'SOIL ORGANIC PHOSPHORUS'

A thesis submitted by

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"The last step of reason is to recognize that there are infinitely many things which go beyond it."

- (Blaise Pascal, C17)

"The natural sciences educate us in modesty and humility. It is a well-known fact that the more thorough an investigator is, the humbler and more modest he becomes, for he realises his limitations the more."

- (Prof. C.E. Luthardt, C19)

Therefore, "if any man lacks wisdom let him ask of GOD who gives generously and ungrudgingly, without finding fault, and it will be given to him. But the request must be made in faith, without doubting (that God can do it)."

- (James 1:5,6; The amplified Bible, C20)

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ABBREVIATIONS USED IN THIS THESIS

Apart from the symbols and abbreviations listed in Chapter 7 (Physicochemical Symbols) of the "Handbook for Chemical Society authors", The Chemical Society Special Publication No. 14, 1961, the following abbreviations have been used throughout the text:

P_t	=	Total phosphorus
P_i	=	Inorganic phosphorus
P_o	=	Organic phosphorus
PP_i	=	Inorganic pyrophosphate
OM	=	Organic matter
K_d	=	Distribution coefficient (Gelotte, 1960).

N.B.:- The term 'organic phosphorus' (P_o) is considered to mean the sum of the P_o in the soil organo-mineral complex, plus the P_o in microorganisms (whether dead or living), plus the P_o in partly decomposed plant materials.

SUMMARY

This thesis deals with the extraction, fractionation and characterization of organic phosphorus from soil.

An effective and rapid method to extract organic phosphorus from soils has been developed using an acid pretreatment followed by ultrasonic vibration in the presence of sodium hydroxide. The results obtained by this method compared favourably with those by other published extraction procedures. The method is suitable for both analytical and preparative extractions.

A simple gel chromatographic procedure which fractionated the phosphorus in the crude extracts was then developed. This resulted in a fraction containing the inositol polyphosphates in a form suitable for further separation by electrophoresis or anion-exchange chromatography.

The gel chromatographic results also revealed that the major portion of the organic phosphorus extracted from all the soils behaved as a macromolecule. This fraction in the Urrbrae soil had the nature of a polysaccharide and was partially degraded with acid to yield three main organic phosphates which did not contain

inositol. Two of these compounds were reducing sugar phosphates which gave a characteristic colour reaction with the carbazole method for uronic acids. The third component was a non-reducing compound.

These results have opened the way for a detailed characterization of the various components of a major and, as yet, unidentified portion of organic phosphorus in the Urrbrae red-brown earth.

DECLARATION

Some of the preliminary results described in Appendix 3 are based on material presented by the author to the University of Adelaide in partial fulfilment of the requirements for the degree of Bachelor of Agricultural Science with Honours.

This thesis contains no other material which has been accepted for the award of any other degree or diploma in any University, and to the best of the author's knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

John H. Steward
Adelaide
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INTRODUCTION

Soil genesis involves the colonization of parent rock by micro-fauna, micro-flora and plants. As such the system usually contains insufficient phosphorus to support regular plant growth; as well losses of phosphorus occur by leaching and secondary-mineral formation. Because inorganic phosphorus is recognized to be an essential element for cell metabolism most soils used in agriculture, horticulture or forestry are regularly given phosphorus fertilizer.

Of the fertilizer phosphorus that is added during crop seeding as little as 10% is recovered in the ensuing harvest. A considerable proportion of the remaining phosphorus may be 'fixed' in the soil. Some of it is precipitated by Fe, Al and Ca or sorbed by inorganic colloids, but some may be biologically 'fixed', i.e. converted to organic forms - by the metabolism of plants, animals and microorganisms. These organic phosphates are later released into the soil by exudation or by decomposition of the organ(-ism) following death. The compounds are retained in the soil by interactions with the clay-organic matter complex.

Very little phosphorus is removed from grazed pastures and organic phosphates accumulate in pasture soils to a greater extent after phosphorus fertilization than in the corresponding cropped soils.

Most organic phosphorus is not directly available to plants. Until inorganic phosphorus is released by mineralisation the compounds act as a store of the element in the soil. Under certain conditions an economically significant proportion of added fertilizer phosphorus may thus accumulate. Some of the particular organic phosphates involved are yet to be identified, therefore the processes responsible for their buildup are also undefined. The potential of these compounds as a future source of inorganic phosphorus in the soil is correspondingly uncertain.

In this thesis the literature review lays a background for fresh approaches to an understanding of the organic phosphorus in soils. Succeeding chapters describe current progress in extracting and isolating the organic phosphates from some representative soils of Eastern Australia and from the major soil of the South Australian Wheat Belt.

REVIEW OF THE

LITERATURE

I. GENERAL

Soil organic phosphorus has been the subject of intense study since 1930. Prior to the 1950's the methods for investigating P_o were limited and progress was slow (Black & Goring, 1953; Ulrich & Benzler, 1955). Subsequent developments have been summarised by Anderson (1967) and Cosgrove (1967). The more recent progress was stimulated by the introduction (Smith & Clark, 1951, 1952) and development (Caldwell & Black, 1958) of a new fractionation technique, and the inception of several effective extraction procedures (Mehta, Legg, Goring & Black, 1954; Saunders & Williams, 1955; Kaila & Virtaanen, 1955).

II. MEASUREMENT OF ORGANIC PHOSPHORUS

Soil organic phosphorus is measured indirectly by two different procedures:

1. Ignition

Organic P corresponds to the increase in the P_i which can be extracted by acid from soil following the destruction of the OM by ignition. Usually untreated and ignited samples are extracted separately and

P_o = P_i extracted from ignited sample
- P_i extracted from untreated sample.

Black & Goring (1953) discussed the problems associated with the use of this approach and numerous publications have since confirmed the existence of difficulties which may each affect the final P_o figure.

There are six sources of variation in the method:

(a) Variation between the two samples (untreated and ignited) which are to be extracted.

(b) Incomplete extraction of P_i from the untreated and/or the ignited sample.

(c) Hydrolysis of P_o during extraction from the untreated soil (Walker & Adams, 1958; Anderson, 1960).

(d) Incomplete oxidation of P_o during ignition (Legg & Black, 1955; Dormaar & Webster, 1964).

(e) Volatilization of P at high ignition temperatures (Dormaar & Webster, 1964).

(f) Alteration of the solubility of native P_i as a result of ignition (Williams & Walker, 1967; McKercher & Anderson, 1968a; Williams, Syers, Walker & Rex, 1970).

The various methods proposed use a variety of ignition temperatures, as well different concentrations of acid are used for the P_i extraction (e.g. Legg & Black, 1955 ignited at 240° and extracted with hot conc HCl cf. Kaila, 1962 who ignited at 600° and extracted with 0.1M H_2SO_4). Usually the P_o figures obtained by an ignition procedure exceed the figures for P_o extracted from the corresponding soils (Legg & Black, 1955; Saunders & Williams, 1955; Anderson, 1960; Kaila, 1962; McKercher & Anderson, 1968a). However the use of more than one ignition procedure on the same soil shows the considerable variations that different ignition conditions evoke (Dormaar, 1964; Bornemisza & Igue, 1967; Williams et al., 1970).

There is little literature on the use of ignition to determine P_o in Australian soils. However the investigations of Norrish (1968) on plumbogummite minerals showed

that moderate igniting temperatures could produce erroneously high P_o contents in Australian (and many other) soils.

The variations caused by steps (a)-(f) above may produce positive and negative errors in the course of determining P_o by the ignition procedure. It is impossible to predict which errors will predominate in any soil and considerable research is thus entailed before the method can be used with confidence on a particular soil type. A sounder basis for widespread use of the method appears to be necessary than the approach of a number of workers which was summarised by Legg & Black (1955) when they stated that "the advantages of the ignition procedure lie in the realm of convenience and time rather than accuracy and precision".

2. Extraction

Organic P is the difference between the total and the inorganic P in an extract of a soil. The P_i is measured before and after destruction of the OM in the extract and

$$P_o = P_i \text{ after destruction} - P_i \text{ before destruction.}$$

Usually only an aliquot of the extract is destroyed (by acid digestion or Na_2CO_3 fusion) for the determination and it is thus possible to obtain both quantitative and

qualitative information from the same extract (cf. the total destruction of the sample by ignition).

Possible sources of error in this approach are incomplete extraction of P_o and subsequent hydrolysis of P_o to P_i during the extraction. In quantitative terms the most effective method of extracting P_o is that which gives the highest numerical P_o value; this does not mean however that partial degradation of the organic moiety attached to the phosphate has not occurred.

