soil extracts, sometimes deeply colored, on alkaline solutions, and also on solutions containing oxalate and the results have been wholly satisfactory. No more thorough test could be made of the applicability of our method to the analysis of soil extracts. Furthermore, this investigation could not have been carried out without such a method for phosphorus determination.

If it should prove that the organic P of the humic acid precipitate is of different identity then we shall have isolated a definite fraction of the soil organic phosphorus which in any case amounts to almost 50 per cent of the total. The importance of this in regard to studies on the identity of the organic P compounds is obvious.

9. Conclusions

Previous workers have concluded that the organic P separated from the soil by extraction with NH_3 and treatment of the humic acid filtrate with alcohol was of nucleic acid or nucleotide nature. As the present work progressed, however, it became increasingly evident that the organic phosphorus compounds present were much more stable than nucleic acid or the simple purine nucleotides.

For a long time therefore, there was no indication of just what organic compound or compounds were present. It was thought that the ordinary nucleotides might form a complex with the humic acid or soil organic matter in such a way that the ordinarily labile nucleotides were

now quite resistant to dephosphorylation, since it was impossible to purify the isolated organic P from the humic acid material.

The inefficiency of the early attempts to separate organic P from soil (7--13% recovery) was due to a variety of causes and we may now enumerate some of these.

One or even two cold ammonia extractions removes only a part of the soil organic P, usually less than 50%. Probably the most important single factor causing low recovery was the carrying down of organic P by the humic acid precipitate, only 5 to 30% remaining in the filtrate. Incomplete precipitation of organic P by 80% alcohol also accounted for a loss of organic P in some cases. The final product contained so much contaminating humic material that filtration and collection of the precipitate was often quite difficult. Most of these difficulties have now been overcome.

It has been known for a long time that preleaching the soil with HCl increases the amount of organic P subsequently extracted by alkali. Schollenberger (1918) assumed that this was due to the removal of Ca. Thus the insoluble Ca humates and other Ca salts were decomposed and the humic acid, etc. would then be dissolved by the alkali.

Hot ammonia treatment (preceded by leaching) extracts over 80% of the total organic P of the soil, thus increasing greatly the efficiency of extraction. Smith (1939) has shown that in some soils there are indications that a small fraction (10% or less) of the organic P is labile to boiling NH_3, but that this effect was negligible at temperatures almost approaching the boiling point. In most cases the temperature of extraction was approximately 75% so that it would seem that little decomposition of organic P occurred here, certainly not over 10--15% (since extraction was over 80% in most cases).

Nucleic acid and its derivatives, and phytin are all soluble in ammonia and should be extracted by ammonia from the soil. Treatment with hot ammonia would decompose Fe phytate with the formation of soluble ammonium phytate. If the soil was not leached free from Ca by the preliminary acid treatment then the phytin might be partially reprecipitated as the slightly soluble phytate. Thus ammonia or alkalies in general should dissolve the greater part of the soil organic P.

The results of the extraction experiments show quite definitely that preleaching with HCl is necessary, and further that this is principally because of its effect in rendering the soil organic matter soluble in the subsequent alkali extraction.

The data on the extraction of organic phosphorus from soil by various methods, showed that the ratio of the amount of organic phosphorus extracted to the amount of carbon extracted, remained practically constant regardless of the method used or the degree of extraction. The work of Hobson and Page (1932) leads to a similar conclusion in regard to the extraction of carbon and nitrogen from the soil. Their work showed also that the proportion of easily hydrolyzable N compounds (as percentage of total soil N) was much higher if the organic matter was first brought into solution by suitable means, (NH_4OH, NaOH, etc.).

Our results and those of Hobson and Page both indicate that the organic matter is bound in the soil in such a way that even the soluble constituents may not be removed without dissolving or peptizing the whole of the organic matter complex.

One possible explanation of this fact is that the organic matter containing soluble and insoluble compounds in a more or less homogeneous mixture, is deposited in the clay-humus colloid, or is adsorbed on the soil particles, and then is incorporated in the soil particles with the colloidal clay or clay-

humus colloidal material which coats the particles. A large part of the soluble material would thus be out of contact with the soil solution, or any extracting solvent, and would behave as an insoluble material unless the clay-humus gel were first peptized. This theory agrees with the experimental results. Similarly the organic matter of the inner particle layers would be relatively unavailable to the action of the soil micro-organisms unless the whole colloidal structure was first attacked.

Formerly, it was believed that most of the organic P remained in solution when humic acid was precipitated from the ammonia extracts by acidification. The results with the Macdonald College soil (p. 22) show that this is not so. This is probably the main reason why such poor results were obtained in the early isolation experiments. This low recovery of organic P in the humic acid filtrate may be due in large part to the more complete extraction of organic P compounds from the soil. It is probable that the more difficultly extractable compounds are also more readily precipitated with humic acid. (It now seems likely that ferric phytate is more completely dissolved by hot ammonia, and that ferric phytate is reformed on acidifying the solution and precipitates with the humic acid).

Experiments with different soils showed that the partition varied, the recovery being very low with the mineral soils (8--15%) and higher with the muck soils (30%).

The addition of oxalate was found to increase the soluble organic P by large amounts in all soils (now 40--50%). This effect may be partly due to the precipitation of Ca but it seems more likely to be due to the binding of Fe in an unionized complex with the oxalic acid (α-OH cpd.) Reprecipitation of the humic acid now yields almost no further amount of organic P although it still contains about 50% of the ammonia soluble organic P in most cases. With the Halliday soil the partition varied only

from 52--53% in small or large scale experiments. This would seem to indicate a fractionation of two distinct P compounds. It seems then that without the presence of oxalate the organic P precipitating with the humic acid contains a large amount of ferric phytate. The titration of phytin by $FeCl_3$ at the thiocyanate endpoint (see later) would indicate that the dissociation constants of ferric phytate, and ferric thiocyanate were almost the same. Also it is probable that that of ferric oxalate is of similar magnitude. Thus it may be that some ferric phytate is still being precipitated with the humic acid.

The method of extracting soil with strong HCl (6 N) gave yields of only about 10% of the soil organic P. The strong HCl would probably decompose ferric phytate thus allowing some phytin to dissolve. The acid had very little dissolving action on the organic matter thus the only plausible reason for the small amount of organic P extracted seems to be that the organic P is protected by, or buried in the soil organic matter or colloidal material so that the acid cannot bring it into solution.

Since the oxalate treatment was satisfactory in increasing the recovery of organic P, the method was used in the separation of larger amounts of soil organic P. The results were quite satisfactory and allowed the isolation of 35--46% of the total organic P of the Halliday soil (compared with yields of 5--15% with former methods). The distribution of the organic P in the extracts was determined and all was accounted for. The acid extract (1 N HCl) contained only about 3% of the soil organic P. This is probably composed of soluble nucleotide compounds and perhaps a small amount of phytin. After hot ammonia extraction the soil residue contained only 6--20% (by difference) of the soil organic P. This includes any organic P decomposed by the hot ammonia treatment and the remainder is presumably not dissolved. If Ca were not leached completely from the soil, it is probable that Ca phytate would precipitate at this point. In addition, ferric phytate may not have been completely decomposed.

The humic acid precipitate contained 38--40% of the soil organic P. A large part of this is probably ferric phytate, even though exalate ion was present in solution as mentioned above. Probably the remainder is made up largely of nucleotide compounds. These may be adsorbed on the humic acid colloids, or chemical complexes with the humic acid or lignin humus may actually be formed. The humic acid precipitate contains a very high proportion of NaOH-stable organic P (see later section). Thus the pyrimidine nucleotides may be adsorbed to greater extent than the purines. But since the humic acid filtrate contains just as much NaOH-stable organic P it seems likely that there is not as much purine nucleotide as pyrimidine in the soil. Again it is probable that the simple nucleotides are not present in appreciable amounts. The 25 per cent enzyme-stable residue of Gulland and Jackson is probably the principal nucleotide constituent of the soil organic phosphorus. Since its constitution or behavior to NaOH is unknown, we may only say that its properties are probably in accord with the above facts. Since phosphoproteins are very labile to hot alkalies it seems unlikely that any large portion is present in this fraction.

The organic phosphorus of the humic acid filtrate was almost completely precipitated by acid 80% alcohol, only about 3% remaining in solution. This is probably due to incomplete precipitation of phytin and nucleotide P. The fraction precipitated by alcohol contained 35--46% of the total soil organic P. Later examination of this precipitate shows that it is composed of about equal amounts of phytin and nucleotides. A small amount of xanthylic acid was found in the nucleotide fraction and the remainder appeared to be made up of pyrimidine nucleotides.

It appears that, although the oxalate method gave a big increase in total yield of soil organic P, this increase is probably almost all due to

phytin so that the amount of nucleotide material isolated may be practically the same.

The above discussion is intended to interpret as fully as possible the data on the extraction and fractionation of soil organic phosphorus on the basis of present knowledge of the phosphorus compounds involved and the nature of the colloidal complex with which they are intimately associated.

B. ANALYSIS AND CONSTITUENTS OF THE SOIL 'NUCLEOTIDE' PREPARATION.

1. Soil 'Nucleotide' Purification

An attempt was made to remove some of the contaminating humic acid by dissolving the substance in ammonia and then precipitating the humic acid by acidification with HNO_3. This process was tried using 1 g. ppt. Ga but it was found that no organic material was removed.

Accordingly the material was purified by reprecipitation in acid alcohol. One such experiment is as follows: 3 g. ppt.(G)$_1^{(A33)}$ were dissolved in about 125 ml. H_2O + 3 ml. NH_4OH. The solution was filtered, a 5 per cent excess of HCl was added and the liquid was poured into absolute alcohol such that the final concentration of alcohol was 80 per cent. After standing the precipitate was filtered off, triturated with a small amount of absolute alcohol and dried in a vacuum desiccator over concentrated H_2SO_4.

1 g. ppt.(G) yielded 0.454 g. ppt. (10p) containing 4.53% P while
3 g. ppt.(G) yielded 1.915 g. ppt. (11p) containing 4.20% P.
5.79 g. ppt.(H) yielded 3.607 g. ppt. (13p) containing
5.88% P after the reprecipitation was repeated 3 times. The recovery of organic phosphorus was 90 per cent.

In each case the alcoholic filtrate contained only a very small amount of organic P (1 mg. P or less). Ppt.(F) probably contained oxalic acid as an impurity since the alcoholic filtrate contained fairly large amounts of oxalate ion. (Shown by low recovery of added inorganic phosphate). When the precipitate from the first alcoholic precipitation was dissolved in water and then acidified with 5 per cent HCl, part of the material was thrown out of solution as a gel. This agrees with the fact that nucleic acids and the hydrochlorides of the individual nucleotides are precipitated in strongly acid solutions. Phytin in fairly concentrated solution is also precipitated under these conditions.

After three reprecipitations the yield from ppt. Fa was 3.739 g. material (12 P) contained 4.50 per cent P (91% recovery of organic P).

It was evident that further reprecipitation did not increase the purity appreciably.

2. Analysis of the Preparations

(a) Nitrogen Content

The nucleotides contain nitrogen as well as P, so the purified preparations were analyzed for their N content.

The micro-Kjehldahl method according to Pregl (1937) was used for this determination. The sample used was about 25 mg. and 2 ml. of Campbell and Hanna's (1937) digestion mixture was used for each sample. The digestion was continued for about 1 to 1½ hours, the time being prolonged in some cases by the addition of a drop of alcohol as recommended by Pregl. Approximately N/50 acid (HCl) and base (NaOH) were used. The blank was found to be 0.02 mg. N. The results are shown in Table X.

TABLE X. NITROGEN CONTENT OF PREPARATIONS

SAMPLE	WEIGHT	PERCENT N FOUND	
Sucrose	25.0 mg.	0.090	(blank = 0.02 mg. N)
Ammonium oxalate	20.4	19.54	(Theoretical = 19.44)
	20.0	19.44	
Ppt. 12p	21.1	2.89	
"	22.4	2.82	2.84 (≠ 0.03)
"	31.9	2.81	
Ppt. 13p	23.2	1.91	
"	27.4	1.81	
"	23.5	2.04	1.92 (≠ 0.06)
"	30.9	1.93	

The N content of the preparations was very low. The N/P ratio for preparation 12p is 0.63 and for 13p, 0.33, while that for yeast nucleic acid is 1.69. It is thus evident that the N content is much lower than would be the case if the organic P consisted only of simple nucleotides.

Supposing the purine nucleotide to be xanthylic acid and the pyrimidine to be uridylic acid and these present in the ratio found in the next section, only 34 per cent of the P would be accounted for in ppt. 13p and only 69 per cent in ppt. 12p.

Phytin seemed to be the only other organic P compound likely to be present and an attempt was made later to confirm the presence of phytin in the material.

(b) Purine Nitrogen Content

The determination of purine N enables us to estimate the proportion of purine and pyrimidine nucleotides, assuming that the N present is all nucleotide N.

Purine N was determined by a micro-modification of the methods of Tipson and Levene (1939) and of Leven/e and Jorpes (1930).

A sample of the material (about 200 mg.) was hydrolyzed by refluxing with 10 ml. of 5 per cent H_2SO_4 for four hours, the solution being heated by means of a micro-burner. To the hot solution was added a hot solution of Ag_2SO_4 (approx. 1 per cent) until the purines were completely precipitated. The solution was cooled and allowed to stand overnight in the refrigerator. The precipitate of silver purines was then centrifuged off and washed twice with Ag_2SO_4 soln. The bases were dissolved in an excess of hot 1 N HCl solution. The precipitated AgCl was filtered off and washed with hot water (slightly acidulated with HCl). The filtrate was made to 50 ml. and N was determined in 25 ml. aliquots by the micro-Kjehdahl method.

The determination was also carried out on a sample of yeast nucleic acid.

The Ag purine precipitate obtained with the soil nucleotides contained considerable dark colored material. It was found that the Ag purine salts were not completely precipitated unless the solution was allowed to stand overnight in the refrigerator. The results are given in Table XI.

TABLE XI. PURINE N CONTENT OF PREPARATIONS.

SAMPLE	WEIGHT MG.	PURINE N FOUND %	PURINE N, % OF TOTAL N.
Yeast nucleic acid	102.2	9.14	66.0 (theoretical = 66.7)
Ppt. 12p	202.8	0.21	7.4
Ppt. 13p	292.6	0.218	11.4

In the case of ppt. 13p the N remaining in the filtrate from the precipitation of Ag purines was determined and found to correspond to 1.71 per cent N (of original material). This agrees with the total N minus purine N, 1.70 per cent.

The results for yeast nucleic acid show very good agreement with the theoretical:

Found, 66.0 per cent purine N, theoretical, 66.7 per cent.

The purine N content of the nucleotide preparations is very low, only 7 to 11 per cent of the total N. This indicates that the preparation contains a much higher proportion of pyrimidine than purine nucleotides, provided that all the total N is nucleotide N.