NaOH is the single most effective extracting agent of P_o from soil, but the yields are usually greater when polyvalent cations are first removed (Jackman & Black, 1951a; Black & Goring, 1953; Mehta et al., 1954; Saunders & Williams, 1955; Anderson, 1960). Some common extraction procedures that use NaOH are outlined in Table 1. The main differences between these methods are in the pretreatments - whereas some workers used a rather concentrated acid for the pretreatment (Mehta et al., 1954; Kaila & Virtaanen, 1955; Kaila, 1962; Greb & Olsen, 1967) Saunders and Williams (1955; Saunders, 1959) used dilute acid. Later workers became convinced of the importance of avoiding hydrolysis of acid-labile phosphates (Anderson, 1960; Harrap, 1963; Dormaar & Webster, 1963b).

TABLE 1.

Comparison of various procedures for the extraction of organic phosphorus from soil

Reference	Pretreatments	Extraction Procedure (0.5M-NaOH)
Mehta et al., 1954	⁂conc HCl - 10 mins 70°; stand 1 hr	stand 1 hr (30:1); 8 hr, 90° (60:1)
Anderson, 1960	0.3M-NaOH - 16 hr shake; followed by ⁂	stand 1 hr (30:1); 8 hr, 90° (50:1)
Saunders & Williams, 1955	0.1M-HCl - 1 hr, 70°	shake 16 hr (400:1)*; repeat (200:1)
Saunders, 1959	0.1M-HCl - 1 hr, 70°	shake 16 hr (400:1)*; repeat (200:1); then stand 16 hr, 90° (200:1)
Kaila & Virtaanen, 1955	2M-H_2SO_4 - stand 18 hr	stand 16 hr, (60:1); 4 hr, 90° (60:1)
Kaila, 1962	2M-H_2SO_4 - stand 18 hr	stand 16 hr (100:1)
Dormaar & Webster, 1963b	Shake each for 4 hr, 1:1 acetone; 0.3M-NaOH; 2M-H_2SO_4	stand 16 hr (60:1); 4 hr, 90° (60:1)
Harrap, 1963	5% Na_2EDTA, shake 2 hr	shake 1 hr (30:1); stand 8 hr, 90° (60:1)
Greb & Olsen, 1967 (calcareous soils)	4M-HCl, 10 mins 70°	stand 16 hr (100:1); 2 hr, 80° (25:1)

* 0.1M-NaOH.

Temperature is room temperature unless quoted.

Solution:soil ratios for NaOH extraction are shown in brackets.

[A few entirely different extraction procedures have also been proposed (Boswall & de Long, 1959; MacLean, 1965a; Halstead, Anderson & Scott, 1966; Thomas & Bowman, 1966). In the writer's opinion none of these methods appear to have any proven advantage or effectiveness over NaOH extraction.]

Distinct limitations of each procedure listed in Table 1 are the long times of extraction and the large solution to soil ratios which are used. These make routine analyses tedious and, on such a scale, qualitative investigations on the extract are rarely possible. Another difficulty is that none of the methods is consistently superior to the others in extracting P_o from soils. With no "standard" method comparison of results in the literature is not strictly valid where different extraction methods have been used (Dormaar, 1964, 1967; MacLean, 1965b; Bornemisza & Igue, 1967).

3. Amounts

In the surface horizons of most soils P_o constitutes a significant proportion of P_t (Table 2). Where the soil pH exceeds 7 the amount of P_o often represents a smaller proportion of the P_t (van Diest & Black, 1959; Williams & Steinbergs, 1958; Williams, Williams & Scott, 1960;

TABLE 2.

Amounts of organic phosphorus extracted from the surface layer of various soils

Location	Reference	No. of soils	ORGANIC PHOSPHORUS (ppm) Range	(ppm) Mean	(% of P_t) Range	(% of P_t) Mean	Method of extraction (From Table 1)
East. Aust.	Williams & Steinbergs, 1958	155	24-580	84	11-61	22	Williams, 1950
Gold Coast	Nye & Bertheux, 1957	67[α]	7- 70	27	- -	20	Williams, 1950
		21[β]	11-270	99	- -	30	Williams, 1950
Scotland	Williams et al., 1960	100	245-1056	695	24-61	52	Saunders & Williams, 1955
New Zealand	Wells & Saunders, 1960	136	270-850	-	13-79	54	Saunders & Williams, 1955 *
Nth. America	Ridley & Hedlin, 1962	68	307-382	360	45-55	51	Saunders & Williams, 1955
Nth. America	Greb & Olsen, 1967	23[γ]	60-330	180	8-37	23	Greb & Olsen, 1967
Nigeria	Enwezor & Moore, 1966b	14[α]	41-125	110	17-29	25	Mehta et al., 1954
		5[β]	106-292	211	31-72	50	
Costa Rica	Bornemisza & Igue, 1967	12	- -	789	34-77	52	Mehta et al., 1954
Ireland	Hanley, 1965	30	288-850	446	38-70	58	Mehta et al., 1954
Canada	John et al., 1965	38	21-802	236	2-78	23	Mehta et al., 1954
Finland	Kaila, 1963	213	100-940	340	17-68	35	Kaila, 1962
U.K.	Harrap, 1963	17	50-680	330	11-58	34	Harrap, 1963

α Savannah vegetation. β Forest vegetation. γ Calcareous soils.

* Ignition.

Hance & Anderson, 1962; Harrap, 1963; John et al., 1965), due to a lower sesquioxide content of the soil or sometimes less OM. A different soil microflora may influence this change in P_o at high pH (Thompson, Black & Clark, 1948). Liming may also raise the pH and cause significant short-term decreases in the P_o content of a soil (Ghani & Aleem, 1942; Thompson et al., 1948, 1954; Williams & Steinbergs, 1958; Williams et al., 1960; McConaghy, 1960; Halstead, Lapensee & Ivarson, 1963; Awan, 1964).

The P_o content of a soil decreases with depth (Williams, 1950; Jackman, 1955a; Barrow, 1961; Kaila, 1963; Dormaar & Webster, 1963b; Enwezor & Moore, 1966b; Garbouchev, Hinov, Nikolov, Neikova & Raikov, 1968) but Ballard (1970) recently suggested that this change is less noticeable when the increase in bulk density down the profile is considered.

Movement of P_o through a profile occurs by leaching (McDonnell & Walsh, 1957; Westin & de Brito, 1969) and is significant in both tropical (Nye & Bertheux, 1957; Enwezor & Moore, 1966b) and temperate soils (Ridley & Hedlin, 1962; Saunders, Taylor & Gold, 1963; Jackman, 1964). Some evidence suggests that soil P_o may be transported with microbial cells and cellular debris (Hannapel

et al., 1964a & b) as well as in the form of simple organic phosphates linked to polyvalent cations (Bowman, Thomas & Elrick, 1967). Both Jackman (1955b) and Martin (1970a) found that there was a decrease in organic carbon relative to P_o down the profile in New Zealand soils and the same has been reported in a tropical soil (Bornemisza & Igue, 1967). These results suggest that a large amount of free P_o can be leached into the lower layers of soil.

In the topsoil P_o accumulates under grazing conditions as a result of regular fertilizer applications and minimal losses of P (Williams, 1950; Donald & Williams, 1954; Jackman, 1955a, 1959; Bromfield, 1961; Williams & Lipsett, 1961). Some New Zealand pasture soils immobilize as much as 60%, though usually it is 20%, of added P by conversion into organic forms (Jackman, 1955a). Similar trends are evident in Australian soils (Williams, 1950; Donald & Williams, 1954; Williams & Donald, 1957).

There is considerable evidence that the mineralisation of P_o in cultivated soils exceeds that in virgin soils (Cosgrove, 1967). Some of the differences that have been determined are shown in Table 3. The P_o fraction of these soils is apparently more resistant to cultivation than the OM (but cf. Nye & Bertheux, 1957). The decrease of P_o in the cultivated soil may be due in part to an even

TABLE 3.

Amounts of organic phosphorus in comparable virgin and cultivated soils

Reference	Location	No. of soil pairs	VIRGIN			CULTIVATED		
			P_o	% of P_t	C:P_o	P_o	% of P_t	C:P_o
Paul, 1954	British Guiana	1	280	37	114	200	33	77
Thompson *et al.*, 1954	Iowa, U.S.A.	20	276	43	142	217	41	120
Grunes *et al.*, 1955	North Dakota, U.S.A.	8	166	31	118	147	27	99
Williams & Lipsett, 1961	N.S.W., Aust.	55	59	27	278	49	22	231
Kaila, 1963	Finland	10	320	55	201	320	34	138
MacLean, 1965b	New Brunswick, Canada	6	390	55	-	339	35	-
Greb & Olsen, 1967	Colorado, U.S.A.	4	165	27	128	140	26	80

distribution throughout the plough layer compared with the accumulation of P_o in the top few cm of virgin soils. Actual losses of P_o from the system may occur by leaching, erosion and crop removal. In the latter case net mineralisation of P_o must first occur (Thompson, Black & Zoellner, 1954; Acquaye, 1963; MacLean, 1965a; Greb & Olsen, 1967; Cosgrove, 1967; Sekhon & Black, 1969). Cultivation and cropping does not always result in net mineralisation of P_o however (Williams, 1950; Ridley & Hedlin, 1962; Enwezor, 1967; van Diest, 1968). The amount of fresh plant material being returned to the soil and its P_i content are important controlling factors in these situations (Friend & Birch, 1960; Birch, 1961; Black & Goring, 1953).

Cosgrove (1967) has reviewed the chemical processes likely to be involved in transformations of organic phosphates in soil. Until the individual compounds involved are identified and their sources in soil are understood (if plant, animal or microorganism) it is impossible to predict the chemical changes effected by various agronomic treatments.

III. INOSITOL POLYPHOSPHATES

1. Introduction

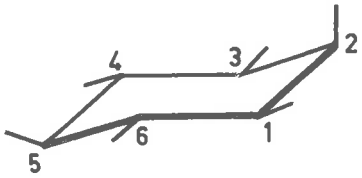
The organic phosphates that have been extracted from soil and identified are nucleic acids and nucleotides, phospholipids and inositol polyphosphates. The latter are the only compounds known to contribute significantly to the P_o in soil. (In most soils at least half of the P_o is yet to be identified.)

Inositol polyphosphates are phosphate esters of inositol (hexahydroxycyclohexane); the predominant one in nature is myoinositol hexaphosphate. The phosphate ester linkages (Fig. 1) are very stable, particularly in strongly alkaline media. The phosphate groups can be hydrolysed by chemical or enzymic means resulting in a series of lower phosphates, the end-products being free inositol and orthophosphate (Cosgrove, 1966a; Posternak, 1965).

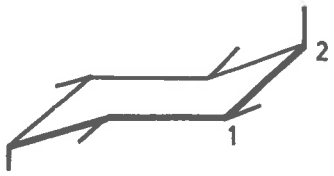
2. Presence in the soil

In 1940 inositol and materials having the properties of inositol hexaphosphate were isolated from soil (Dyer, Wrenshall & Smith, 1940; Yoshida, 1940). Since that time four of the five stereoisomers shown in Figure 1

INOSITOL CONFORMATIONS

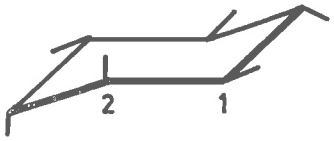


MYO-

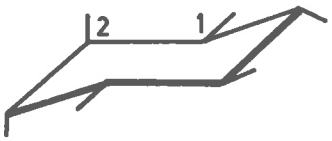


NEO-

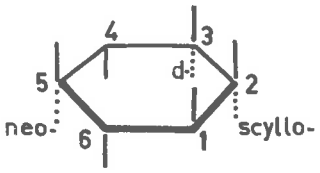
SCYLLO-



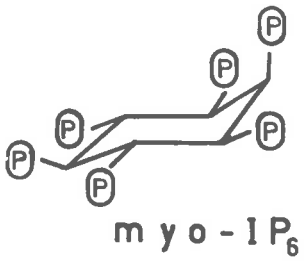
D (+)



L (-)



myo-



myo-IP_6

Ⓟ = OPO_3H_2

(H- bonds not shown)

Figure 1.

have been extracted from soil as the hexaphosphate : *myo*-, *scyllo*-, D(*chiro*)- and *neo*- (Cosgrove, 1963; Cosgrove & Tate, 1963; Cosgrove, 1969b).

The likely sources of these compounds in soil are currently the subject of considerable discussion. Small amounts of *myo*inositol hexaphosphate have been found in many vegetative parts of plants, including the roots (Knowles & Watkin, 1932; Earley & de Turk, 1944; Makarevich, 1953; Johri & Kehar, 1962; Sobolev, 1962; Cosgrove, 1964; Tewari & Singh, 1964; Roberts & Loewus, 1968; Martin & Molloy, 1971). These parts are often returned to the soil either directly (Jones & Bromfield, 1969) or via grazing animals (Donald & Williams, 1954; Bromfield, 1961; Bromfield & Jones, 1970). In so doing they may contribute significant amounts of *myo*inositol hexaphosphate to the soil.

However soil inositol polyphosphates are more complex than if they were derived from plant sources alone. Micro-organisms are a probable source of the D(*chiro*)-, *neo*- and *scyllo*- inositol hexaphosphates. The discussion to follow shows that there is a growing body of indirect evidence to support this theory, evidence which implicates both micro-organisms in the digestive tract of animals and micro-organisms from the soil.

(a) Microorganisms within the animal -

There is now no doubt that animal manures contain inositol polyphosphates (Caldwell & Black, 1958; Peperzak, Caldwell, Hunziker & Black, 1959; Martin & Molloy, 1971). Before this was known McAuliffe & Peech (1949), using ^{32}P-labelled orthophosphate, had shown that there was a considerable synthesis of acid-soluble P during the metabolism of plant materials in a sheep's rumen. Unfortunately the labelled products in the manure were not fractionated. Recently Bromfield & Jones (1970) found that, depending on the nutritional status of the sheep, considerable amounts of P_o were synthesised in the rumen; but at other times there was a net mineralisation of P_o. As well they measured considerable synthesis of acid-soluble P_o in the course of experiments in the laboratory on dung decomposition.

These results show that the rumen microorganisms of sheep can serve as a source of P_o and thus could give rise to inositol polyphosphates. The fact that Martin & Molloy (1971) found only myoinositol hexaphosphate in one sample of sheep faeces may only reflect the particular rumen and intestinal flora in the sheep and/or its nutrition at that time, and is not conclusive evidence that hexaphosphates of the other inositol isomers are not to be found in sheep manure.

(b) Microorganisms in the soil -

There is virtually no recycling of manure and other organic materials in virgin or highly cultivated soils and the presence of inositol polyphosphates has to be explained in some other way. The microorganisms present in the soil may be responsible for the synthesis of these compounds. Both Smith & Clark (1951) and Caldwell & Black (1958) have presented good evidence for the production of inositol hexaphosphates in soils incubated with microorganisms.

The most convincing demonstration of the ability of a microorganism to synthesise any compound is by detecting that compound after growing the organism in pure culture on a synthetic medium. Most of the experiments using this approach have been summarised by Cosgrove (1967) who concluded that "many investigations with microorganisms in pure culture have been unsuccessful, others at best have been inconclusive".

Cosgrove did not discuss some Russian work in which Gotovtseva (1956, 1957) extracted inositol polyphosphates from the fungus Penicillium chrysogenum but omitted to consider the possible introduction of myoinositol polyphosphate from the maize extract which was used as a growth medium. It is clear that the conditions of culture, type of medium, time of harvest (Gotovtseva, 1957) and

methods of analysis all influence the final results of such experiments, and these problems may account for the lack of convincing evidence from pure cultures.

Dr. S.G. Williams has unequivocally demonstrated the synthesis of myoinositol hexaphosphate by Neurospora crassa grown in pure culture on a synthetic medium (Waite Institute Annual Report 1966-1967). The Na salt of ^{32}P-labelled myoinositol hexaphosphate was crystallized from a perchloric acid extract of an inositol-less mutant of N. crassa which had been cultured on a glucose C-source with ^{32}P-labelled orthophosphate (S.G. Williams - personal communication).

A substantial amount of survey work is required if the important inositol polyphosphate-producing micro-organisms from soil are to be identified. Ikawa, Borowski & Chakravarti (1968) measured up to 0.6% of myoinositol on a dry weight basis in some fungi and algae. They used a method which releases all of the inositol from inositol hexaphosphates and a logical extension of this work would be to examine a wide range of microorganisms for the presence of D(chiro)-, scyllo- and neo- inositols.

3. Extraction and purification

Inositol polyphosphates are extracted from soil in the same way as the whole P_o i.e. a suitable pretreatment is followed by alkali extraction(s).

The published methods for the purification of inositol polyphosphates can be divided into a number of groups:

(a) Those which depend upon the relative stability of the fully substituted inositol hexaphosphate towards procedures designed to remove extraneous matter -

(i) Precipitation of coloured materials (humic acids) at low pH --

This approach was used in the early work (Yoshida, 1940; Anderson, 1956; Caldwell & Black, 1958) before Anderson (1961) showed that "below pH 0.4 a small and constant proportion of the soil P_o is precipitated with the humic materials". The major danger in this approach is the likelihood of hydrolytic degradation of the P_o as a result of prolonged exposure to acid.