This suggests that the purine nucleotides are absorbed to a large extent by the humic acid or that they are easily decomposed in the soil.

3. Purine Constituents

An adaptation of the methods given by Schmidt and Levene (1938), Levene and Jorpes (1930) and Levene and Bass (1931, p. 110) was selected as the best for this purpose.

A quantity of soil nucleotide preparation was first hydrolyzed as for the determination of purine N above. The purines were precipitated by Ag_2SO_4 and the Ag precipitate decomposed with HCl also in the same manner as above. The procedure of Levene and Bass was used for the separation of adenine and guanine. The HCl solution of the purines was neutralized to Congo red by the addition of strong NaOH solution. The guanine thus precipitated was filtered off while still warm. It was dissolved in dilute HCl and reprecipitated by NaOH (to remove traces of adenine). The filtrate was concentrated and while hot was treated with a saturated solution of picric acid. The precipitate of adenine picrate was then filtered off.

This procedure gave satisfactory results with a yeast nucleic acid hydrolysate. A good precipitate of guanine was obtained when either NaOH or NH_3 was used for neutralization. A crystalline precipitate of adenine picrate was also obtained in good yield.

In the case of the hydrolysate of the soil nucleotide, a gelatinous precipitate was obtained immediately on adding NaOH, but this precipitate redissolved again at the Congo red neutral point. No precipitation occurred on standing. Guanine therefore appeared to be absent. On adding picric acid solution, no crystals were formed so that adenine also appeared to be absent.

It seemed possible that the gel which first appeared might be xanthine. This purine forms salts with strong mineral acids but they are very easily hydrolyzed. The base then precipitates being insoluble

in dilute acids and water. It is soluble in alkalies and so dissolves on neutralizing a solution with NaOH. Thus the separation of xanthine from other purines is possible because of its insolubility in dilute HCl, the hydrochlorides of the other purines being stable and soluble under these conditions.

A few drops of 2 N NaOH were added to the hydrolysate of 13p. A small amount of gel precipitate appeared which on centrifuging off appeared quite brown in color, having removed considerable coloring matter from solution. This precipitate was insoluble in dilute H_2SO_4 but very soluble in NaOH. (It appeared to be humic acid mixed with some xanthine.)

On adding a few drops more NaOH to the solution a colorless gel was precipitated and the solution after centrifuging off the gel was almost colorless. This precipitate was soluble in NH_3 and could be reprecipitated by H_2SO_4 or 1 N HCl. Therefore it is not hypoxanthine or guanine. (Guanine is soluble in dilute acids and hypoxanthine is insoluble in NH_3.)

NaOH was added to the solution until it was just acid to Congo red and a further quantity of gel was precipitated. It was centrifuged off. On making the solution just alkaline a very small amount of red-brown gel precipitate separated. This appeared to be composed mainly of the remaining coloring matter of the solution along with a very small amount of gel (possibly a trace of guanine).

This experiment confirms the presence of xanthine.

Again no precipitate was obtained with picric acid so adenine was absent.

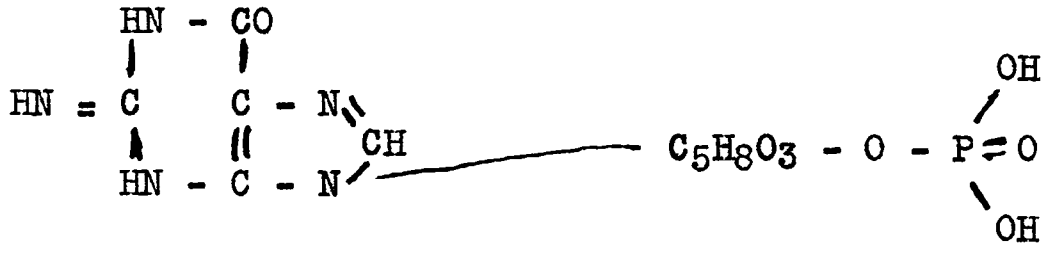
The xanthine reaction (Murexide test) (Levene & Bass 1931, p.112) was used extensively in these experiments to indicate the presence of the purines. The reaction is given by xanthine and guanine but not by adenine.

The purine solutions above gave a positive xanthine test and the xanthine precipitate gave a very strong reaction and also responded to Wiedel's test - (a modified xanthine test not given by guanine). The amount of xanthine precipitate obtained was too small to allow making further analyses.

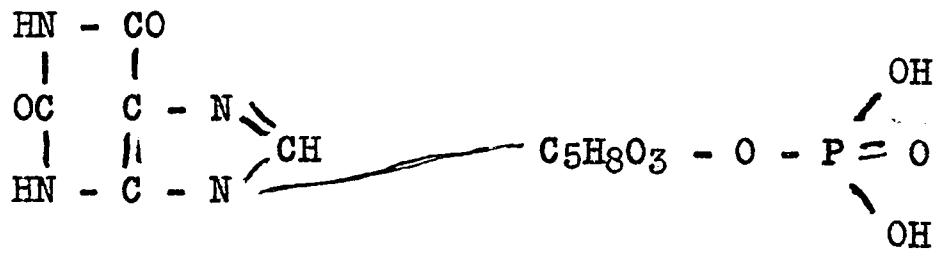
The accumulated evidence indicates that the purine in this soil preparation is xanthine. The presence of free xanthine in the soil has been reported by Schreiner and Shorey (1910). Its presence would be accounted for by the decomposition of adenylic or guanylic acid and oxidation of the purine constituent to xanthine. Xanthylic acid has not been reported in soil up to this time.

Its presence in soil could be accounted for by the deamination of guanylic acid (see formulae)

Guanylic Acid



Xanthylic Acid



There are certain enzymes which are present in soil micro-organisms (Waksman 1932, p. 421) that carry out this process. However, the origin of xanthylic acid in soil may be due to a specific synthesis of this substance by micro-organisms.

Under the conditions of isolation the adenylic or guanylic acids do not give xanthine as a hydrolytic product, so the data indicate the presence of xanthylic acid in the soil. Further, adenine and guanine were obtained in the control experiment with yeast nucleic acid so it seems that adenylic and guanylic acids must be absent from the soil preparation.

Adenylic acid was the only purine nucleotide found by Bottomley (1919) and by Wrenshall and McKibbin (1937) in experiments on peat and muck soils.

It is not contended that the A_1 layer of the Halliday podsol is representative of all Quebec podsols but that probably the nucleotide constituent varies in different types of soils.

4. Pyrimidine Constituents.

The method cited in Levene and Bass (1931, p.58) was used.

The material is hydrolyzed for 2 hours at 175° C. in an autoclave with 25 per cent H_2SO_4. After hydrolysis sulphuric and phosphoric acids are removed by $Ba(OH)_2$, Ba ions are removed quantitatively by H_2SO_4. The solution is acidified and the purines are precipitated by Ag_2SO_4 solution. After filtration the filtrate is saturated with $Ba(OH)_2$. The precipitate consists of the Ag salts of the pyrimidines. This precipitate is suspended in water and decomposed by H_2S. AgS and excess H_2S are removed and the solution is concentrated under reduced pressure. (Thymine now separates out if present.) Ba ions are removed by H_2SO_4. The warm filtrate is treated with a saturated solution of picric acid. The precipitate of cytosine picrate is filtered off and a second crop obtained by further evaporation.

The excess picric acid in the filtrate is removed by H_2SO_4 and ether, and then the excess H_2SO_4 is removed by $Ba(OH)_2$ and the solution is concentrated under reduced pressure. Uracil precipitates on cooling.

5 g. of yeast nucleic acid were hydrolyzed in the above manner and also 3 g. of the soil nucleotide (2 g. ppt. 12p / 1 g. ppt. 13p). A good yield of cytosine picrate was obtained from the yeast nucleic acid but none could be detected in the soil nucleotide hydrolysate. Similarly, uracil crystals were obtained from the yeast nucleic acid but none were obtained from the soil nucleotide. The cytosine and uracil from the yeast nucleic acid both gave very strong positive reactions with the Wheeler and Johnson test for pyrimidines (Levene and Bass 1931, p.61) but the soil nucleotide hydrolysate gave no response. These negative results are somewhat inconclusive. It may be technically impossible to identify the pyrimidines by this experiment in view of the small quantity of preparation available for hydrolysis (3 grams) and the relatively low nitrogen content of the preparation. On the other hand there is a distinct possibility that pyrimidines are actually absent from this preparation. It is true that a high proportion of the nitrogen present is not purine nitrogen, but there is no direct experimental evidence that it is pyrimidine nitrogen, other than separation of a precipitate of Ag salt from alkaline solution. It is noteworthy that the Wheeler and Johnson test for pyrimidines was negative.

5. Carbohydrate Constituents

If the compound contains nucleotides, these would be split by hydrolysis into the component purines, pyrimidines, sugar, and phosphoric acid. Various sugar tests were applied to the solution of 12p and 13p after hydrolysis to detect the presence of any sugars liberated by the hydrolysis.

The Molisch test (Hawk and Bergeim 1927, p.71) was positive and Bial's and Tollen's tests (p.86) for pentoses were also definitely positive indicating the presence of pentoses. The Feulgen and Pine shaving tests (Levene and Bass 1931, p.259) for desoxy sugars were negative - thus showing that the sugar is probably ribose.

6. Other Constituents

Phosphoric acid is liberated on hydrolysis of the compound by H_2SO_4 as evidenced by positive colorimetric tests for phosphate ion.

Since the compound contains nitrogen it is of interest to determine if the compound contains any proteinaceous material.

Accordingly the various biochemical tests for protein were applied. (Hawk and Bergeim 1922, p.162)

Negative results were obtained with Millon's Test, Biuret Test, xanthoproteic reaction and the glyoxylic acid reaction, on solutions of the materials 12p and 13p - either before or after hydrolysis. Thus the preparations are assumed to be free from protein.

7. Conclusions

It has been shown that the isolated preparation contains phosphoric acid, a pentose sugar (probably ribose) and nitrogen.

It is quite possible that some humic acid or lignin-humus material is contained in the preparation and these might account for a part of the N. Xanthine has been shown to be present thus it is concluded that xanthylic acid is present. Since the purine N accounted for only 10 per cent of the total N, the remainder is probably present, as pyrimidine nucleotides - cytidylic or uridylic acid, as lignin-humus nitrogen, or as some other unidentified nitrogen compound.

As stated elsewhere the 25% undecomposed residue of Gulland and Jackson does not fit in with the present day structure of nucleic acid (Levene's formula) and thus it is of unknown constitution. It may not, therefore, conform to the ordinary reactions of the purine and pyrimidine nucleotides and thus it is possible that the ordinary schemes for separation and hydrolysis of these nucleotides may not be applied to the material under investigation, although they are applicable to nucleic acid which contains this fraction. The identification of xanthylic acid is a new result, and as outlined above it is quite reasonable that this compound should occur in the soil.

Adenylic acid was not isolated and it seems impossible that this compound may remain with the humic acid precipitate. No evidence could be obtained for the presence of pyrimidine nucleotides, but since such small quantities of material were available no significance is attached to this finding.

It is now obvious that the pyrimidine N (precipitable by Ag_2SO_4 in saturated $Ba(OH)_2$ solution)should have been determined as well as the purine N. We would then have an indication of the amount of non-nucleotide N present.

However this does not alter the fact that the most significant conclusion obtained from the examination of the isolated organic phosphoric material is that the nucleotides do not account for all the organic phosphorus, and that the other organic phosphorus compound may by phytin.

C. NaOH DECOMPOSITION OF ORGANIC PHOSPHORUS

1. Introduction

Smith (1939) has found that boiling dilute NaOH hydrolyzed a definite portion of the soil organic P (about 45 per cent being decomposed in the Halliday soil). This result suggests that there may be two or more distinct compounds comprising the soil organic phosphorus.

A pertiment question was now the determination of whether the partition of organic P between the humic acid precipitate and filtrate was related to the susceptibility of decomposition by NaOH of the organic P of each of these fractions.

2. Method

In the preliminary experiments amounts of solution corresponding to 1g. of soil were placed in 100 ml. Erlemeyer flasks, evaporated to 25 ml. and 1.25 g. NaOH was added to make a 5 per cent NaOH solution. The solution was then refluxed under a reflux condenser for 4 hours. Although the flasks had been thoroughly weathered by boiling with acid and alkali it was found that the P content of the blanks was very high amounting to nearly 25 per cent of the organic P content of the extracts. Thus the results obtained were not very satisfactory. Short pyrex test-tubes which loosely fitted the mouth of the Erlemmeyer flasks were found to be more suitable than the ordinary reflux condensers. A stream of cold water was kept circulating through these tubes.

With this arrangement it was found that the P content of the blanks was quite constant for a given time of refluxing. Refluxing for a period of 4 hours was found sufficient by Smith, and this period was used throughout.

In order to reduce the relative magnitude of the blank aliquots corresponding to about 5 g. soil were used in the later experiments. The blank then amounted to about 5 per cent of the organic phosphorus being hydrolyzed.

3. Decomposition of Various Fractions

(a) Humic Acid Precipitate and Filtrate

Humic acid filtrates and precipitates were prepared from the Halliday soil as described above. A solution of the humic acid in dilute ammonia was used for hydrolysis. Aliquots of solution corresponding to 5 g. soil were refluxed 4 hours as above. The solution was then filtered and the filtrate and washings made to 250 ml. after which inorganic phosphate was determined. The results are given in Table XII.

This experiment shows that the organic P of the humic acid filtrate is quite stable to hydrolysis by NaOH. The hydrolysis found, 4.5 per cent is quite small and could be accounted for by the slow partial hydrolysis of resistant compounds.

The organic P of the humic acid precipitate is decomposed to the extent of 27.4 per cent. Thus there is a definite difference in the stability of the organic P of the two extracts but not enough to postulate the separation into two definite P compounds.

In this soil Smith (1939) found about 68 mg. org. P per 100 g. soil stable to NaOH. 48 mg. of this has been separated in the humic acid filtrate. The remaining 20 mg. resistant organic P presumably remains with the humic acid. The analyses show that 22 mg. (30-8) undecomposable organic P remain in this fraction, so the results check very satisfactorily. Thus we have removed only about 70 per cent of the NaOH stable fraction of the organic P from the humic acid.

(b) Nucleotide Precipitate.

The decomposability of the organic P in the isolated fraction was then determined. 0.615 g. ppt. G was dissolved in 2 ml. dilute NH_4OH and water was added to make 100 ml. Aliquots of 25 ml. were used for hydrolysis which was carried out as in the above experiments.