(ii) Alkaline hypobromite oxidation --

Hockensmith and coworkers introduced alkaline hypobromite oxidation to decolourize extracts before determining P_i (Whitney & Gardner, 1936). Subsequently Wrenshall & Dyer (1941) and Bower (1949)

used this treatment to purify extracts containing inositol polyphosphates. Both schools found < 5% release of P_i when calcium phytate preparations were treated in the same way as the soil extracts. Wrenshall & Dyer (1941) pointed out the particular danger of hydrolysis of the P_o when the mixture was boiled under acidic conditions to remove the excess bromine.

The use of improved fractionation procedures leaves no doubt that considerable degradation of the higher inositol polyphosphates may occur during alkaline hypobromite oxidation (Anderson, 1964; Halstead, 1965; Moyer, 1968).

(iii) Prolonged alkaline hydrolysis --

A prolonged extraction of acid-leached soil with boiling 3M-NaOH gives a greater yield of free inositol polyphosphates than a shorter extraction at 60° (Anderson & Hance, 1963; Halstead & Anderson, 1970). It has been proposed that linkages between the inositol polyphosphates and carbohydrates or proteins are broken.

(b) Those methods which depend upon the high charge density of the inositol polyphosphate molecules -

(i) Precipitation with polyvalent cations --

Ferric chloride in acid solution or barium acetate at alkaline pH are most commonly used (Anderson, 1967).

(ii) Ion exchange chromatography --

Smith & Clark (1951, 1952) introduced ion-exchange chromatography in which adsorption of the phosphoesters on an anion-exchange resin allows them to be separated from uncharged and cationic compounds. The phosphates are then eluted from the column in bulk, in groups or as individual components.

Anion-exchange chromatography is the method which has been most responsible for the recent developments in the isolation and fractionation of soil inositol polyphosphates (e.g. see Cosgrove, 1969a & b).

(c) Approaches based on molecular size -

Thomas and coworkers have used Sephadex gel chromatography to fractionate extracts of soil (Thomas & Bowman, 1966; Moyer & Thomas, 1970). However they omitted to consider the strong anion exclusion effect on dextran gels eluted with distilled water (Neddermeyer & Rogers, 1968) and the resulting skewed elution profiles make the results difficult to interpret and impossible to quantitate.

No single method has yet been found to yield analytically pure inositol polyphosphates from crude soil extracts. The complex nature of the starting material usually requires combinations of groups (a) and (b) to achieve satisfactory fractionations. Martin (1964) introduced a non-oxidative procedure which replaced

preliminary chemical purification with a resin column fractionation, but later work demonstrated that the method has some significant limitations (Martin, 1970 a & b).

4. Amounts

Wide variations in the inositol polyphosphate contents of soils have been reported. The published figures for the penta- and hexa- phosphates in a number of surface soils have been listed in Table 4. At least one-fifth of the P_o in most soils is inositol polyphosphate P.

Accumulation of these compounds in soil in the presence of large numbers of phytase-producing organisms (Greaves & Webley, 1965, 1969; Cosgrove, 1967; Cosgrove, Irving & Bromfield, 1970) reflects the limited amount of phytate-P which is in the soil solution (Jackman & Black, 1951, 1952; Theodorou, 1968; Bornemisza & Igue, 1967). The stability to hydrolysis of the inositol polyphosphates in the soil is enhanced by adsorption of the compounds and/or the enzymes on clay minerals and sesquioxides (Goring & Bartholomew, 1950; Jackman & Black, 1951a; Mortland & Gieseking, 1952; Anderson & Arlidge, 1962; Bowman et al., 1967; Martin & Cartwright, 1971).

TABLE 4.

Content of inositol penta- and hexa- phosphates as determined by anion-exchange chromatography of extracts from various soils

Reference	No. of soils	Location	Soil sample	INOSITOL POLYPHOSPHATES (ppm) Range	(ppm) Mean	(% of P_o) Range	(% of P_o) Mean
Caldwell & Black, 1958	10	Iowa	Surface α	28- 85	56	7-25	16
	10	Iowa	β	22- 68	40	9-25	16
	24	Other U.S.	"	4- 67	29	3-52	18
	4	Canada	"	51- 99	66	19-24	22
Thomas & Lynch, 1960	9	Canada	A-horizon	9-156	55	2-22	12
	9	Canada	B-horizon	2- 10	5	1- 6	3
McKercher & Anderson, 1968a	18	Canada	Surface	20- 71	38	11-23	17
	12	Canada	Subsoil	18- 43	26	11-19	17
McKercher & Anderson, 1968b	4	Canada	Surface	31- 71	54	13-23	18
	4	Britain	Surface	90-460	240	31-50	44
Anderson, 1960	3	Britain	Surface	192-292	233	25-34	30
Anderson, 1964	17	Britain	Surface	56-460	199	24-58	40
Martin, 1970	4	N. Zealand	Topsoil	132-266	205	28-62	47
Cosgrove, 1963	3	Australia	Surface	92-334	183	12-16	14
Williams & Anderson, 1968	47	Australia	Surface	1-356	39	1-38	16
	14	Australia	Surface α	2- 21	12	17-29	19
	14	Australia	γ	7- 47	21	8-23	16
	10	Australia	Surface α	7- 16	11	11-21	15
	10	Australia	β	6- 12	9	13-25	19

$\alpha \frown \beta$ represents paired virgin and cultivated soils.

$\alpha \frown \gamma$ represents paired virgin and leguminous pasture soils.

IV. UNIDENTIFIED ORGANIC PHOSPHATES

In 1958 Robertson reported the separation by paper chromatography of two phosphates in citric acid extracts of some soils. There was no evidence that they were organic phosphates however. Other workers have since detected unidentified organic phosphates during anion-exchange chromatography of the P_o from NaOH extracts of soil.

Considerable amounts of P_o from the fulvic fraction of NaOH extracts which did not contain inositol were loosely bound to anion-exchange resins (Thomas & Lynch, 1960; Dormaar, 1967). Similar compounds were found in extracts obtained by prolonged alkaline hydrolysis of soils (Halstead & Anderson, 1970). Other organic phosphates did not adhere to anion-exchange resins at all. McKercher, using an extract prepared in the same way as Halstead & Anderson (1970), recorded that in one soil extract 28% of the total P_o was not held by the resin (McKercher, 1968; McKercher & Anderson, 1968c). The material was carbohydrate in nature and was similar to a fraction detected in extracts from a different soil by Moyer (1968).

Using extreme chemical pretreatments (overnight alkaline hypobromite oxidation, 6M-HCl hydrolysis) Cosgrove (1963) found two organic phosphates which were loosely bound to Dowex Cl^-. They were extremely acid resistant and did not contain inositol, carbohydrate, amino acids or nucleotides.

All of these unidentified organic phosphates were incidental by-products of chromatographic investigations on soil inositol polyphosphates. Losses during fractionation (Dormaar, 1967) and hydrolysis in the course of chemical pretreatments with alkaline hypobromite (Ghani, 1942; Bower, 1945, 1949; Smith & Clark, 1951; Eid, Black, Kempthorne & Zoellner, 1954; van Diest & Black, 1959) or hot alkali (Plimmer, 1913; Wrenshall *et al.*, 1940; Ghani, 1943; Mehta *et al.*, 1954) suggest that the amounts determined for these unknown compounds are minimal. The need to isolate and identify such a significant portion of the P_o in soils is obvious.

AIMS OF THE

EXPERIMENTAL WORK

I. THE EXTRACTION OF ORGANIC PHOSPHORUS

The current methods of extracting P_o from soils have two major disadvantages:

Long extraction times which increase the ever-present possibility of hydrolytic degradation of the P_o and impede the progress of routine surveys; and

Large solution to soil ratios which make large-scale extractions impracticable and generally render qualitative observations impossible.

Ultrasonic vibration is an effective means of dispersing soil particles (Olmstead, 1931; Edwards & Bremner, 1964, 1967). It enables the rapid extraction of organic materials from soils (Greenland & Ford, 1964; Swincer, Oades & Greenland, 1968) and rock fragments (McIver, 1962).

The extraction of P_o from soils at low solution to soil ratios using ultrasonic vibration was therefore investigated. The best results were compared with similar extractions by two common procedures (Mehta *et al.*, 1954; Harrap, 1963).

II. FRACTIONATION OF ORGANIC PHOSPHATES

The successful isolation of inositol polyphosphates from crude extracts of soil to date has required the use of preliminary chemical fractionations. These chemical treatments remove extraneous OM, interfering inorganic compounds and much of the unidentified P_o. The treatments may also degrade the inositol polyphosphates.

Column chromatography, a chemically mild means of fractionation, has failed to produce satisfactory separations of P_o compounds in crude extracts. In view of the wide acceptance of Sephadex gel chromatography in the fractionation of organic materials from soil (Posner, 1963; Mehta, Dubach & Deuel, 1963; Lindqvist, 1967; Bailly & Margulis, 1968; Dubin & Fil'kov, 1968; Ladd, 1969; Dorado, 1969) surprisingly few applications have been made to the fractionation of soil P_o.

Separations on Sephadex gels are achieved on the basis of the size, shape and charge of molecules (Flodin, 1962; Determann, 1968); this suggested that relatively small organic phosphates could be separated from the large humic materials on a suitably chosen grade of gel. A basis for fractionating P_o in crude extracts of soil was obtained by first developing an understanding of the behaviour of known phosphate compounds when chromatographed on Sephadex gels.

III. THE UNIDENTIFIED COMPOUNDS

This fraction represents 80% of the total P_o in most soils (Table 4) and it commands a growing interest among soil chemists. Not until the chemical nature of this fraction is known can the dynamics of P_o in the soil be seriously investigated.

It was the prime aim of this work to locate, isolate and identify this fraction of the soil P_o.

THE EXTRACTION OF ORGANIC PHOSPHORUS FROM SOIL

I. MATERIALS

Soils were obtained from two sources:

1. Urrbrae series (U..)

Six sites from the C1 permanent rotation trial at the Waite Institute were sampled to a depth of 6.5 cm in the summer of 1967. Fifty cores were taken from each site using the zigzag pattern recommended by Jackson (1958). Overnight airdrying of the soils was followed by grinding (< 2 mm); then a representative subsample, obtained by quartering, was ground to fine powder in a Sieb-teknik mill. The soils were stored at room temperature in sealed plastic jars.

The C1 trial has been maintained in its present form since 1950 (Waite Institute Annual Report, 1966-1967) on the Urrbrae fine sandy loam, a red brown earth of pH 5.5-6.5, clay content 20% (Piper, 1938).

2. Eastern Australia

Seven soils were kindly supplied by Dr. C.H. Williams, CSIRO Division of Plant Industry, Canberra. Four of the soils were the same as those used by Williams & Anderson (1968), viz 237, 250, 253 and 315; the other three were closely related to soils used by the same authors, viz ROB - same site as 531, 350 - same site as 348, CH - same paddock as 641.

II. METHODS

1. General

(a) All the soils were analysed for total C using a Fisher Carbon Induction furnace (Young & Lindbeck, 1964). The C of Soils 250 and 350 was corrected for carbonate content. P_t for each soil was determined by X-ray fluorescence spectrography using pressed borate discs (Norrish & Hutton, 1964).

(b) Total P in the extracts was measured after perchloric acid digestion by Usher's adaption (Usher, 1963) of the method of Bartlett (1959a). Inorganic P was determined by the method of John (1970) after the humic materials in the extract had been precipitated in 0.005M-H_2SO_4 at pH 2 for 10-20 mins (Anderson, 1961).

The difference between P_t and P_i in the extract was called P_o; all P values are quoted as ppm P on an air-dry-soil basis. Differences between soils of less than 20 ppm P were considered to be not significant.

(c) Ultrasonic vibration was performed with a Branson S-75 ultrasonic generator operating with a power output of 75 watts and a frequency of 20 kc/sec at the probe tip. The tip was placed 5-10 mm below the liquid surface and

all samples were exposed to the vibrations for 3 min.

(d) Quantities of soil extracted -

(i) 5 g lots of soil were extracted with 0.5M-NaOH (25 ml) in a 50 ml plastic centrifuge tube.

(ii) Lesser amounts of soil ($\frac{1}{2}$-2 g) were extracted with 0.5M-NaOH (6 ml) in a 20 ml flat-bottomed polythene tube.

All extracts were centrifuged and the supernatant solution was retained for analysis.

2. Comparison of extraction by ultrasonic vibration with extraction by shaking

Individual 5 g lots of soil were extracted with NaOH in four different ways:

(a) Shake 16 hr, 25°.

(b) Shake 16 hr, 70°.

(c) Ultrasonic vibration 3 min, room temp.

(d) Ultrasonic vibration 3 min, 70°.

Similar extractions were made on soils that had been pretreated by shaking for one hour with 5% (w/v) Na_2EDTA (25 ml).

3. Development of the extraction procedure using ultrasonic vibration

For the quantitative analyses required in this section the P_o calculation included an adjustment for that material remaining entrained in the soil residue after decanting the supernatant extract. The weight of solution entrained was measured and its P_o concentration was considered to be identical to the P_o in the supernatant extract. The adjustment was calculated assuming a density of 1.0 g/cc for the extract (see Sections (a), (b) and (c) below).

Later, the need for such adjustments was eliminated by washing the soil residue twice with 0.1M-NaOH and adding the washings to the extract (Section (d)).

(a) Solution to soil ratio -

The effect of varying the solution to soil ratio from 3:1 to 12:1 on extraction by ultrasonic vibration was determined.

(b) Variation in the temperature -

Fresh, duplicate samples of soil were extracted by ultrasonic vibration. One sample was extracted at room temperature, the other was stood in an oven at 70^{o} for 30 min immediately prior to extraction.

(c) Effect of a sequestering agent -

The extractions in (b) were repeated using 0.5M-NaOH which contained 5% (w/v) potassium gluconate.

(d) Use of a pretreatment -

Soils were shaken for 1 hr with 1M-HCl (15 mls), washed twice with dist H_2O and extracted by ultrasonic vibration at room temp with 0.5M-NaOH. After centrifuging, the residue was washed twice with 0.1M-NaOH. The pre-treatment, extract and washings were combined and the pH was made alkaline with NaOH

(e) Other extraction procedures used -

Duplicate 1 g lots of each soil were extracted by the methods of Mehta et al., (1954) and Harrap (1963) respectively. The extracts in each step of these methods were analysed individually.

III. RESULTS AND DISCUSSION

1. The effectiveness of extraction by ultrasonic vibration

Three minutes of ultrasonic vibration was as effective in extracting P_o from a soil as an overnight (16 hr) shaking at room temp or at an elevated temp (Table 5). The rapidity of extraction during ultrasonic vibration led to experiments designed to determine the best conditions for P_o extraction by this method.

TABLE 5.

Phosphorus (ppm) extracted from soil U16 with a solution to soil ratio of 5:1

Pretreatment :	NIL				5% EDTA			
Extraction :	Continuous Shaking		Ultrasonic Vibration		Continuous Shaking		Ultrasonic Vibration	
	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
Starting temp:	25°	70°	16°	70°	25°	70°	16°	70°
P_t	156	190	146	180	178	200	164	198
P_i	87	120	76	94	108	128	99	113
P_o	69	70	70	86	70	72	65	85

N.B.:- Shaking was carried out at constant temp. At the end of the ultrasonic vibration the temp had risen to 45° and 90° for the 16° and 70° treatments respectively.

2. The effect of variation in the solution to soil ratio on the extraction of organic phosphorus by ultrasonic vibration

Changing the solution to soil ratio produced the sort of result that is shown in Table 6. An optimum ratio was found for each soil above which there was no significant increase in P_o extracted.

TABLE 6.

The extraction of phosphorus (ppm) by ultrasonic vibration from four soils at three solution to soil ratios

(Values corrected for entrained solution.)

Solution:soil ratio			3:1	6:1	12:1
Soil	U16	P_t	242	237	276
		P_i	121	105	145
		P_o	121	132	131
	U29	P_t	320	307	329
		P_i	159	151	170
		P_o	161	156	159
	315	P_t	795	1362	1424
		P_i	108	291	334
		P_o	687	1035	1090
	237	P_t	1131	1266	1481
		P_i	753	792	920
		P_o	378	474	561

For the soils in the Urrbrae series 3:1 proved to be an adequate solution to soil ratio. The Eastern Australia soils had a greater moisture adsorbtion capacity and to achieve efficient dispersion higher solution to soil ratios were required (6:1 for 250 and CH, 12:1 for the others).

Ultrasonic vibration is most effective when a small volume of liquid surrounds the probe tip. Up to the point where viscosity becomes limiting, the transmission of energy increases as the amount of solid material present increases. The resulting efficiency of dispersion explains why the optimum solution to soil ratios for this method are so low when compared with those used in shaking procedures (Table 1).