TABLE XII. NaOH DECOMPOSITION OF ORGANIC PHOSPHORUS IN HUMIC ACID PRECIPITATE AND FILTRATE

EXTRACT		INORG. P ORIGINAL	INORG. P. AFTER RE-FLUXING	BLANK	DECOMPOSITION	
		mg. P per 100 g. soil				% Org. P*
Humic Acid Filtrate	1	16.5	21.4	2.6	2.3	4.8
	2	16.5	20.7	2.0	2.2	4.6 4.5(±0.5)
	3	16.5	20.8	2.6	1.7	3.5 (Blank = 5.2%)
	4	16.5	20.9	2.0	2.4	5.0
Solution of Humic Acid Precipitate	1	1.4	11.6	2.6	7.6	25.4
	2	1.4	11.2	2.0	7.8	26.1
	3	1.4	12.3	2.6	8.3	27.8 27.4(±1.7)
	4	1.4	12.5	2.0	9.1	30.4 (Blank = 7.7%)

*Org. P Humic acid filtrate = 48.0 mg. P per 100 g. soil, humic acid soln. = 29.9

The data in Table XIII show that 27.7 per cent of the organic P was decomposed.

4. Conclusions

The results of these experiments, although not wholly satisfactory, show that the product isolated contains a large proportion of the NaOH-stable organic P.

The purine nucleotides are easily hydrolyzed by NaOH but phytin is much more resistant (Plimmer 1913) and is probably in the NaOH-stable class of organic phosphorus compounds. The phosphoprotein type of organic phosphorus compound is easily decomposed by NaOH (Plimmer 1913) so that it is probable that only small amounts of this compound exist in the soils.

TABLE XIII. NaOH HYDROLYSIS OF NUCLEOTIDE PRECIPITATE

	INORG.P Original	INORG.P After Refluxing	BLANK	DECOMPOSITION	
	% Nucleotide ppt.				% Org.P*
1	0.04	0.970	0.065	0.865	27.4
2	0.04	0.974	0.049	0.885	28.1)27.7(±0.3)
3	0.04	0.974	0.065	0.869	27.6**

*Org. P = 3.15%

**Blank = 2% org. P.

D. SOIL DECOMPOSITION EXPERIMENTS

1. Introduction

The evidence of culture solution experiments (see Introduction) indicate that organic P must first be hydrolyzed to inorganic phosphate before any appreciable amounts may be absorbed by plant roots. Schulow (1931), however, indicates that a small amount of phytin may be assimilated directly by plant roots.

If the large store of organic P in our soils is to be of use to plants then it must be brought actively into the P cycle. It almost seems that in our infertile podsol soils, the organic P is not being continuously synthesized and decomposed as in fertile soils but represents an accumulation of stable organic P.

The more we know about this organic P in our soils, the more we will know how to render this great store of P available for plant use. Therefore we have designed experiments with the object of studying the relative stability of various organic P compounds when they are added to the soil.

The synthesis and decomposition of organic phosphorus compounds in the soil is probably almost completely a micro-organic process, the hydrolysis of organic P compounds being effected by enzymes and the synthesis by assimilation of inorganic phosphate by the bacterial cell.

There are no experiments cited in the literature on the actual decomposition of organic P compounds in the soil itself. Hilbert et al (1938), found that Na glycerophosphate added to the soil was rapidly changed to an insoluble form. They concluded that it was hydrolyzed by the soil micro-organisms and then the phosphate was fixed by the soil. It would seem that this type of compound is so readily attacked that it could not remain in the soil for any length of time and therefore hardly needs to be considered.

Lecithin represents only a small fraction of the soil organic P (2% or less) and therefore cannot be a factor in its accumulation.

Phosphoproteins, as casein, are very readily attacked by micro-organisms (Dox 1911). Kelley (1915) shows that casein is completely decomposed in 7 days in the soil. Therefore it seems unlikely that this material would accumulate in the soil.

Nucleic acid is very readily decomposed by phosphatase enzymes as are the individual nucleotides. Stoklasa (1911) assumed that the inorganic and organic phosphorus added to the soil by plant residues was mineralized and then assimilated by the micro-organisms into the microbial protoplasms(principally in the form of nucleotides and nucleic acid). The nucleic acid was then decomposed on dissolution of the dead microbial bodies and became available to the plant or to other micro-organisms.

As stated before various workers have shown that nucleic acid is readily decomposed and assimilated by plants in culture solutions and sand cultures. If nucleic acid is only 75 per cent dephosphorylated in soils, then it seems probable that the enzyme stable residue would accumulate and thus the accumulation of organic P in soil would occur. The identity or structure of the residue is still unknown and so we may only postulate that it is nucleotide in nature.

Phytin has not been detected in soil up to the present. As mentioned above Potter and Snyder (1918) found similar hydrolysis curves for phytin and the soil organic P and so thought that phytin might be present. Later workers however, have left phytin almost entirely out of consideration. Auten (1923) assumes that since it is so easily hydrolyzed by phytase that it could not accumulate in the soil. Again the decomposition of phytin in the soil itself has not been investigated.

The above discussion shows that the organic P compounds most likely to be present in soils are nucleic acid or one or more of its derivatives. This is rendered more probable because of the known presence of adenylic and uridylic acids in certain soils.

Therefore experiments were designed with the object of studying the decomposition of the above organic P compounds in the soil itself and determining whether any evidence could be obtained for the decomposition or synthesis of organic P in the soil itself.

2. First Soil Decomposition Experiment - Nucleic Acid, Soil Nucleotide Inorganic Phosphate, $Ca(OH)_2$ and Glucose

In the first experiment, nucleic acid, soil 'nucleotide' and inorganic phosphate were added to a St. Chlothilde muck soil with without treatments with $Ca(OH)_2$ and glucose. Since liming a soil decreases the organic P (Vincent 1937), $Ca(OH)_2$ was added to obtain the maximum decomposition of organic P. Glucose, on the other hand, was added to supply energy material for the stimulation of micro-organisms in order to obtain a synthesis of organic P.

The rate of treatment of the soils and plan of the treatment was as follows:

Plan of Treatments

	Nil	$Ca(OH)_2$	Glucose
Nil	1, 11	2, 12	3, 13
Nucleic Acid, Yeast	4, 14	5, 15	6, 16
Nucleotide, Soil	-	10, 20	-
$Ca(H_2PO_4)_2 \cdot H_2O$	7, 17	8, 18	9, 19

Rate of Treatment

Glucose, 1% of dry soil (0.3833 g. per 100 g. wet soil)

$Ca(OH)_2$. The equivalent of 2 tons $CaCO_3$ per acre (2,000,000 lbs.) (0.0567 g.)

$Ca(H_2PO_4)_2.H_2O$. 0.2% P of dry soil (0.3115 g.)

Yeast nucleic acid. 0.248% P of dry soil (0.4875 g.)

Soil nucleotide. 0.1% P of dry soil.

For each test, 100 gm. of soil was placed in a glass tumbler and the required amount of amendment added. Water (25 ml.) was then added to bring the soil to approximately the field moisture content and the tumblers were then stored in a dark room at fairly constant temperature (about 23.5° C.). Water was added from time to time to replace that lost by evaporation.

Samples of the treated soils were taken immediately and dried in the oven at 100° C.

To follow the decomposition dilute acid extractions (Quebec method) for readily soluble phosphate were used to indicate whether any decomposition of organic P, or vice versa, had taken place.

For the determination of readily soluble phosphate 0.5 gms. of the dried soil, ground to pass a 40-mesh sieve, were extracted with 100 ml. of Quebec solution (pH 3.0) (Wrenshall and McKibbin 1935) by shaking for 30 minutes. After filtering, inorganic phosphate was determined on the filtrate by the method of Dyer and Wrenshall (1938). The results are given in Table XIV.

The high results for yeast nucleic acid show that some decomposition has taken place either by the extracting acid or by heating the samples when they were being dried. To test this, a small amount of nucleic acid was heated to 100° C. and then extracted with Quebec

solution. The extract was found to have a very high phosphate content while nucleic acid extracted without heating yielded only a small amount of phosphate.

TABLE XIV

No.	Treatment		Readily Soluble P mg. P per 100 g. soil
1	Nil	Nil	8.00
11	"	"	8.00
2	"	$Ca(OH)_2$	6.68
4	Yeast N.A.	Nil	20.4
5	"	$Ca(OH)_2$	18.9
10	S. nucleotide	$Ca(OH)_2$	8.00 6.80
7	$Ca(H_2PO_4)_2.H_2O$	Nil	123.2*
8	"	$Ca(OH)_2$	125.0
9	"	Glucose	108.0

*Inorganic Phosphate added - 200 mg. P per 100 g. soil

The values for inorganic phosphate treatment show that one extraction is insufficient to extract completely the added inorganic phosphate. (54-62% extraction)

Since heating in the oven has resulted in the decomposition of yeast nucleic acid, the above results were considered to be unreliable. In order therefore to obtain initial values for comparison with the incubated soil, a new series of treatments was made up in exactly the same way as in the original case.

However, no glucose or $Ca(OH)_2$ was added since no differences were indicated in the above.

After bringing to the same moisture content as before, samples were taken and dried at about 35° C. for a few hours, then overnight at room

temperature. The soils were ground to 40-mesh, and moisture and Quebec soluble phosphate determinations were made. The results are given in Table XV. (Immediate)

Comparison of the values with those in Table XIV show that the soil should not be heated to a high temperature for drying.

After 45 days samples were again taken and analyzed as before. The results are reported in Table XV (45 days).

TABLE XV. SOLUBLE PHOSPHATE EXTRACTED BY QUEBEC SOLUTION
mg. P per 100 g. soil

Treatment	Time: Immediate	45 days
1. Nil Nil	3.54	2.55
2. " $Ca(OH)_2$	-	2.70
3. " Glucose	-	2.35
4. Nucleic Acid Nil	4.43	161.2
5. " $Ca(OH)_2$	-	161.8
6. " Glucose	-	163.0
7. $Ca(H_2PO_4)_2$ Nil	142.6	113.0
8. " $Ca(OH)_2$	-	109.0
9. " Glucose	-	108.3
10. Soil Nucleotide $Ca(OH)_2$	5.90	11.35

Table XV shows that the $Ca(OH)_2$ or glucose treatments did not affect the amount of soluble phosphate. This is in contrast to the usual finding that liming decreases organic P in soils. (Vincent 1937) Numerous instances might be cited where liming was found to give an increase in the available phosphate status of soils and also that calcareous soils generally have a lower organic P content. A decrease in soluble phosphate was obtained in two cases (nil, and inorganic phosphate). This may be due to a fixation of inorganic phosphate by the soil.

Table XVI shows the increase insoluble phosphate in the P treated soils over the nil, leaving the $Ca(OH)_2$ and glucose treatments out of consideration. Table XVII shows the increase in soluble phosphate due

to the mineralization of added organic P during 45 days. This is expressed also as percentage of the organic P added.

The results show that the yeast nucleic acid is decomposed to a large extent while the soil nucleotide was relatively very stable. The actual percentage decomposition figures are not very accurate since only between 55 and 70% of the added inorganic phosphate was recovered and so it is probable that the recovery of decomposed organic P is also low.

TABLE XVI. INCREASE IN SOLUBLE PHOSPHATE OVER UNTREATED SOIL DUE TO MINERALIZATION OF THE ORGANIC PHOSPHORUS ADDED
mg. P per 100 g. soil

TREATMENT	IMMEDIATE	45 DAYS
Yeast Nucleic Acid	0.89	159.5
Soil Nucleotide	2.36	8.82
Inorganic Phosphate	139.0	109.6

TABLE XVII. INCREASE IN SOLUBLE PHOSPHATE DURING 45 DAYS OVER THE IMMEDIATE VALUES DUE TO MINERALIZATION OF ORGANIC PHOSPHORUS

TREATMENT	INCREASE	ORGANIC P ADDED	% DECOMPOSITION
	mg. P per 100 g. soil		
Yeast Nucleic Acid	158.6	248	63.8
Soil Nucleotide	6.46	100	6.5
Inorganic Phosphate	-29.4	200*	-14.7

*Inorganic phosphate added

3. Second Soil Decomposition Experiment - Yeast, Nucleic Acid, Soil Nucleotide, Individual Nucleotides, Manure and Inorganic Phosphate

The results of the first decomposition experiment have shown that the soil 'nucleotide' behaves very differently from yeast nucleic acid.

Only derivatives of adenylic and uridylic acid had been obtained from the soil up to the time of this experiment and there was no evident reason why only two of the four nucleotides should persist in the soil. Therefore the next step was to prepare quantities of the nucleotides of ribonucleic acid, and to compare the decomposition of these compounds in the soil itself.

(a) Preparation of Nucleotides from Yeast Nucleic Acid.

The method of Levene (1931, p. 218) was used for the preparation of the nucleotides. 100 gms. of yeast nucleic acid was hydrolyzed in an autoclave with 550 ml. of 5 per cent ammonia at a temperature of 115° C. for one hour. Guanylic acid was precipitated by pouring into alcohol and it was purified as outlined by Levene. The other three nucleotides were separated by fractional crystallization of their brucine salts. In order to get complete separation nine recrystallizations are necessary, and the guanylic acid has also to be carried through this crystallization to purify it from uridylic acid. Each of the four fractions of purine salts was then decomposed by NH_4OH, precipitated by lead acetate and the lead salts were decomposed by H_2S. After repeating the lead precipitation several times, the solution of the free acid was evaporated at low temperature (Below 40°C.) and allowed to crystallize.

Difficulty was experienced in obtaining a crystalline product and after attempts to obtain crystalline uridylic acid and its diammonium salt had failed, the calcium salt was prepared. A small amount of $Ca\ Cl_2$ was added to the solution of uridylic acid and the solution was neutralized with $Ca(OH)_2$ solution. On concentration of the solution, the crystalline Ca salt was obtained.

The yields and analyses of the products are given in Table XVIII.

TABLE XVIII. PHOSPHORUS CONTENT OF NUCLEOTIDES

Substance	Yield Gms.	Inorg. Water Sol. PO_4, % P	Total P %	Organic P %	Org. (Theoret.) % P
Adenylic acid	7.2866	1.13	8.86	7.43	8.54
Guanylic acid	3.7164	2.10	8.60	6.50	8.94
Cytidylic acid	1.1589	3.07	10.21	7.13	9.59
Ca-uridylate	3.5346	0.354	6.25	5.90	8.56

The nucleotides contain small amounts of inorganic phosphate originating probably from hydrolysis during the preparation. The analysis shows that pure compounds were not obtained but the products are sufficiently pure for the present purpose.

It was observed that the solutions of the compounds were stable to the molybdate sulphuric acid solution (0.4 N H_2SO_4) used in the phosphate determination.

(b) Nucleotide Decomposition Experiment

An experiment was then planned to determine the behaviour of these nucleotides when they are added to the soil. Any decomposition of organic phosphorus compound added to the soil liberating inorganic phosphate, should produce a corresponding increase in the easily soluble phosphate in the soil. Accordingly, extractions of phosphate soluble in Quebec solution (pH 3) were made on the samples. It was found, on account of the high content of soluble phosphate with some treatments, that an additional extraction was necessary to recover all the readily soluble phosphate.

The soil treatments were as follows - nine tests being carried on. 1. Nil. 2. Yeast nucleic acid. 3. Soil nucleotide. 4. Guanylic acid. 5. Adenylic acid. 6. Cytidylic acid. 7. Ca uridylate. 8. Inorganic phosphate. 9. Manure. The manure treatment was started at a later date than the others and this necessitated a separate nil, 10. An amount of each phosphorus compound containing 25 mg. of phosphorus (P) was added to 50 g. samples of a Macdonald College soil (20 mesh). Thorough mixing was achieved by grinding the material with a few grams of soil then thoroughly incorporating this mixture with the remainder of the soil. The treated soil was placed in glass tumblers and 15 ml. of water was added to give a moisture content similar to that existing under field conditions.

The samples were then stored in a dark room, kept at a fairly constant temperature (23° C.). Water was occasionally added to replace that lost by evaporation. At intervals, a sample of about 3.5 g. (moist weight) was taken from each tumbler for analysis. Each sample was spread out in a thin layer on a watch glass and allowed to air dry at room temperature for about two days. The soil was then ground to pass a 40-mesh sieve and placed in a stoppered bottle. The moisture content of the samples was determined and all samples were found to have practically uniform moisture content (2.30 per cent H_2O) which corresponded almost exactly with the original moisture content of the soil, so that for comparative results the very slight differences were considered as not significant and were neglected.

Duplicate 1 g. samples of each soil were weighed out and extracted with 200 ml. Quebec solution by shaking for one-half hour as in the regular procedure. The residue was then re-extracted with 200 ml. Quebec solution as before. The concentration of inorganic phosphate in each extract was determined. The results are shown in Table XIX.

TABLE XIX. SOLUBLE PHOSPHATE EXTRACTED BY QUEBEC SOLUTION FROM SAMPLES TAKEN AT INTERVALS (COMBINED FIRST AND SECOND EXTRACTS)-mg. P per 100 g. soil

Treatment		Time - weeks 0	1	2	4	9	12
Nil	1	7.28 (0.63)	19.05	22.60	14.99		16.12
Yeast Nucleic Acid	2	7.98 (3.24)	59.88	67.63			550.50
Soil Nucleotide	3	8.14 (3.06)	23.26	26.30	19.39		20.04
Guanylic Acid	4	19.95 (24.65)	57.91	60.18			45.54
Adenylic Acid	5	17.86 (48.14)	59.47	63.46			48.78
Cytidylic Acid	6	22.09 (27.76)	59.64	62.54			47.42
Ca Uridylate	7	11.03 (37.12)	61.04	62.97			49.90
Inorganic Phosphate	8	46.70 (1.00)	61.61	62.96			50.12
Manure	9	58.19	40.29			43.22	
Nil	10	15.03	15.74			15.74	

The figures given represent the total phosphorus removed by the two extractions. The samples for 0 time were taken directly after moistening the samples, and the other samples were taken after the periods indicated in the table.

Table XX shows the increase in soluble phosphate over that of the untreated (nil) soil, at each period of sampling. This subtraction of the soluble phosphate values of the untreated soil from those of the treated soils should eliminate any difference caused by varying conditions of drying the samples, and of extraction, etc., which might alter the proportion of the soil phosphorus rendered soluble.

Table XXI shows the increase in soluble phosphate over the initial values, at each sampling period. The soluble phosphate values for 0 time in Table XX are subtracted from the values for each period. This corrects for the inorganic phosphate added along with the organic. The values given in Table XXI should show the amount of inorganic phosphate liberated from the added organic (phosphorus) compounds at the respective time intervals.

The phosphorus treatments of the soils were made on the basis of the total phosphorus content of the materials with which the treatment was made. Inorganic phosphate was also present in most of these materials and the actual amount of organic phosphorus added with each treatment is tabulated in Table XXII.

In Table XXIII the increase in soluble phosphate extracted by the Quebec solution over the initial value (Table XXI)is expressed as per cent of the organic phosphorus added in the treatments. The percentage recovery of inorganic phosphate is also shown for comparison.

The similar behaviour of the yeast nucleic acid and the individual nucleotides is well shown, while the soil nucleotide, on the other hand, acts altogether differently. There was no significant difference in the behaviour of the individual nucleotides, either in the rate or extent

TABLE XX. INCREASE IN SOLUBLE PHOSPHATE OVER UNTREATED SOIL DUE TO MINERALIZATION OF THE ORGANIC PHOSPHORUS ADDED - mg. P per 100 g. soil.

Treatment	Time - weeks 0	1	2	4	9	12
2	0.70	40.83	45.03			34.38
3	0.86	4.21	3.70	4.40		3.92
4	12.67	38.86	37.58			29.42
5	10.58	40.42	40.86			32.66
6	14.81	40.59	39.94			31.20
7	3.80	41.99	40.37			33.78
8	39.42	42.56	40.36			34.00
9	23.17	24.55			27.48	

TABLE XXI. INCREASE IN SOLUBLE PHOSPHATE OVER THE INITIAL VALUES DUE TO THE MINERALIZATION OF THE ORGANIC PHOSPHORUS ADDED - mg. P per 100 g. soil.

Treatment	Time - weeks 1	2	4	9	12
2	40.13	44.33			33.68
3	3.35	2.84	3.54		3.06
4	26.19	24.91			16.75
5	29.83	30.28			22.08
6	25.78	25.13			16.39
7	38.19	36.57			29.98
8	3.14	0.94			-5.42
9	1.38			4.31	

TABLE XXII. AMOUNT OF ORGANIC AND INORGANIC PHOSPHORUS ADDED WITH EACH TREATMENT - mg. P per 100 g. soil

Treatment No.	Organic P	Inorganic P
1	-	-
2	50.0	-
3	48.44	1.56
4	37.80	12.20
5	43.40	6.60
6	34.90	15.10
7	47.22	2.78
8	-	50.00
9	12.00	38.00

TABLE XXIII. INCREASE IN SOLUBLE PHOSPHATE OVER THE INITIAL VALUE (TABLE XXI) EXPRESSED AS PERCENT OF THE TOTAL ORGANIC PHOSPHORUS ADDED.

Treatment		Time - weeks 1	2	4	9	12
Yeast Nucleic Acid	2	80.26	88.66			67.36
Soil Nucleotide	3	6.92	5.87	7.63		6.32
Guanylic Acid	4	69.25	65.95			44.61
Adenylic Acid	5	68.80	69.75			50.84
Cytidylic Acid	6	73.82	72.00			46.96
Ca Uridylate	7	80.95	77.45			63.50
Manure	9	11.50			35.95	
Iorganic Phosphate*	8	85.12	80.72			68.00

*Percent recovery of inorganic phosphate added.

of decomposition. The data do not suggest any reason why only the adenine and uracil nucleotides have been found in the organic soil phosphorus material isolated.

A 100 per cent recovery of decomposed phosphate was not expected since some fixation has taken place, as evidenced by the incomplete recovery of inorganic phosphate. The amount of phosphate recovered from the nucleotide compounds approaches very nearly that recovered from inorganic phosphate, thus indicating that the organic phosphorus was almost totally decomposed. Further, the results show that this decomposition took place within a week, which is quite remarkable. It might be thought that the decomposition occurred during the extraction or during the determination of phosphate. In Table XIX in the column for 0 time, the amount of organic phosphate in the extracts is given (in brackets). This shows a large proportion of organic phosphorus in the extracts. The nucleotides are very soluble in the Quebec solution, whereas yeast nucleic acid and the soil nucleotide are almost insoluble.

In contrast to the nucleotides the soil 'nucleotide' was only decomposed to the extent of about 6-7 per cent, and showed almost no change from its value at 2 weeks. The difference in behaviour of the soil 'nucleotide' and ordinary nucleotides is thus definitely established.

Gulland and Jackson (1938) observed that in the enzymic hydrolysis of yeast nucleic acid by various enzymes and mixtures of enzymes never more than 75 per cent of the total phosphate was liberated. The decomposition of organic phosphate in the soil is probably almost entirely an enzymic process so that if the above is true there should be a residue amounting to 25 per cent of the organic P which can not be decomposed by the enzymes and therefore would accumulate in the soil. It seems very probable that this is the reason for a large part of the nucleotide P accumulation in soils.

According to Waksman (1932, p.244) when organic matter is added to the soil the activity of the micro-organisms is stimulated to a marked extent. The fungi and not the bacteria are the most active. It is possible that the fungi may build up a part of the phosphorus into compounds which may not be hydrolyzed by the soil agencies, as suggested above. However, it is rather difficult to credit such an hypothesis, since the organic phosphorus content of some soils may be considerably reduced by tilling and liming the soil (Vincent 1937) and this would not be possible if the organic soil phosphorus consisted mainly of a non-hydrolyzable compound. It is possible that the 25 per cent residue observed by Gulland and Jackson may be decomposed to some extent under certain soil conditions, and that it is this fraction which makes up a large part of the soil organic phosphorus. It would seem that this problem should be investigated further by a study of the properties of the isolated soil nucleotides and a purification of this material should be effected to determine if it is a single compound of a nucleotide nature. This was impossible at this stage in our investigation since the methods available were too inefficient to allow the isolation of sufficient nucleotide.

Another point which is brought out in Table XXIII is the appreciable decrease of the soluble phosphate after 12 weeks. This could be due to two processes. Firstly, a fixation, or reversion of soluble phosphate could have taken place, or, secondly, phosphate may have been assimilated by micro-organisms and built up into their protoplasm in organic form, i.e. a biological absorption of phosphorus, as Stoklasa (1911) termed it, may have occurred.

If the decrease was due to fixation of inorganic phosphate, then this phosphate should be recovered by the use of a stronger acid for the extracting agent.

The 12 week samples were extracted with 0.4 N H_2SO_4 solution. The procedure used was exactly the same as that used in the extraction by Quebec solution, and a re-extraction of the samples was also made as with the Quebec solution. The results aregiven in Table XXIV.

The increase in inorganic phosphorus due to the decomposition of the organic phosphorus is approximately equal to the amount of organic phosphorus added, in the case of the nucleotides and yeast nucleic acid, while the soil nucleotide is only decomposed to the extent of 27.3 per cent according to the results of this experiment. The recovery of inorganic phosphorus is almost 100 per cent. Therefore the results indicate that no biological absorption of phosphorus has occurred but that that the decrease was due to fixation of inorganic phosphate.

Table XXIII shows further very interesting results for the manure treatment. At nine weeks the soluble phosphate is still increasing in spite of the fact that a marked micro-organic stimulation has taken place soon after the beginning of the experiment. Vandecaveye and Villanueva (1934) report that in a manured soil the fungi are very active at first, but their numbers decline after 30 days and are reduced to normal after about 40-50 days. Thereafter the Azotobacter increase. Azotobacter are capable of decomposing the remains of the fungi and utilizing the organic phosphorus contained in their dead bodies. In this process part of the organic phosphorus is liberated as inorganic phosphate and this may account for the observed increase in soluble phosphate. Also the Azotobacter should attack any organic phosphorus contained in the manure.

TABLE XXIV. PHOSPHATE SOLUBLE IN 0.4 N H_2SO_4 (12 WEEK SAMPLES)(COMBINED FIRST AND SECOND EXTRACTS)- mg. P per 100 g. soil.

Treatment		Phosphate Extracted	Increase over Nil(A)	Inorg. P Added(B)	Org.P recovered as Inorg. (A-B)	Org. P Added	Recovery of Org. P added %
	1	76.0	-	-	-	-	-
Yeast Nucleic Acid	2	139.2	63.2	0	63.2	50.0	126.4
Soil Nucleotide	3	90.8	14.8	1.56	13.2	48.44	27.3
Guanylic Acid	4	123.1	47.1	12.20	34.9	37.8	92.4
Adenylic Acid	5	130.7	54.7	6.60	48.1	43.4	110.9
Cytidylic Acid	6	129.8	53.8	15.10	38.7	34.9	110.9
Ca Uridylate	7	124.7	48.7	2.78	45.9	47.22	97.3
Inorganic Phosphate*	8	125.8	49.8	50.0	-0.2	0	99.6*

* Recovery of Inorganic Phosphate added.

This experiment has shown that the soil 'nucleotide' behaves very differently from the ordinary nucleotides and possible reasons for this difference have been discussed. The results showed that a method of obtaining a sufficient quantity of soil nucleotide was needed. This method should allow a practically 100 per cent recovery of organic phosphorus from the soil, at least in some cases, to ensure that no fractionation of the soil organic phosphorus takes place during the extraction, and that the whole of the organic phosphorus complex is being investigated.

4. Third Soil Decomposition Experiment - Nucleic Acid and Soil Nucleotide on Two Soil Types

At this stage the oxalate method of separation of organic P had been developed, and since a much larger proportion of the soil organic P was now being isolated, it was deemed advisable to test the decomposition of the product isolated by this procedure. A series of decomposition experiments were carried out on two different types of soils, one, the Macdonald College soil (calcareous loam) previously used, and the other, a podsol soil from the Eastern Townships. These two types were selected since ordinarily organic P accumulates in the podsol and is decomposed to a greater extent in the calcareous soil.

The soil samples were passed through a 20-mesh sieve to insure uniformity of sampling. Amounts of material containing 25 mg. P were added to 50 g. soil (air-dry) contained in a glass tumbler. Thorough mixing was attained by the methods used in the former experiments.

Three treatments were used - nil, yeast nucleic acid and isolated soil nucleotide - arranged as follows:

TREATMENT	MACDONALD COLLEGE SOIL	PODSOL SOIL
Nil	1	4
Yeast nucleic acid	2	5
Soil nucleotide	3	6

Eastman Kodak Co. nucleic acid and the soil nucleotide (expt.^{G}) were used in making up the treatment.

After thorough mixing of the samples, a dry sample (3.5 g.) of each treatment was taken for analysis of original moisture and soluble phosphate content.

15 ml. water was added to each culture and the series of soils was stored in a dark room kept at a fairly constant temperature, 22-23^{o} C.

Distilled water was added to the cultures periodically to keep the moisture content nearly constant.

At intervals 5 g. samples were taken. These samples were spread out to air-dry for about 36 hours and were then ground sufficiently to pass a 40-mesh sieve. Determinations of moisture and soluble phosphate were made on each sample. The procedure for the latter was the same as for the determination of Quebec soluble phosphate except that 0.4 N H_2SO_4 was used as the extracting solution and two successive extractions were made.

0.4 N H_2SO_4 was used instead of Quebec solution for the extraction since the results of the last experiment showed that this acid completely extracted the added inorganic phosphate while the Quebec solution gave incomplete extraction.