3. The effect of high temperature on the extraction of organic phosphorus by ultrasonic vibration

The preliminary results of Table 5 suggested that there may be some effect of temperature on the yield of P_o extracted from soil U16. Table 7 shows that the P_o extracted from any of the soils at 70° was never significantly higher than the amount extracted at room temperature.

TABLE 7.

The extraction of phosphorus (ppm) by ultrasonic vibration with 0.5M-NaOH at room temp and at 70° in the presence and absence of potassium gluconate

Soil Series:			URRBRAE				EASTERN AUSTRALIA			
Extractant :			NaOH alone		NaOH with 5% K gluconate		NaOH alone		NaOH with 5% K gluconate	
Temperature:			Ambient	70°	Ambient	70°	Ambient	70°	Ambient	70°
Soil	1	P_t	149	141	149	152				
		P_i	84	77	88	93				
		P_o	65	64	61	59				
	5	P_t	165	167	180	209				
		P_i	95	103	119	138				
		P_o	70	64	61	61				
	15	P_t	231	231	271	294				
		P_i	111	116	151	193				
		P_o	120	115	120	101				
	16	P_t	259	252	268	293				
		P_i	133	121	149	187				
		P_o	126	130	119	106				
	17	P_t	209	224	242	240				
		P_i	120	116	144	152				
		P_o	89	108	98	82				
	29	P_t	313	295	335	343				
		P_i	150	135	182	188				
		P_o	163	160	153	155				
Soil	250	P_t					42	152	113	120
		P_i					7	100	47	49
		P_o					35	52	66	71
	CH	P_t					216	192	207	219
		P_i					77	72	78	86
		P_o					139	120	129	133
	237	P_t					1481	1485	1559	1555
		P_i					920	908	1043	1041
		P_o					561	577	516	514
	315	P_t					1424	1333	1387	1421
		P_i					334	328	332	330
		P_o					1090	1005	1055	1091
	253	P_t					609	613	1030	940
		P_i					320	336	660	575
		P_o					289	277	370	365
	ROB	P_t					1200	1089	1320	1283
		P_i					423	385	450	500
		P_o					777	704	870	783
	350	P_t					817	1072	1724	1173
		P_i					607	851	1262	789
		P_o					210	221	462	384

In two cases (315, ROB) less P_o was extracted at the higher temperature: this drop reflected a decrease in the P_t extracted. It was probably caused by evaporation of moisture whilst standing in the 70° oven with a consequent lowering of the solution to soil ratio below the optimum.

By contrast there was a suggestion of P_o hydrolysis in two soils (350, 250) where the high temperature caused more P_t to be extracted without any significant rise in P_o.

4. The use of a sequestering agent with the extractant

Gluconate effectively sequesters Ca, Al and Fe in NaOH solutions (Prescott, Shaw, Bilello & Cragwall, 1953; Mehltretter, Alexander & Rist, 1953; Pecksok & Sandera, 1953; Watts & Utley, 1956; Pickering & Miller, 1958); it also dissolves freshly precipitated ferric hydroxide in alkali (Ferlin, Snikeris & Karabinos, 1958). Since polyvalent cations precipitate phosphates in soil the addition of gluconate could aid in the solubilization of some organic phosphates during alkali extraction.

The effect of adding 5% (w/v) potassium gluconate to the 0.5M-NaOH during extraction of P_o is shown in Table 7. The P_o extracted from the Urrbrae soils and soil CH was

not altered by gluconate; the results with Soil 315 were variable but suggested that there was no significant response. The depression of yields in Soil 237 was inexplicable.

Soils 250, 253, ROB and 350 did yield more P_o when potassium gluconate was used with the NaOH. Table 8 shows that the effect was not solely the result of a high salt concentration in the NaOH. The additional effect on extraction (compared with 5% KCl) was attributed to the sequestering ability of K gluconate at high pH.

TABLE 8.

The extraction of phosphorus (ppm) from four soils by ultrasonic vibration with 0.5M-NaOH at room temp and the effect of adding potassium chloride, rather than potassium gluconate, to the extractant

Soil	NaOH alone			NaOH + KCl			NaOH + Kgluc		
	P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o
250	42	7	35	64	19	45	113	47	66
253	609	320	289	700	372	328	1030	660	370
ROB	1200	423	777	1145	386	759	1320	450	870
350	817	607	210	1196	872	324	1729	1262	462

5. Incorporation of an acid pretreatment in the extraction

In the absence of consistent, positive effects of high temperature or K gluconate the use of an acid pretreatment was investigated. When the cold, 1M-HCl pretreatment was bulked with the NaOH extract for analysis the results in Table 9 were obtained. These figures clearly show that, in terms of total P_o extracted, use of the HCl pretreatment was superior to NaOH alone or NaOH plus K gluconate (Table 7).

TABLE 9.

The extraction of P (ppm) at room temp by 0.5M-NaOH with and without a cold, 1 hr pretreatment of shaking with 1M-HCl

Soil Series:		URRBRAE				EASTERN AUSTRALIA	
Pretreatment:		Nil	1M-HCl			Nil	1M-HCl
				Soil 250	P_t	42	168
					P_i	7	95
Soil 1	P_t	149	179		P_o	35	73
	P_i	84	109				
	P_o	65	70	CH	P_t	216	226
					P_i	77	100
5	P_t	165	225		P_o	139	126
	P_i	95	150				
	P_o	70	75	237	P_t	1481	2800
					P_i	920	2200
15	P_t	231	318		P_o	561	600
	P_i	111	185				
	P_o	120	133	315	P_t	1424	1512
					P_i	334	425
16	P_t	259	320		P_o	1090	1087
	P_i	133	194				
	P_o	126	126	253	P_t	609	1520
					P_i	320	1060
17	P_t	209	300		P_o	289	460
	P_i	120	193				
	P_o	89	107	ROB	P_t	1200	1430
					P_i	423	514
29	P_t	313	449		P_o	777	916
	P_i	150	266				
	P_o	163	183	350	P_t	817	5702
					P_i	607	5133
					P_o	210	569

6. Comparison of the results with two other extraction procedures

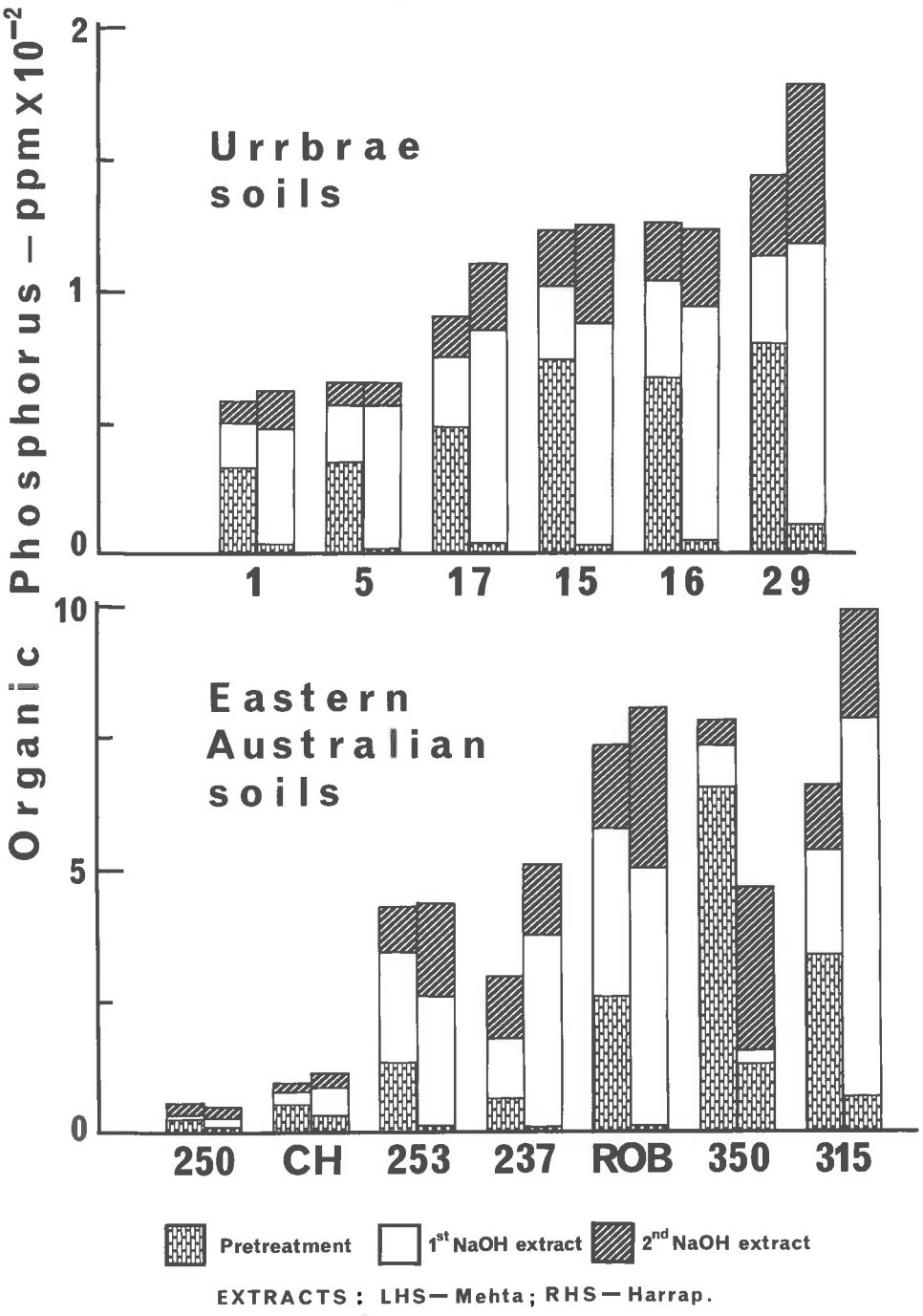
The two extraction procedures chosen for comparison had essentially the same NaOH extraction steps (Table 1). They differed in the type of pretreatment: hot, conc HCl (Mehta et al., 1954) vs. cold, 5% (w/v) Na_2EDTA (Harrap, 1963).