The results of the analyses are given in Table XXV-XXVLLI

In Table XXV corrections have been made for the different moisture contents of the various samples. The table shows that the phosphate extracted from the nil samples remained practically constant throughout the duration of the experiment and that the samples extracted immediately showed almost identical soluble phosphate values.

TABLE XXV. SOLUBLE PHOSPHATE EXTRACTED BY 0.4 N H_2SO_4 FROM SAMPLES TAKEN AT INTERVALS (COMBINED FIRST AND SECOND EXTRACTIONS) mg. P per 100 g. SOIL.

TREATMENT		TIME - WEEKS		
		0	1	3
Macdonald College Soil:				
Nil	1	77.57	79.74*	77.40*
Yeast Nucleic Acid	2	81.08	112.74	115.20
Soil Nucleotide	3	78.85	78.26	78.40
Podsol Soil:				
Nil	4	12.17	12.20	11.86
Yeast Nucleic Acid	5	12.26	15.64	17.03
Soil Nucleotide	6	12.38	12.51	12.21

*Corrected to Moisture Content of original soil.

TABLE XXVI. INCREASE IN SOLUBLE PHOSPHATE OVER UNTREATED SOIL DUE TO MINERALIZATION OF THE ORGANIC PHOSPHORUS ADDED - mg. P per 100 g. SOIL.

TREATMENT	TIME - WEEKS		
	0	1	3
2	3.51	33.00	37.80
3	1.28	-1.48	1.00
5	0.09	3.44	5.17
6	0.21	0.31	0.35

TABLE XXVII. INCREASE IN SOLUBLE PHOSPHATE OVER THE INITIAL VALUES (0-time) DUE TO MINERALIZATION OF THE ORGANIC PHOSPHORUS ADDED - mg. P per 100 g. SOIL

TREATMENT	TIME - WEEKS	
	1	3
2	29.49	34.29
3	-2.76	-0.28
5	3.35	5.08
6	0.10	0.14

TABLE XXVIII. INCREASE IN SOLUBLE PHOSPHATE OVER THE INITIAL VALUES (TABLE XXVII) EXPRESSED AS PER CENT OF THE ORGANIC PHOSPHORUS ADDED.*

TREATMENT	TIME - WEEKS	
	1	3
2	58.18	68.58
3	-5.52	-0.56
5	6.70	10.16
6	0.20	0.28

*Organic Phosphorus added was 50 mg. P per 100 g. soil

The increase in soluble phosphate extracted from the treated samples over that from the untreated samples is shown in Table XXVI. The increase over the initial values due to mineralization of the organic phosphorus added is recorded in Table XXVII. In Table XXVIII this latter is expressed as per cent of the organic phosphorus added.

The yeast nucleic acid was almost 70 per cent decomposed on the Macdonald College soil but only 10 per cent on the podsol soil, and decomposition was still proceeding at the end of 3 weeks in each sample.

These results are in agreement with the well known fact that organic phosphorus is more easily mineralised by calcareous soils. No decomposition was shown by the soil nucleotide compound on either soil. This again demonstrates the great stability of at least a large part of the soil organic phosphorus.

It may be that the most labile fraction of the soil organic phosphorus, becomes decomposed during the isolation precedure or remains in the humic acid fraction and it is possible that it is this fraction which becomes decomposed when a soil is limed.

The results indicate that a large part of the soil organic P must be protected in some way from the action of the soil micro-organisms. This again suggests that the organic P may be 'buried' in the organic matter as in the theory advanced to explain the manner of solution of the soil organic P.

One reason why liming a soil brings about a decomposition of organic phosphorus is probably to be found in the resulting immense stimulation of micro-organic activity. Liming a soil causes the aggregation of the soil colloids which would probably decrease the solubility of the organic matter. This however would result in much better aeration of the soil. The pH would become more favourable for the development of the

soil organisms. Thus the stimulation of the soil micro-organisms would lead to the decomposition of much more organic matter including organic phosphorus compounds and this would probably be much more important than the decrease in solubility of the organic matter.

This however does not explain why the isolated organic phosphorus was not decomposed when incorporated in the soil. Apparently, it is stable to the ordinary soil decomposition agencies under the conditions present in the soils studied. Since (see later) the isolated material probably contains both phytin and nucleotides which are both decomposable by micro-organisms, it is difficult to explain this fact. It seems that the organic phosphorus must be combined with humic acid, Fe, or some other inert material in such a way that the action of the soil decomposing agencies is inhibited. However the slow decomposition of yeast nucleic acid on the podsol soil suggests that conditions are not very favourable for the degradation processes in this type of soil.

5. Fourth Soil Decomposition Experiment - Phytin

This experiment was undertaken when it became evident that phytin was one of the soil organic P constituents. Neubauer (1933) found that phytin was 59% absorbed by rye seedlings in sand cultures, and when soil was added phytin became unavailable. There was no explanation of this behaviour.

The tests in the present experiment were arranged in a similar manner to the last experiment, the same two types of soil being used, a calcareous loam (Macdonald College) and a podsol (Sherbrooke sandy loam).

TREATMENT	MACDONALD COLLEGE SOIL	PODSOL SOIL
Nil	1	3
Phytin	2	4

The soil was prepared as in the previous experiments. Amounts of phytin (prepared from wheat bran by the method of Boutwell, 1917) containing 25 mg. P were added to 50 g. samples of soil. This was mixed with the soil and the samples stored as before.

Samples were taken immediately for the initial analysis and at the stated times thereafter.

The samples were dried and analysed for soluble phosphate (0.4 N H_2SO_4 as the extracting acid) as before. The results are given in Table XXIX.

TABLE XXIX. SOLUBLE PHOSPHATE EXTRACTED BY 0.4 N H_2SO_4 (COMBINED FIRST AND SECOND EXTRACTS) mg. P per 100 g. SOIL

TREATMENT	TIME - WEEKS		
	0	2	8
Macdonald College			
Nil	77.5	73.1	75.1
Phytin	78.1	74.3	101.9
Podsol			
Nil	14.1	12.9	14.5
Phytin	13.7	13.9	16.8

The results show that phytin was not decomposed in either soil in two weeks.

At 2 months, however, about 51 per cent of the phytin had been decomposed in the calcareous soil, while no decomposition took place in the podsol. This agrees with the known facts that organic P tends to be decomposed on liming a soil. The phytin however is only slowly mineralized when compared with nucleic acid compounds.

Since the completion of our results on the isolation of phytin, it would seem that precipitation in the soil of the very insoluble ferric phytate, which is not attacked by enzymes, is the reason for the stability of phytin. This also explains Neubauer's results.

The stability of the soil 'nucleotide' preparation therefore is almost completely explained. Probably, it is composed mainly of enzyme-stable residue of nucleic acid and phytin and these are both stable.

6. Conclusions

Nucleic acid and the four nucleotides of yeast nucleic acid are rapidly dephosphorylated when added to the soil. Phytin and the separated soil 'nucleotide', on the other hand, are relatively stable.

The decomposition of yeast nucleic acid in podsol and calcareous loam soils showed a marked difference. Conditions in the podsol soil were not particularly favorable for the decomposition precesses.

Although it is certain that some soil organic P is taking part in the biological cycle being actively decomposed in the soil and synthesized in the bodies of micro-organisms, it is probable that the greater part represents an accumulation of relatively stable organic P compounds.

The properties of phytin would suggest that it is responsible for a considerable part of the organic P accumulation in acid soils. This will be discussed in more detail in the next section. The nucleotide P of the accumulating fraction of the organic P is probably composed mainly of the 25 per cent enzyme-stable residue of nucleic acid. This enzyme-stable fraction along with phytin probably makes up the major part of

the stable accumulating part of the soil organic P.

Other P compounds, lecithin, phosphoproteins, and purine and pyrimidine nucleotides, probably exist in soil in small quantities, being actively concerned in the life processes of micro-organisms. This fraction represents the dynamic portion of the organic P which is being added to the soil continuously by plant and micro-organic residues and is also being continuously decomposed by the soil micro-organisms, gradually liberating inorganic phosphate for assimilation by plant roots in the process.

There is evidence that phytin and the enzyme-stable fraction of nucleic acid are stable in the soil and there is also the theory that decomposable compounds may become 'buried', so to speak, in the soil colloidal complex and so become perhaps positionally, unavailable. The evidence for this theory has become somewhat weakened since the finding of the unavailability of phytin and the nucleic acid residue, but the manner of solution of the carbon, nitrogen and organic P compounds is not readily explainable by any other means. Thus it would seem that the chemistry of organic P in soils is much more complex than was originally anticipated.

This concept of the division, or perhaps fractionation, of the soil organic P is now being introduced into soil chemistry for the first time. A considerable body of evidence has been brought forth in support of the conclusions, and even if they do not prove to be strictly correct, they do afford a basis from which future research may be orientated and represent a very significant contribution to the knowledge of soil organic P .

E. PHYTIN

I. INTRODUCTION

Very little is known about phytin in the soil. A search of the literature reveals that phytin has not been detected in the soil up to the present. Only one attempted isolation from the soil has been reported. Stoklasa (1911) was unsuccessful in an attempt to obtain a Cu salt.

Plant residues contain up to 85 per cent of their phosphorus in the form of phytin (Rather 1917, Heck and Whiting 1927) so that considerable quantities of phytin are added to the soil annually. Many bacteria, fungi, etc., contain the enzyme phytase and are able to hydrolyze phytin (Kawahara 1930, Dox and Golden 1911), so it would seem very probable that it would be hydrolyzed in the soil by these agencies. One would not expect phytin to accumulate in the soil unless it were de-activated in some way. There are two possible ways in which such a de-activation might occur. First, the phytin might become incorporated along with other undecomposed organic matter with the colloidal material of the soil and become buried - so to speak on the soil particles by further deposition of colloidal material in such a way that further bacterial decomposition would be prevented for the time being. This is the same explanation as that advanced for the persistence of the nucleotide compounds in the soil. Second, phytin forms a very insoluble iron compound, and it seems possible that this compound might be precipitated in the soil and thus remove the phytin from the sphere of bacterial action as above.

The low N content of the preparations obtained in the work on the separation of organic P indicated the presence of phytin. Also Smith (1939) has found that a fraction of soil organic P is very resistant and possibly is phytin. Therefore an attempt was made to confirm the presence of phytin in the material isolated from soil.

2. Estimation of Phytin in the Soil Nucleotide Preparation

The $FeCl_3$ titration method of Rather (1917) was first used. 28.8 mg. of ppt. 12p dissolved in dilute HCl was titrated by $FeCl_3$ (0.5 mg. Fe per ml. in 1 N HCl) in the presence of KCNS. Using the factor 1.19 (Rather 1917) for converting Fe to phytin P, the phytin phosphorus found was 1.34 per cent, although the endpoint was very uncertain. Due to this difficulty, the procedure of McCance and Widdowson (1935) for the determination of phytin was tried. An excess of $FeCl_3$ solution was added to a solution of ppt. 12p, the Fe phytate precipitate was filtered off and washed with N/6 HCl. It was then decomposed by heating 15 minutes on the steam bath with a 1 per cent NaOH solution. After centrifuging off the coagulated $Fe(OH)_3$ and washing with hot water, organic P was determined in the solution. The phosphorus amounted to 2.12 per cent P ($\simeq$49 per cent Total P of ppt. 12p). This procedure was much more satisfactory than the direct titration.

This shows that the isolated material, 12 p. contains almost half its P in an organic form precipitable by $FeCl_3$ in acid solution and therefore almost certainly phytin.

The next step was to prove that this material was phytin. The characterization of phytin is usually made by an isolation of inositol after hydrolysis of the phytin material.

A quantity of the soil 'nucleotide' was therefore treated with $FeCl_3$ in order to separate the phytin.

1.674 g. ppt. 11p / 1.158 g. ppt. 13p, containing 0.1384 g. Total P, was dissolved in 100 ml. dilute NH_3, NH_3 being added until just alkaline. The solution was then made slightly acid with HCl. 50 ml. $FeCl_3$ solution was added and the solution was heated 15 minutes on the steam bath to coagulate the Fe phytate. After cooling, the precipitate was centrifuged off and washed with 20 ml. N/6 HCl. The filtrate was further treated with 50 ml. $FeCl_3$ and a second precipitate was obtained as above.

Each precipitate was dissolved in 50 ml. 1 N NaOH, and heated 20 min. in a boiling water bath. The coagulated $Fe(OH)_3$ was centrifuged off and washed with hot water. The solution was just acidified with N HCl and a reprecipitation with $FeCl_3$ was carried out as above. The final NaOH solutions were united and concentrated under reduced pressure to 20 ml. Analysis of a portion showed 39.2 mg. total P and 35.7 mg. org. P.

On neutralizing the filtrates from the Fe phytate above a small amount of precipitate was obtained. This only precipitated in just acid solution even after reprecipitation and contained only 0.4 mg. P. This might possibly be an Fe salt of inositol triphosphoric acid since Anderson (1912) found that this compound gave an iron salt which was insoluble at a neutral reaction. Its presence could be accounted for by a slow partial decomposition of the inositol hexaphosphate.

3. Attempted Isolation of Inositol

The solution of phytin in NaOH obtained as described above was neutralized with H_2SO_4 and 5 ml. conc. H_2SO_4 added - total volume 25 ml. This was then hydrolyzed for 4 hours in an autoclave under pressure at 150° C. to liberate inosite (Anderson 1915). As a check experiment a solution of 5 g. phytin in 25 ml. of 20 per cent H_2SO_4 was hydrolyzed at the same time.

H_2SO_4 and H_3PO_4 were precipitated from the hot hydrolysate by hot saturated $Ba(OH)_2$ solution. The $BaSO_4$ and $Ba_3(PO_4)_2$ were filtered off and washed with hot water. The excess Ba was precipitated by CO_2 and the $BaCO_3$ filtered off. The solutions were then evaporated to 20 ml. The inosite was then precipitated by the method of Young (1934). 100 ml. acetone and 50 ml. ether were added to each solution and the mixtures were placed in the refrigerator overnight. The pure phytin solution gave a white crystal line precipitate immediately while the soil phytin gave a gugary mass. The inosite precipitate was filtered off; washed with acetone and ether and dried for a short time at 100° C. The pure phytin

yielded 0.352 g. inositol (a) while the soil phytin gave 3.39 g. of impure material, (b).

A portion of (b) gave a negative Scherer's test for inosite, while (a) gave a definitely positive test. This was taken to mean that material (b) was impure since the test works satisfactorily only with relatively pure material.

An attempt was therefore made to purify the material according to the procedure of Young (1934).