The yields of P_o in the pretreatment, cold NaOH and hot NaOH extraction steps of these two methods are shown in Figure 2. Detailed figures for the extractions are given in Appendix 1.

With one exception (350) the total P_o extracted by the Harrap method equalled or exceeded that extracted by the Mehta procedure. The mild pretreatment of Harrap extracted relatively little P_o and considerable amounts were removed by both NaOH extractions.

By contrast a concentrated hydrochloric acid pretreatment removed considerable amounts of P_o (see also Hance & Anderson, 1962; Martin, 1964; Dormaar, 1968) and Figure 2 shows that the second NaOH extraction became considerably less important in terms of P_o extracted. (Similarly contrast the results of Nye & Bertheux, 1957 and Saunders, 1959 with Mehta et al., 1954; Kaila, 1962 and Dormaar, 1968).

Figure 2


Organic Phosphorus – ppm X10⁻²
Urrbrae soils
2
1
0
1
5
17
15
16
29
Eastern Australian soils
10
5
0
250
CH
253
237
ROB
350
315
Pretreatment
1st NaOH extract
2nd NaOH extract
EXTRACTS : LHS— Mehta ; RHS — Harrap.

In Table 10 the total amounts of P extracted by the procedures of Mehta et al. (1954) and Harrap (1963) are compared with the P extracted by the HCl-ultrasonic vibration method. The use of a 1M acid pretreatment with a single vigorous NaOH extraction generally gave values as high as, or higher than, those obtained by the other methods. Thus, in combination with an effective acid pretreatment to remove polyvalent cations, good dispersion enabled rapid extraction of P_o by NaOH and eliminated the need for a second NaOH extraction step (cf. Kaila, 1962).

It was concluded therefore that during shaking a long period was required for the NaOH to penetrate into the hidden recesses of clay particles and extract OM, whereas ultrasonic dispersion provided almost immediate access to adsorbed and concealed organic particles. If a weaker extracting agent is used, e.g. acetylacetone, longer shaking and ultrasonic vibration times are required to effect complete extraction of the P_o (Halstead et al., 1966; Halstead & Anderson, 1970). Thus effective physical and chemical agents are both necessary if rapid and complete extraction of P_o from a soil is to be achieved.

TABLE 10.

Comparison of P (ppm) extracted by the HCl - ultrasonic vibration procedure with extractions by the methods of Mehta *et al*. (1954) and Harrap (1963)

	Method of extraction								
	MEHTA			HARRAP			ULTRASONIC VIBRATION		
	P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o
Urrbrae series:									
U 1	255	197	58	192	130	62	179	109	70
5	314	249	65	242	177	65	225	150	75
15	402	279	123	327	202	125	318	185	133
16	389	263	126	318	195	123	320	194	126
17	348	258	90	303	193	110	300	193	107
29	493	349	144	441	262	179	449	266	183
Eastern Australian series:									
250	183	130	53	104	56	48	168	95	73
CH	223	131	92	230	119	111	226	100	126
237	2553	2257	296	1495	988	507	2800	2200	600
315	1225	567	658	1362	370	992	1512	425	1087
253	2064	1636	428	1038	603	435	1520	1060	460
ROB	1719	985	734	1385	581	804	1430	514	916
350	7038	6257	781	1954	1490	464	5702	5133	569

IV. RECOMMENDED EXTRACTION PROCEDURE

1. The basis of the recommendation

According to the figures in Table 10 considerable variation is possible when P_o is extracted from a soil by different methods (see also Appendix 1). On the basis of P_o yield alone no single extraction method was superior to the others. The choice of one method over another for use in routine extraction of P_o must be influenced by other factors, when the methods are similar in their ability to extract P_o.

The HCl-ultrasonic vibration procedure gains much over other extraction methods because of its effectiveness in such a short time. The pretreatment plus extraction take only 2 hr and this enables analyses to be made on the day of extraction. The number of soils that can be handled at one time is limited only by the capacity of the centrifuge and the speed of analysis.

2. The steps in the proposed procedure

(a) Weigh the soil ($\frac{1}{2}$-2 g) into a 20 ml polythene vial.

(b) Shake for 1 hr at room temp with 1M-HCl (15 ml).

(c) Centrifuge and decant the supernatant. Wash the residue twice with dist H_2O, centrifuge and add the washings to the pretreatment, then neutralize with solid NaOH.

(d) To the soil residue add 0.5M-NaOH (6 ml), stir thoroughly, then vibrate ultrasonically at maximum current for 3 min.

(e) Remove the vial and wash the material adhering to the probe back into the sonified suspension with dist H_2O.

(f) Wash the residue after centrifugation twice with 0.1M-NaOH and add the washings to the NaOH extract.

(g) Analyse the extract and the pretreatment (individually or after bulking them) and calculate the results as ppm P.

3. Adaptations of the method

(a) Where it is desired to follow a quantitative determination with qualitative observations two simple variations in the method greatly increase the concentration of P_o in the NaOH extract -

(i) By keeping the pretreatment and NaOH extracts separate, 1M-HCl extracts with comparatively little P_o can be discarded before the qualitative experiments.

(ii) Omit steps (e) and (f) from the recommended procedure. Instead the P_o in the entrained solution is accounted for by weighing (as described in II.3. p.33) and assuming negligible loss of soil and solution on the ultrasonic probe. (This assumption results in an error of less than 5%.)

N.B.:- All of the subsequent investigations of this thesis were made on NaOH extracts prepared in accordance with steps (i) and (ii) above.

(b) The extraction can be adapted to deal with large quantities of soil by using the flow-through cell described by Ford, Greenland & Oades (1969). A recent example was the extraction of 20 kg of Soil U29 by 0.5M-NaOH using a solution to soil ratio of 4:1. The extraction took 3 days and yielded a large quantity ($\simeq$ 30 g) of material for identification studies.

V. THE SIGNIFICANCE OF THE RESULTS

1. Eastern Australia soils

The soils from Eastern Australia did not show any consistent relationship between their C, P_o and P_t contents (Table 11). Five of these soils were high in OM content and they also contained large amounts of P_o, although in only two of them (315, ROB) did this P_o represent a high proportion of the total soil P.

TABLE 11.

Details of the soils from Eastern Australia

Sample	Soil Group	C(%)	P_t	P_o	C:P_o	P_o:P_t(%)
250	"Alkaline"	1.27	340	73	174	21.4
CH	Podzolic	2.03	500	126	161	25.2
237	Krasnozem	4.04	3,940	600	67	15.2
315	Alpine humus	9.85	2,200	1,087	91	49.4
253	Black earth	3.72	2,240	460	81	20.5
ROB	Krasnozem	5.84	2,420	916	64	37.9
350	"Alkaline (calcareous)"	6.93	13,500	569	122	4.2

N.B.:- P_o was determined by the HCl-ultrasonic vibration method.

2. Urrbrae series

In the case of the Urrbrae soils the $C:P_o$ ratio was relatively constant (Table 12). Apparently the P_o in these soils is an integral part of the OM; a similar conclusion was reached by Oades (1967a) for the carbohydrates in the same soils. The constancy of the $C:P_o$ ratio suggested that selective accumulation of inositol polyphosphates has not occurred in any (of the) particular rotation(s) (cf. Williams & Lipsett, 1961; Williams & Anderson, 1968; Table 3).