The substance was taken up with HCl and 3.0 g. $Ba(OH)_2$ was added. The mixture was heated 5 minutes on the water bath and 40 ml. alcohol was added. After standing 2-3 hours the precipitate was filtered off, washed twice with 5 ml. absolute alcohol and dissolved in 40 ml. hot water. A slight excess of 2 N H_2SO_4 was added, and the solution was heated 45-60 minutes on the steam bath. The filtrate from $BaSO_4$ was evaporated to 3 ml. and precipitated with 30 ml. acetone and 15 ml. ether, as before. 52.1 mg. of a white powder was obtained. 10 mg. gave a negative Scherer test. Recrystallization of the remainder yielded 37.3 mg. This material also failed to give the Fischler and Kurten (1930) test for inosite, while the inositol prepared from phytin gave a very definite test. Apparently the product was still impure, since the test works satisfactorily only on a relatively pure sample of inositol.

Also the Fischler and Kurten test for phytin was applied to ppt. 10p but since so much extraneous colouring matter was present the results were extremely dubious.

The experiments described above were taken as being indicative of the presence of phytin in the soil preparations, but showed that some way must be found to isolate it in a nearly pure state if conclusive tests are to be obtained.

4. Stability of Phytin to Oxidation by Br_2 in Alkaline Solution

The attempts to separate the organic phosphorus compounds from soil have shown that it is very difficult to free the product from humic acid or similar organic material. Lignin, humic acid, protein and other related compounds are very readily oxidized by NaOBr, or NaOCl in alkaline or neutral solution (Feustel and Byers 1936, Norman 1939, Dean 1938). On the other hand phytin and the organic phosphorus material of soil are quite resistant to decomposition in NaOH solution. Thus if phytin should prove stable to oxidation by Br_2 then extraneous organic compounds could easily be removed from the soil phytin preparation.

A sample of phytin prepared from wheat bran by the method of Boutwell (1917) was used in this experiment. Analysis showed 21.33% P (moisture free basis)(Theoretical = 20.96 for calcium inositol hexaphosphate).

To 5 ml. of a 0.5% solution of the phytin in N/10 HCl were added 10 ml. H_2O, 5 ml. 5 N NaOH and 5 ml. saturated Br_2 water. After boiling for various periods, the solution was made acid with 5 ml. 6 N HCl and the excess Br_2 was boiled off. The solution was cooled, diluted to 100 ml., and inorganic phosphate was determined. The results are presented in Table XXX.

TABLE XXX. DECOMPOSITION OF PHYTIN BY ALKALINE Br_2

Solution	Time of Boiling Minutes	Phytin Present mg. P	Inorg. PO_4 Found mg. P	Decomposition mg. P	%
Blank			0.004		
Inorg. P 5 ml. phytin soln.		4.00	0.028		
Total Blank			0.032		
a_1 - 5 ml. phytin soln.	30	4.00	0.139	0.107	2.7
a_2 - " " "	60	4.00	0.194	0.162	4.0
a_3* " " "	40	4.00	0.300	0.268	6.7
a_4** " " "	30	4.00	0.143	0.111	2.8

*a_3 was boiled almost dry after acidification
**A large excess of Br_2 was used in this test.

The results show that phytin is very slowly oxidized by Br_2, only 4% being decomposed by boiling for 1 hour with excess Br_2 in 1 N NaOH solution. (Acid hydrolysis gives a larger decomposition - a3)

It appears that it should be feasible to use Br_2 oxidation in the isolation of soil organic phosphorus.

If the phytin is converted only to inositol pentaphosphate, then 2.75% dephosphorylation represents the decompostion of 16.5% phytin.

5. Separation of Phytin from Soil

A preliminary experiment was undertaken to isolate phytin from soil by oxidizing the NaOH soil extract with Br_2 and precipitating the phytin from the clear acidified solution by $FeCl_3$.

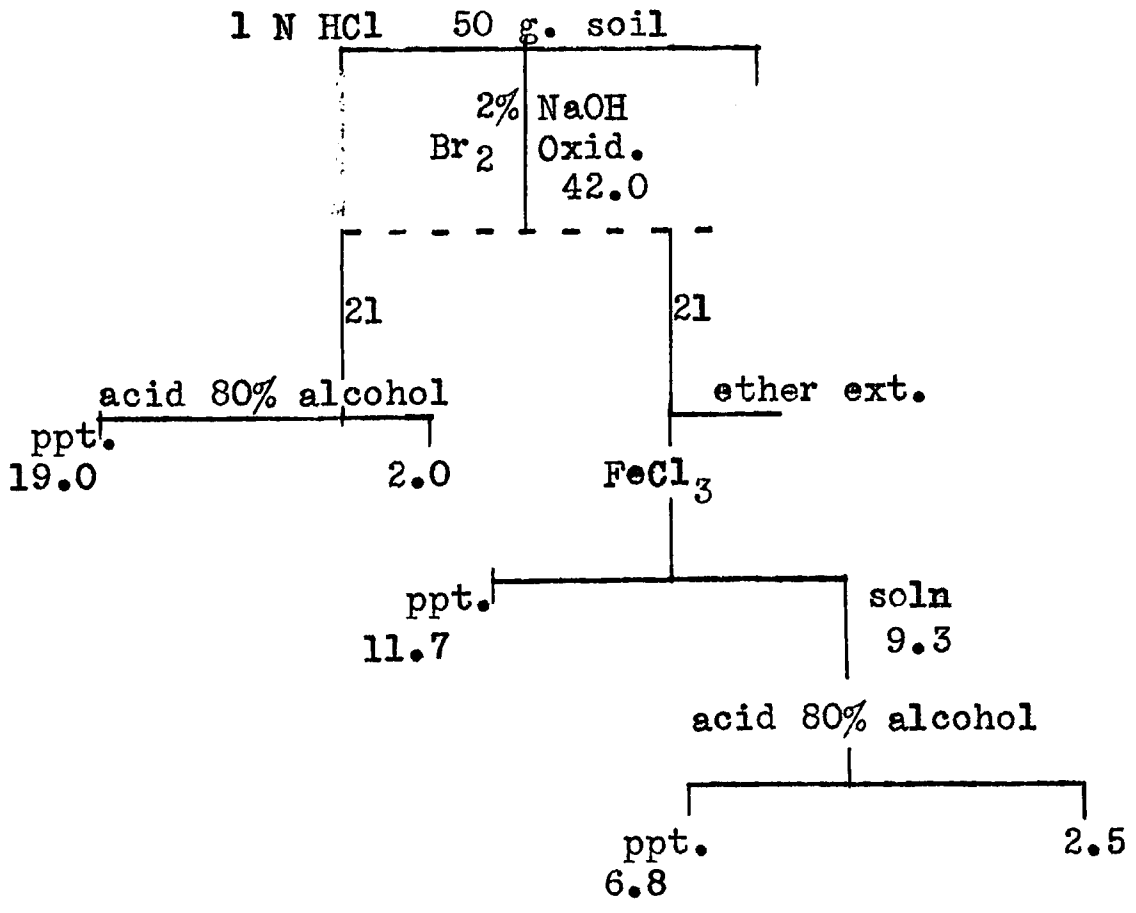
50 g. Halliday soil was leached with 100 ml. N HCl and then washed well with water. The soil was then heated overnight on the steambath with 500 ml. 2% NaOH. The supernatant liquid was decanted off through a cloth and treated with a solution of Br_2 dissolved in the equivalent amount of NaOH. The solution was boiled and Br_2 added until the solution was decolorized. After acidifying with HCl the excess Br_2 was boiled off. A white flocculent precipitate now appeared which was removed. It resembled cellulose but was easily soluble in 2% NaOH giving a dark colored solution containing no phosphorus. The light brown liquid contained 42 mg. organic P.

Feustel and Byers (1936) have shown that a large proportion of the products of bromination of lignin and humic acid are soluble. The liquid was divided into two parts, one of which was extracted successively four times with about 50 ml. ether. A large part of the yellow color was removed and the solution was now much clearer.

50 ml. $FeCl_3$ solution was added to each solution (also 31 ml. 5 N NaOH to make the solution just slightly acid). The solutions were then heated on the steam bath for 20 min.

The ether extracted portion formed a precipitate almost immediately. The solution was cooled and the precipitate was centrifuged off and washed with N/6 HCl. It was a greyish white color and contained 11.7 mg. organic P. On making the filtrate up to 80% alcohol a precipitate was obtained which contained a further 7 mg. organic P.

The non-ether extracted portion gave no precipitate. Feustel and Byers found considerable oxalic acid among the products of Br_2 oxidation of humic acid. It is possible that the α-OH organic acids formed by bromination inactivate the $FeCl_3$ so that ferric phytate is not formed. Evidently ether extraction removes the interfering substances. The organic P was almost completely precipitated from the non-ether-extracted solution when a 5% excess of HCl and alcohol were added to give a final concentration of 80% alcohol. These results are summar ized in the diagram below (The figures show the organic P present in the various fractions):



The ferric phytate is more nearly pure than the alcohol precipitate although the latter gives a greater recovery of organic phosphorus. This experiment showed that the Br_2 oxidation method is practical and so a larger amount of soil was now extracted.

500 g. Halliday soil were extracted with 1000 ml. N HCl, and then heated overnight on the steam bath with 5 litres 2% NaOH. The liquid was then decanted and treated with Br_2 dissolved in NaOH until the extract was decolorized. This procedure took several hours at the boiling temperature. The solution was then acidified with HCl, the excess Br_2 boiled off and a small amount of precipitate was removed after cooling. The solution contained 172 mg. organic P. After acidifying with a 5% excess of HCl, the solution was made up to 80% alcohol. The precipitate of NaBr crystals and organic matter was filtered off, 41 mg. organic P still remaining in the filtrate. The precipitate was suspended in hot water and 50 ml. $FeCl_3$ solution (5 mg Fe/ml. 1 N HCl) was added together with 4 g. NaOH to give approximately N/6 HCl solution. After heating 20 minutes on the steam bath, the ferric phytate was centrifuged off. The light colored precipitate contained 121 mg. organic P. This represents 71% of the organic P present in the oxidized extract and about 25% of the total soil organic P.

The filtrate from the alcohol precipitation containing 41 mg. organic P was treated with 10 ml. $FeCl_3$ soln. After standing 2 days, the precipitate was removed and found to contain all the organic P present.

Thus we have succeeded in separating a large proportion of the soil organic P in the form of an iron salt which is almost certainly ferric phytate.

The various alcohol precipitates from the above experiments were dissolved in 100 ml. N/10 HCl and the solution was treated with $FeCl_3$ solution. The precipitate was collected in the usual way and was then dissolved in hot 1% NaOH solution. The solution was very dark in color and so was decolorized with Br_2 after which ferric phytate was again prepared. The product was now pure white and after washing with alcohol and ether was dried in a vacuum desiccator. It weighed 0.0596 g. (ppt. A).

The ferric phytate precipitates obtained from the soil extract above were now purified by reprecipitation. The material was suspended in 100 ml. hot water and 100 ml. 0.8 N NaOH added. The mixture was heated 20-30 minutes on the steam bath and after cooling the coagulated $Fe(OH)_3$ was centrifuged off and washed with hot water. The filtrate contained 100.2 mg. P. The solution was acidified and ferric phytate prepared as before. It was dissolved in 150 ml. 1% NaOH and $Fe(OH)_3$ removed. The solution was still dark colored so it was treated with Br_2. After acidifying and boiling off excess Br_2, the solution was extracted with ether. This removed traces of Br_2 and the solution was now clear and colorless. Ferric phytate was again precipitated and collected as before. It was a pure white product and contained 48.6 mg. P (see below). This represents a 50% yield after two $FeCl_3$ precipitations, one Br_2 treatment, and two NaOH treatments. The loss is probably due to incomplete precipitation and partly to decomposition by Br_2 oxidation.

6. Analysis of Iron Salts from Soil and from Bran Phytin

Precipitate A was dissolved in 10 ml. hot water and 10 ml. 0.5 NaOH. After heating and cooling the $Fe(OH)_3$ was centrifuged off and washed

with hot water. The solution contained 5.93 mg. P. The Fe $(OH)_3$ was dissolved in HCl and Fe was determined by the dipyridyl method (Parker and Griffin 1939). 8.60 mg. Fe was present.

This corresponds to an analysis of 9.97% P and 14.4% Fe, with a P/Fe ratio of 0.69.

The sodium phytate solution from precipitate B contained 48.6 mg. P and the $FeCl_3$ solution 63.8 mg. Fe. The P/Fe ratio was thus 0.76.

It was found by examination of some of the Fe $(OH)_3$ precipitates above that only traces of phosphorus were precipitated along with the $Fe(OH)_3$.

The P/Fe ratio of ferric phytate found by titration (Rather 1917, Heubner and Stadler 1914) is about 1.20 and this differs widely from the ratio found above. Accordingly ferric phytate was prepared from pure phytin (prepared from bran) for comparison with the above.

0.323 g. phytin (80 mg.P) was dissolved in N/10 HCl and treated with $FeCl_3$. The ferric phytate was decomposed by 1% NaOH and analyses made as above. The Na phytate contained 39.8 mg. P and the $Fe(OH)_3$, 55.8 mg. Fe, giving a P/Fe ratio of 0.71. This value agrees with the value from the soil ferric phytate.

To determine if treatment with Br_2 had any effect on the material, 50 ml. of the above solution of sodium phytate from phytin was treated with Br_2. The solution was acidified and excess Br_2 boiled off. It was extracted with ether, precipitated by $FeCl_3$ and the ferric phytate solution contained 9.55 mg. P and the $Fe(OH)_3$ 14.0 mg. Fe.

Thus the P/Fe ratio is 0.68, in agreement with the above.

Thus the P/Fe ratios of the soil ferric phytate preparation are identical with those of ferric phytate prepared from pure phytin.

A sample of ferric phytate (air $/^{dry}$) prepared by Miss E. Knight by precipitation of phytin with $FeCl_3$ (phytin definitely in excess) was now analyzed.

Moisture was determined by drying a sample of 48.5 mg. at 120° C. for 2 hours. The sample onntained 13.8% H_2O. The sample was then ignited with $Mg(NO_3)_2$ and P and Fe was determined after solution of the residue in HCl. (No interference of Fe with the P determination could be observed.) The solution contained 8.30 mg. P and 7.25 mg. Fe. Thus the ferric phytate contained (oven-dry basis) - 19.8% P and 17.3% Fe. and shadea P/Fe ratio of 1.14.

203.3 mg. of the ferric phytate sample were washed with alcohol and ether and dried in a vacuum desiccator. The sample still retained 9.64% H_2O. This is in accord with the results of Posternak (1921) who found that all phytin salts contained large amounts of water of crystallization which was very difficult to remove.

101.1 mg. of this dried preparation was decomposed with NaOH and the $Fe(OH)_3$ was removed as before. The sodium phytate solution contained 17.4 mg. P while the $Fe(OH)_3$ had 15.75 mg. Fe. Thus the preparation contained :

Ether dry Basis - P..17.2% Fe..15.6%

Oven dry basis - P..19.1% Fe..17.3% — P/Fe..1.11

This shows that the two methods of analysis give identical results.

The P/Fe ratio of this preparation differs widely from the preparations above and corresponds with the titration ratio. This is explained in that an excess of $FeCl_3$ was not used and therefore the iron salt formed was the same as that formed in the titration procedure (see later.)

To check the above results, another sample of phytin was treated with excess $FeCl_3$ and the ferric phytate precipitated was decomposed in NaOH and P and Fe determinations were made as before. After drying with alcohol and ether the preparation still contained 17.5% H_2O. On an ovendry basis the results showed:-

- P..15.6% , Fe..23.6%, P/Fe..0.67

Thus we have isolated two iron salts, both precipitated in acid solution, but one containing much more Fe than the other.

No data on these salts could be found in the literature.

7. Titration by $FeCl_3$ of Sodium Phytate from Soil and from Bran Phytin

The fact that the P/Fe ratios obtained by analysis did not agree with the titration values given in the literature was now investigated.

Heubner and Stadler (1914) first introduced the method of titration of phytin by $FeCl_3$. Rather (1917) Harris and Mosher (1934) Averil and King (1926) and others have introduced various modifications all designed to allow a more accurate determination of the endpoint. Rather's direct titration method using NH_4CNS as an indicator was used in this work.

10 ml. of sodium phytate solution was made up to 50 ml. (approx.) with enough HCl to make a 0.6% HCl solution. 2 ml. of 0.3% NH_4CNS was added. The solution was then titrated with $FeCl_3$ solution (0.5 mg. Fe/ml. 1N HCl) until a brownish color, permanent for 5 minutes, was obtained. A blank determination required 0.15 ml. $FeCl_3$ solution. Results on the sodium phytate solutions from soils and from phytin are given below in Table XXXI.

TABLE XXXI. TITRATION OF PHYTIN SOLUTIONS

Solution	P present mg. P	Fe added mg. Fe	P/Fe	
5ml. soil Naphytate	1.41	1.10	1.27)	) 1.23
5ml "	1.41	1.19	1.19)	)
10ml phytin " (a)	1.47	1.32	1.12)	1.21
10ml. " " (b)	3.51	2.74	1.27)	) 1.20
10ml " " (c)	3.51	2.92	1.20)	)

These values for the P/Fe ratio agree very well with those found in the literature - Heubner and Stadler (1914) gave the value, 1.19, and Rather (1917), 1.21. There is practically no difference between the sodium phytate prepared from phytin and that prepared from the soil product.

Another titration was made with the phytin dissolved in just acid solution. The soil sodium phytate gave a value for the P/Fe of 1.04 while the bran phytin sodium phytate gave 1.03. Again the results are practically identical, although differing from the value obtained in acid solution. Apparently the acidity has some effect on the amount of iron taken up by the phytin.

8. Discussion of Iron Salts of Phytin.

The P/Fe ratios found by analysis and those found by titration are summarized in Table XXXII.

TABLE XXXII. P AND Fe CONTENT OF PHYTATE PREPARATIONS

Preparation	Analysis			Titration P/Fe		
	%P	%Fe	P/Fe ratio	(P present mg.)	0.6% NCl	slightly acid soln.
Fe phytate, soil			0.76	1.41	1.27	
				1.41	1.19	
				2.43		1.03
Fe phytate, soil (2 Br_2 treatments)	9.97	14.4	0.69			
Fe phytate, bran phytin (1)			0.71	1.47	1.12	
Fe phytate, bran phytin (2)	12.9 (15.6)[x]	19.5 (23.6)[x]	0.67	3.51 3.51	1.20 1.27	
Fe phytate, bran phytin (Br_2 treated)			0.68			1.04
Fe phytate - unsat'd	17.2 (19.1)[x] (19.8)[o]	15.6 (17.3)[x] (17.3)[o]	1.11 1.14			

x These values are on oven dry basis. The others are analyses of ether dry material.

o Analysis by ashing with $Mg(NO_3)_2$.

The average P/Fe ratio by analysis is 0.72 for the soil preparation and 0.69 for the phytin preparation, so that the two agree very well. If one assumes a moisture content of 37.5% for the soil ferric phytate analyzed then the P content is 15.9% P and the iron 23.1% Fe which is almost identical with the composition of the phytin salt.

As to the theoretical composition of ferric phytate, we have very little data on which to base our conclusions.

Rather (1917) assumed that phytin was a salt of inositol pentaphosphoric acid and assumed the iron salt formed by titration was a heptaferric salt $\left[C_6H_6OH.H_3(PO_4)_5\right]_3 Fe_7$.

On the basis of Anderson's conclusion that phytin is a salt of inositol hexaphosphoric acid, it appears to the writer that the iron salt would be represented best by the octaferric salt $\left[C_6H_6H_4(PO_4)_6\right]_3 Fe_8$.

The iron saturated salt would be represented by the tetraferric salt - $C_6H_6(PO_4)_6 Fe_4$.

The P and Fe contents and P/Fe ratio of these compounds are given in Table XXXIII below.

TABLE XXXIII. COMPOSITION OF FERRIC PHYTATES

COMPOUND	% P	% Fe	P/Fe
$C_6H_6(PO_4)_6Fe_4$	21.3	25.7	0.83
$(C_6H_6H_4(PO_4)_6)_3Fe_8$	23.2	18.6	1.24
$(C_6H_6OH\ H_3(PO_4)_5)_3Fe_7$	20.8	17.5	1.19

The theoretical iron saturated salt contains a higher percentage of P and Fe than that found in either the soil phytin or phytin preparations. Posternak (1921) found that phytin salts in general adsorbed

very high amounts of water and also he found that it was very difficult to remove this water from the salts. If it is assumed that these preparations contained adsorbed water (as the soil phytate preparation certainly did) then the analysis approaches very nearly that of the theoretical. The P/Fe ratio shows that our preparations contained more iron than the tetraferric phytate (P/Fe 0.71 - 0.83) Assuming the preparation to contain $C_6H_6(PO_4)_6Fe_4$ + $Fe(OH)_3$ + H_2O then calculation shows the bran phytin preparation should contain 1.03 mols. $Fe(OH)_3$ and 11.6 mols. H_2O per mol. of $C_6H_6(PO_4)_6Fe_4$, and the soil phytin 0.82 mols. $Fe(OH)_3$ and 5 0.6 mols. H_2O. This H_2O content is of the same order as Posternak obtained for sodium phytate and other phytin salts, and it would seem reasonable that the highly colloidal ferric phytate precipitate could adsorb a molecule of $Fe(OH)_3$ even in acid solution.

The ferric salt formed by titration and that precipitated in a solution with excess phytin agrees very well with either the octaferric salt of inositol hexaphosphoric acid or the heptaferric salt of inositol pentaphosphoric acid, on the basis that the prepared salt again contains adsorbed water.

Regardless of whether the compounds agree with the theoretical or not the soil preparation agrees almost exactly with the bran phytin in every respect.

Two different ferric salts of phytin have been prepared and it is obvious that more work is needed on the chemistry of these phytin compounds.

9. Fischler- Kurten Test for Phytin.

The Fischler and Kurten (1932) test for phytin was applied to the sodium phytate solutions from pure phytin and from the soil preparations 10ml.

sodium phytate solution (containing approx. ¼mg. phytin P per ml.) was evaporated almost to dryness (syrupy) in a 80 ml.Erlenmeyer Flask. Each solution gave a yellow-green color at this point. A knife point of Na_2O_2 was added, mixed well with the residue, and one to two drops of water added to form a paste. The flask was then carefully heated for 1-2 minutes over a flame until a spot of carmine-red appeared which spread over the whole surface. A definite carmine-red color test was obtained from both solutions.

Thus the Fischler Kurten test indicated the presence of phytin in the soil preparation.

10. Enzyme Dephosphorylation.

Enzymatic hydrolysis should give further proof of the identity of the product isolated from soil. If it is phytin then phytase should hydrolyze the organic phosphorus whereas phosphatase enzymes should not attack the compound. These two enzymes were allowed to act on the sodium phytate solution and the extent of hydrolysis of organic P was determined.

(A) Action of Phosphatase.

An enzyme extract was prepared by extracting the mucosa of pig's intestine with water. It was allowed to autolyze for 24 hours and was then filtered. This extract should contain both diesterases and monoesterases (Levene and Dillon 1937). 10ml. of this extract was added to 5ml. of test solution and the solution was then brought to about pH 8.5 (Just alkaline to phenolphthalein). 1ml. of toluene was added to each tube, and the samples were then incubated at 35°C. The test solutions were as follows:

1. 5 ml. water(blank)

2. 5 ml. 1% yeast nucleic acid soln.

3. 5 ml. Na phytate (soil prepn.)

4. 5 ml. Na phytate (bran phytin)

At intervals 1 ml. of solution was removed from each tube, diluted to 10 ml., and inorganic phosphate determination made. The results are given in Table XXXIV below.

TABLE XXXIV. PHOSPHATASE DEPHOSPHORYLATION

SUBSTRATE	Org.P added Mg.P	Inorg. P found mg. P			DECOMPOSITION mg. P			DECOMPOSITION % org. P		
		0 days	3	6	10	3	6	10	3	6	10
1.Blank	-	0.18	0.55	0.58	0.58	-	-	-	-	-	-
2.Nucleic Acid	4.85	0.18	3.09	4.00	4.23	2.54	3.42	3.65	52.4	70.5	75.0
3.Na phytate (soil)	1.22	0.19	0.66	0.82	0.91	0.11	0.24	0.33	9.0	19.6	27.0
4.Na phytate (phytin)	1.43	0.18	0.52	0.60	0.66	-0.02	0.02	0.08	-1.4	1.4	5.6

The intestine extract contained some organic phosphorus and so the value for the blank was substracted from those of the [illegible] test solutions to give the amount of added organic P hydrolyzed. The decomposition of nucleic acid proceeds to 75%, thus showing that the enzymes were active. This result is in accord with Gulland and Jackson's observation that yeast

nucleic acid is only 75% dephosphorylated by enzymes. The importance of this in regard to the accumulation of organic phosphorus in soils has already been mentioned. The Na phytate from phytin was not attacked at all and that from soil only slightly, thus leaving no doubt that the soil phytate preparation is principally non-nucleotide in nature.

A nitrogen estimation on the soil Na phytate solution showed that it contained no nitrogen, so that the stability of the organic phosphorus can not be due to its being composed of the 25% undecomposable fraction of nucleic acid.

(B) Action of Phytase.

A phytase enzyme extract was prepared by extracting bran with about 5 times its weight of water for an hour and filtering off the bran (Plimmer 1913). 3ml of this extract was then added to 10ml. of test solution which had been adjusted to about pH 4.7 (just colorless to p-nitrophenol). Kawahara (1930) states the optimum pH for the phytase enzyme is 4.67. The solutions were made up to 15ml., 1ml. of toluene was added to each and the solutions were incubated at about 35°C.

The test solutions were as follows:

1. 10ml. water
2. 10ml. water
3. 10ml.Na phytate (bran phytin)
4. 10ml. Na phytate (soil)
5. 10ml. Na Phytate (phytin Br_2 treated)
6. 10ml. Fe phytate (" " ")
7. 3ml. 1% nucleic acid.

At intervals 1 ml. samples were removed, diluted to 50ml. and phosphate determinations were made. The results are given in Table XXXV

TABLE XXXV. PHYTASE DEPHOSPHORYLATION

SUBSTRATE	Org. P added mg. P	Inorg. P found mg. P			DECOMPOSITION			
					mg. P		% org. P.	
		0 days	3	7	3 days	7	3	7
1.Water	-	0.84	1.12	1.12	-	-	-	-
2.Water	-	0.86	1.12	1.12	-	-	-	-
3.Na phytate (phytin)	2.86	0.88	3.45	3.56	2.33	2.44	81.5	85.3
4.Na phytate (soil)	2.44	0.87	2.84	3.30	1.72	2.18	70.5	89.4
5.Na phytate (phytin Br_2)	1.47	0.89	2.34	2.52	1.22	1.40	83.0	95.1
6.Fe phytate (phytin Br_2)	1.47	0.81	1.10	1.16	-0.02	0.04	-1.4	2.7
7.Nucleic acid	2.91	0.86	3.22	3.22	2.10	2.10	72.2	72.2

The phytin solutions contained practically no inorganic phosphate as the initial phosphate content was approximately the same in all cases.

The bran extract contained some phytin and so the water and enzyme solution was used as a blank.

All the sodium phytate preparations, from soil or phytin, were dephosphorylated, from 85-95% of the organic P being decomposed, there being no difference between the soil and phytin solutions.

These results considered with those of the phosphatase decomposition experiments leave no doubt that the soil phytin is the same as the pure phytin in its behaviour toward enzymes.

The phytase also decomposed nucleic acid (again almost 75%) so that the extract must have contained some phosphatase active in acid solution. Plimmer (1913) also found that bran extract partially hydrolized nucleic acid. This is unfortunate in that we may not distinguish between phytin and nucleic acid by the phytase enzyme from bran. However the phytin was decomposed to a greater extent than the nucleic acid.

A result of very great significance is that ferric phytate was not decomposed. It is quite possible that this is due to the insolubility of ferric phytate. It seems likely that in soils, the majority of which are slightly acid and contain Fe in an active state, that phytin will be precipitated as ferric phytate which is not decomposed by the soil enzymes, and thus phytin would accumulate in the soil.

11. Conclusions

The results of our investigation of phytin in soil have been very significant. One experiment showed that a sample of the organic P preparation isolated from soil contained 49% of its P as phytin and in another experiment, about 25% of the total soil organic P was obtained in the form of phytin. The isolation was certainly not quantitative so that a large part of the organic P of some of our soils must be present as phytin. The significance of this will be discussed later.

It was found that the ferric phytate prepared from soil extracts by precipitation with $FeCl_3$ in acid solution contained too much contaminating organic matter, which could not be removed by ordinary means, to allow the characterization of phytin compounds.

Phytin has been found to be quite stable to oxidation with Br_2

in alkaline solution, and this treatment was found to be very effective in removing the extraneous organic matter and leaving the phytin, although some loss of phytin undoubtedly occurred. The following procedure was found to be effective for the isolation of phytin. The soil, leached with 1 N HCl as usual, was extracted with hot 2% NaOH for several hours. The extract was forced from the soil residue and oxidized with Br_2 in boiling alkaline solution until an excess of Br_2 was present. The extract was then acidified, excess Br_2 boiled off, and the phytin precipitated in acid 80% alcohol solution. The precipitate was dissolved in N/10 HCl and precipitated with $FeCl_3$. The ferric phytate was collected, decomposed with NaOH and again treated with Br_2 to remove extraneous organic matter completely. Excess Br_2 was removed as before and the colorless acid solution was treated with $FeCl_3$. The pure white ferric phytate was collected. Although this procedure was not quantitative, the product obtained was quite pure and analysis showed that it was identical with ferric phytate prepared from a pure sample of bran phytin.

The ferric phytate preparations contained considerable water of hydration and when this is taken into account, the P and Fe content of the prepared ferric phytate agrees very well with the theoretical value, although they contain more Fe, which could be accounted for by absorbed Fe.

Analysis of the sodium phytate solutions by titration with $FeCl_3$ to the thiocyanate endpoint gave results agreeing with the theoretical value, and the value for soil phytin again agreed with the bran phytin.

Two iron salts were prepared, one corresponding to the iron saturated salt $Fe_4C_6H_6(PO_4)_6$ and the other to the partially iron saturated salt $\left[C_6H_6H_4(PO_4)_6\right]_3\ Fe_8$.

Enzyme dephosphorylation studies with phosphatase and phytase enzyme extracts confirm the presence of phytin in the soil preparation. Phosphatase hydrolyzed nucleic acid (75%) while it had little action on bran

phytin or the soil phytin preparations. Phytase decomposed all three substances, the soil phytin and bran phytin to the same extent (85-95%) and nucleic acid to about 72%. Since the soil preparation contained no nitrogen the dephosphorylation must be due to hydrolysis of phytin.

Gulland and Jackson's observation of a 75% enzymatic hydrolysis of nucleic acid was fully confirmed, and it seems probable that this undecomposed fraction may account for the greater part of the accumulation of soil organic phosphorus which is not due to phytin.

The presence of phytin in the soil, and the fact that ferric phytate is not hydrolyzed by the phytase enzyme, is very important. The phosphorus of plant residues is present to a large extent as phytin. When this is added to soils, the majority of which are slightly acid, very probably ferric phytate would be precipitated and thus would be in a form in which it would not be attacked by the enzymes of the soil micro-organisms. Thus a large part of the accumulation of organic phosphorus in soils would be accounted for.

We may speculate that in calcareous soils, where ordinarily organic phosphorus shows a lesser tendency to accumulate, the insoluble ferric phytate either would not be formed or would be decomposed (since it is unstable in alkaline solution), thus liberating the organic phosphorus for the action of micro-organisms. The beneficial effect of liming acid soils may be due, in part, to the liberation of phytin phosphorus. This is substantiated by the finding that phytin was partially decomposed on a calcareous soil.

Similarly, Na_2CO_3 treatments of soil would result in the hydrolysis of ferric phytate, thus rendering the phytin more subject to micro-organic attack. Also, particularly in podsols, the iron liberated from the ferric phytate would probably form complexes with the α- OH organic acids present in these soils, and would thus be rendered available for plant absorption. In this way the increased yields and increased iron content of plants grown

on sodium carbonate treated soils (DeLong 1939, Dyer and McFarlane 1938) may be explained.

SUMMARY OF NEW RESULTS

The methods of separation of organic phosphorus from soil in use at the outset of this investigation were found to be very inefficient, allowing only a separation of 5 - 13 percent. This was unsatisfactory since it was almost impossible to obtain any appreciable amount of soil organic phosphorus with a reasonable amount of time and labor, and further it was considered that the small fraction obtained might not be at all representative of the principal organic phosphorus compounds present.

A new method of extraction using 6 N HCl was also tried but the results were again unsatisfactory, giving yields of from 2 - 12 percent.

Investigation of various extracting solutions and methods showed that extraction with hot ammonia was much superior to cold ammonia or Na_2CO_3. Hot ammonia preceded by leaching with HCl removed 90 - 100 percent of the soil organic phosphorus. In all cases the amount of organic matter dissolved paralleled the amount of organic phosphorus dissolved. The addition of $Ca(NO_3)_2$ to the ammonia solution before removing the soil residue was shown to have a specific precipitating action on organic phosphorus.

When hot ammonia extraction was used in an attempt to separate organic phosphorus material from the soil it was found, that after humic acid was precipitated by acidifying the ammonia extract, only about 10 percent of the organic phosphorus remained in solution.

This organic phosphorus distribution between the humic acid precipitate and filtrate was found to be of the same order in several soils. In four podsol soils from 7.8 to 14.8 percent of the organic phosphorus of the ammonia extract was obtained in the humic acid filtrate. The Halliday podsol A_1 layer yielded 10.1 percent, a calcareous loam 8.4 percent and the St. Chlothilde muck 30.4 percent, thus showing a difference between the muck and the other soils.

It was found that if ammonium oxalate was added to the ammonia extract before acidification that the recovery of organic phosphorus in the humic acid was very much increased (20 - 400%); for the muck soil, from 30.4 - 36.6 percent, a podsol, from 10.3 - 28.4 percent, Halliday podsol, A_1 layer, from 10.6 - 52.0 percent. The organic matter content of the solution remained practically the same. The optimum concentration was found to be about 0.15 g. ammonium oxalate per extract from 1 g. soil.

The hot ammonia extraction method with the addition of oxalate was used to separate the organic phosphorus from large amounts of Halliday soil. From 38 - 46 percent of the total soil organic phosphorus was actually separated as a 'nucleotide' precipitate. About 38 - 40 percent remained in the humic acid precipitate, about 3 percent in the acid extract (leachings) and in the alcoholic filtrate, and from 6 - 20 percent remained in the soil residue.

The nitrogen content of the purified 'nucleotide' preparations was found to be much lower than would be the case if the organic phosphorus consisted of nucleotide compounds. The nitrogen accounted for only 34 percent and 69 percent of the organic phosphorus in two soil preparations. This suggested the occurrence of phytin.

About 10 percent of the total N was accounted for by purine N.

Xanthylic acid was the only purine nucleotide which could be detected in the 'nucleotide' preparation and attempts to identify pyrimidine nucleotides were unsuccessful. Ribose sugar and phosphoric acid were present.

Hydrolysis with NaOH showed that 75-95 percent of the organic phosphorus of the humic acid precipitate, filtrate and the separated 'nucleotide' material was stable to NaOH.

Soil decomposition experiments, in which organic phosphorus compounds were added to soil and the mineralization of organic phosphorus determined

by acid extractions of the soil at suitable intervals, showed that yeast nucleic acid and the four nucleotides prepared from nucleic acid were rapidly dephosphorylated on muck and on calcareous loam soils. Addition of $Ca(OH)_2$ or glucose had no effect on the decomposition. The separated soil 'nucleotide' was stable under these conditions. The recovery of added inorganic phosphate was approximately the same as that of nucleic acid phosphorus indicating almost complete decomposition.

Another experiment showed that nucleic acid was rapidly decomposed on a calcareous loam while it was only slowly decomposed on an acid podsol soil. The soil 'nucleotide' was stable on both soils. This suggests that podsol soil conditions are not very favorable for the decomposition of nucleotide compounds.

A further experiment showed that phytin was stable on the podsol soil while it was slowly decomposed on the calcareous loam. These experiments show conclusively that the soil 'nucleotide' does not behave like the ordinary nucleotides. Titration of a solution of the soil 'nucleotide' with $FeCl_3$ showed the presence of material precipitable by $FeCl_3$. A phytin determination according to the method of McCance and Widdowson showed that about 49 percent of the organic phosphorus was present as phytin. An attempted isolation of inositol from a hydrolyzed portion of the soil 'nucleotide' was unsuccessful, very probably due to the presence of extraneous organic material.

Phytin was found to be stable to oxidation by Br_2 in alkaline solution. Phytin was isolated from soil by treating NaOH soil extracts with Br_2 and precipitating phytin from the acidified solution by $FeCl_3$ (in some cases by 80 percent alcohol). The preparation was purified by reprecipitation and further treatment with Br_2 and was obtained as a white precipitate of ferric phytate, in amount corresponding to about 25 percent of the total organic phosphorus of the original soil.

This soil ferric phytate preparation was analyzed and its Fe and P content (P/Fe ratio - 0.72) corresponded with ferric phytate prepared from pure phytin (wheat bran)(P/Fe - 0.69). Two iron salts were prepared, the composition of which corresponded approximately to tetraferric inositol hexaphosphoric acid, $C_6H_6(PO_4)_6Fe_4 \cdot XH_2O$, and octaferric inositol hexaphosphoric acid, $[C_6H_6H_4(PO_4)_6]_3\ Fe_8 \cdot XH_2O$.

Titration with $FeCl_3$ of sodium phytate solutions prepared from the soil and bran phytin ferric phytate gave values for the P/Fe ratio of 1.23 for the soil phytate and 1.20 for the bran phytin. These differed by less than the experimental error from the values of 1.19 to 1.21 given in the literature.

The Fischler and Kurten test for phytin was applied to the above sodium phytate solutions and was positive, thus indicating that phytin was actually present.

Enzyme dephosphorylation studies showed that the sodium phytate from the soil ferric phytate was hydrolyzed to only a slight extent by a phosphatase enzyme system, while it was readily decomposed by a phytase enzyme (85 - 95 percent). Nucleic acid was 75 percent decomposed and ferric phytate was not attacked at all.

The evidence for the presence of phytin in the soil may be summed up as follows.

1. Low N/P ratio of separated organic phosphorus.
2. The presence of organic phosphorus stable to decomposition by NaOH or Br_2.
3. The presence of organic phosphorus precipitable from acid solution by $FeCl_3$.
4. The P/Fe ratios, obtained either by titration with $FeCl_3$ or analysis of the iron salt, of the phytin preparations from the soil and from pure bran phytin were identical.

5. The Fischler and Kurten test was positive.

6. The soil phytin preparation contained no nitrogen.

7. The soil phytin preparation was rapidly dephosphorylated by phytase enzyme but was not attacked by phosphatase enzymes.

8. The presence of phytin explains the behavior of the soil organic phosphorus: stability and accumulation in soil, effect of oxalate on the precipitation of organic phosphorus with humic acid, etc.

GENERAL CONCLUSION

In the introduction it was stated that the organic phosphorus of soils was thought to consist mainly of nucleic acid type compounds. A small amount of phospholipids (2 % or less of the organic phosphorus) and small amounts of nucleotide material containing adenylic and uridylic acids had been isolated, but apart from this there was no definite information as to the identity of the organic phosphorus compounds making up a large fraction of the soil organic phosphorus.

In this investigation, about 25 percent of the total organic phosphorus present in a podsol soil was isolated as phytin. About 40 percent of the organic phosphorus of the same soil was separated as a 'nucleotide' fraction. This was subsequently shown to contain about half its phosphorus as phytin so that the actual nucleotide phosphorus separated was only about 20 percent of the soil organic phosphorus. The 'nucleotide' product obtained by previous investigations also probably contained phytin. This was actually found to be the case with the product obtained by Wrenshall and McKibbin (1937). Thus their conclusion that 65 percent of the ammonia soluble organic phosphorus was nucleotide in nature was not valid.

Thus the identity of a considerable fraction of the soil organic phosphorus has been determined.

Phytin in acid soils is probably present as the insoluble ferric phytate. This compound is not acted on by the phytase enzyme and this probably explains the accumulation of large amounts of phytin in the soil. Calcium phytate is relatively insoluble in alkaline solution, and it is possible that phytin might also accumulate to some extent in calcareous soils.

A considerable part of the nucleotide fraction of the organic phosphorus may be made up of the enzyme stable residue of nucleic acid. As yet this material is of unknown constitution, and, until its structure and properties are more clearly defined, it can only be stated that the existence of such a residue affords an explanation for the accumulation of nucleotide compounds in the soil. The continual decomposition and synthesis of nucleic acids or nucleoproteins by the bacterial cell would favor the accumulation of such an enzyme stable residue. Certainly the nucleotide compounds in natural soil are more resistant than those prepared from nucleic acid, although evidence was obtained that conditions were not particularly favorable for the decomposition of nucleic acid in one podsol soil.

A small amount of nucleic acid is undoubtedly present in soil in the undecomposed bodies of micro-organisms, but this can only account for a small fraction of the total. Perhaps a small amount of the ordinary purine or pyrimidine nucleotides exist in the soil 'buried' in the soil particles or colloidal coatings such that they are protected from the decomposition agencies.

Apart from the isolated material there are indications that a considerable part of the remainder may be phytin phosphorus along with more of the nucleotide phosphorus.

Thus very significant contributions have been made to the fractionation of soil organic phosphorus.

This conception of the soil organic phosphorus explains its behaviour in the field and in the laboratory remarkably well.

Ferric phytate is decomposed by alkalies and the phytin released along with the nucleotide compounds is then dissolved. If Ca is present part of the phytin probably reprecipitates as calcium phytate and the

dissolution of the nucleotides is probably also hindered (partly, at least, due to the insolubility of calcium humates). If Ca is first removed then the phytin and nucleotides are dissolved. Hot ammonia decomposes ferric phytate more completely and thus gives better extraction. This explains the results of Williams and of Dean who found that a large part of the organic phospharus could not be dissolved in alkali without first removing exchangeable calcium.

The increasing solubility of organic phosphorus in acid as the strength of the acid is increased is due to the hydrolysis of ferric phytate and of calcium compounds, allowing phytin and nucleotides to dissolve.

The precipitation of organic phosphorus with the humic acid precipitate is largely due to the precipitation of ferric phytate in acid solution and the addition of oxalate partially prevents this by the formation of unionized complexes of Fe with the α - OH acid.

The stability of phytin to hydrolysis by NaOH explains the stability of the soil organic phosphorus to this treatment. The resistance of phytin to oxidation by Br_2 accounts for Dean's finding that only a small fraction of organic phosphorus was lost by this treatment.

The presence of phytin and the enzyme stable nucleotide explains the accumulation and resistance to decomposition of the organic phosphorus in the soil. This also explains why plants may not readily use the soluble soil organic phosphorus, since there is little evidence that appreciable amounts of organic phosphorus may be assimilated without first being hydrolyzed.

The well known fact that liming decreases the organic phosphorus of an acid soil is probably explained by the decomposition of ferric phytate in local alkaline spots in the soil and the consequent liberation of phytin for the action of the soil micro-organisms. This is corroborated

by the finding that phytin was partially decomposed in a calcareous soil. The effect of sodium carbonate treatments in increasing the soluble iron of a podsol soil is explained by the formation of soluble complexes of the iron liberated from the ferric phytate with the α - OH organic acids of the soil.

In conclusion a word might be said of the significance of these conclusions as regards the practical problems of composting and manuring practices. If plant residues and manure are added directly to the soil, their phytin phosphorus, which may amount to 50 percent or more of the total phosphorus, would probably be fixed almost immediately as ferric phytate and thus become unavailable. The same thing may happen where soil is added to organic material in the making of composts. Thus theoretically,for a maximum utilization of phosphorus, the material should first be allowed to decompose so that the phytin phosphorus would be hydrolyzed and converted to nucleotide phosphorus of the micro-organic tissue, in which form it would probably be more available when added to the soil.

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