TABLE 12.

Characteristics of the soils from the Urrbrae series

Plot	Rotation	C(%)	P_t	P_o	$C:P_o$	$P_o:P_t$(%)
U 1	WF	1.06	500	70	151	14
U 5	WPF	1.22	575	75	163	13
U15	WWPPPP	2.07	730	133	156	18
U16	PPPPWW	2.08	725	126	165	17
U17	W	1.62	670	107	151	16
U29	P	2.88	930	183	157	20

N.B.:- (1) Each rotation was sampled in the last phase shown.

(2) W=Wheat crop, F=Fallow, P=pasture.

(3) Plots 1, 5, 17 have been maintained since 1925; 15,16, 29 have been maintained since 1950.

(4) P_o determined by HCl - ultrasonic vibration method.

3. Urrbrae soils sampled by other workers

Four soils referred to by Williams (1950) were also examined. Although the method by which they were sampled is not known, the soils were taken from a natural pasture experiment 250 yards south-east of, and on the same soil type as, the C1 permanent rotation trial. The soils were analysed by the same methods used to obtain the results in Tables 11 and 12; the determinations are shown in Table 13*.

---

* A check of the records showed that corrections were needed to two statements made in part II of Williams (1950).

1. The P applied to the soils in Table 4 of Williams (1950, part II) was equivalent to 40 lb P_2O_5/ac/an and not 20 lb. (Total application equivalent to 23 yr x 40 lb = 920 lb P_2O_5/acre, i.e. 450 kg P/ha; Waite Agric. Res. Inst., Annual Report 1943-1947).

2. A cross-check of the labels on the bottle with the Waite Agric. Res. Inst., Annual Report 1925-1932 showed that soil U61 in Table 5 of Williams (1950, part II) had received no fertilizer, and soil U57 had received fertilizer to the equivalent of 120 lb P_2O_5/acre (50 kg P/ha).

TABLE 13.

Characteristics of Urrbrae soils (0-10 cm) growing natural pasture before and after fertilization with superphosphate

Soil Code (Williams, 1950)	Year of taking sample	Total P added in super (kg/ha)	C(%)	P_t (ppm)	P_o* (ppm)	$C:P_o$	$P_o:P_t$(%)
U 61	1927	NIL	1.20	370	58	207	16
U 57	1927	58.3	1.00	390	65	154	17
U622	1947	NIL	1.82	425	82	222	19
U618	1947	450.6	1.80	800	106	170	13

* P_o was determined by the HCl - ultrasonic vibration procedure.

Soil U61 was the best available example of the original virgin Urrbrae red brown earth. In the course of grazing the unfertilized plot for 20 yr a significant rise in fertility occurred (U622). (All the plots in this experiment were grazed annually from May to November and the sheep must have been responsible for the addition of P and possibly some C to the plots.) The $C:P_o$ ratio however remained above 200. By contrast a plot which received the equivalent of 451 kg P/ha as superphosphate over the same 20 yr showed a similar increase in fertility, but the $C:P_o$ ratio was considerably less and fell almost into the range for the other plots which have received superphosphate (Table 12).

4. Total phosphorus determinations

The P_t value of 370 ppm for soil U61 (Table 13) was much higher than values for similar samples determined by Piper (1938) and Williams (1950): 284 and 240 ppm respectively. This discrepancy probably reflects the different procedures for the analysis of total soil P. X-ray fluorescence should give a higher figure than the conc HCl extraction used by Piper and Williams.

Too little attention has previously been paid to the method of determining P_t in a soil. Table 2 shows the results from twelve independent papers where the P_o was reported as a % of P_t. In only two of the cases was P_t determined by the same procedure (Williams & Steinbergs, 1958; Williams *et al.*, 1960). Muir (1952) and Sherrell & Saunders (1966) have shown that variations in P_t occur when different methods are used for its determination. A suitable method that effectively determines P_t in all soils is therefore highly desirable.

A test of the reliability of the measurement of P_t by X-ray fluorescence spectrography is given in Table 14. This table was prepared by computing the phosphorus added to the soil as superphosphate fertilizer and the phosphorus removed in harvested grain (Waite Agric. Res. Inst., Annual Report 1966-1967; see Table 15). The effect of grazing

animals during the pasture phase of plot 5 was assumed to be negligible and losses of phosphorus by erosion, leaching and removal in the above-ground vegetative parts of plants were assumed to be small (Woodroffe & Williams, 1953). A background P_t of 425 ppm (U622, Table 13) was used for the computed P_t in Table 14.

TABLE 14.

Total soil phosphorus (ppm P) in three rotations from plots cropped with wheat and fertilized with superphosphate since 1925

Soil	Plot Rotation	P added in fertilizer	P harvested in grain	Net gain in P	Computed P_t*	Measured P_t[b]
1	WF	131	45	86	511	500
5	WPF	183	38	145	570	575
17	W	274	37	237	662	670

* Determined for 0-10 cm.

[b] Determined for 0-6.5 cm.

These results indicated that phosphate balance sheets for soils are possible providing that the methods used for determining P_t are reliable and that the limitations of the assumptions are understood.

5. The conversion of phosphorus from fertilizer into organic forms

Considerable accumulation of P_o has occurred in the top-soil of plots from the Urrbrae series which had relatively high accumulations of OM (Table 12, plots 15, 16, 29). Table 12 also suggested that as the fertility increases a greater proportion of the P_t is converted to organic forms.

By comparison with Soils U61 and U57 (Table 13) the fertility of plots U1 and U5 has changed little in more than fifty years although addition of superphosphate has reduced the $C:P_o$ ratio to $\simeq 160$; Soil U17 (which does not have a fallow phase) has shown some increase in fertility and P_o. Farm records which have been carefully maintained since 1925 (Waite Agric. Res. Inst., Annual Report 1966-1967) have enabled the efficiency of utilization of added fertilizer in plots U1, 5 and 17 to be calculated (Table 15).

Applied phosphorus was most efficiently utilized in the WF rotation and least efficient utilization was in the soil growing wheat each year. In part this was due to an accretion of P_o in U17, an indication that added P was accumulating in organic forms to the detriment of the plant. Out of all the rotations the least efficient

TABLE 15.

Balance sheet of phosphorus added in superphosphate fertilizer and phosphorus removed in harvested grain, for soils which have included wheat in the rotation since 1926

Soil	Rotation	Gain in Fertilizer			Loss in Grain Harvest					Efficiency of use of fertilizer phosphorus %
		Total super (kg/ha)	P added (kg/ha)*	P added (ppm/ha-15 cm)≠	Ave ann yield (kg/ha)	Years of harvest (since 1926)	Total grain produce (kg/ha)	P removed (kg/ha)♣	P removed (ppm/ha-15 cm)≠	
1	WF	2889	289	131	1884	21	39,564	99	45	34
5	WPF	4019	402	183	2376	14	33,264	83	38	21
17	W	6029	603	274	767	42	32,214	81	37	14

* Assumes P content of super is 10%.

≠ 1 ha-15 cm ≈ 2.2×10^6 kg soil, thus 1 ppm = 2.2 kg P/ha-15 cm.

♣ Assumed P content of grain 0.25% (Woodroffe & Williams, 1953; Piper & De Vries, 1964).

utilization of fertilizer phosphorus was in the permanent pasture soil. This soil has accumulated 113 ppm P_o in excess of the P_o in the WF rotation, an excess of P_o which is equivalent to 25 bags of superphosphate ($\simeq$ \$50)/ha-15 cm. Such a buildup demands studies of the chemical nature of the accumulated compounds so that mechanisms for the efficient use of this P_o, and of further added phosphorus, may be suggested.

GEL CHROMATOGRAPHY

OF ORGANIC PHOSPHATES

I. MATERIALS

Sephadex gels (Pharmacia AB) of grades G-10, -15, -25, -50 (coarse and medium), -100 and -200 were used. Each gel was equilibrated in eluant buffer before use; initially the gels were swollen by standing in dist H_2O. Column size and elution conditions are described in the relevant places of the text and in Appendix 2.

II. METHODS

1. General

The determination of the relative mobility of a compound chromatographed on a Sephadex gel (the distribution coefficient, K_d) is described in Appendix 2.

2. Standard phosphates

The K_d values of a number of pure phosphates were determined by gel chromatography under a number of different elution conditions. Details of the compounds are given in Appendix 2.

3. pH of elution

Columns were equilibrated and eluted with solutions of various pH values.

(a) pH 7 :- solutions were dist H_2O or aq. LiCl.

(b) pH 3 :- 0.05M-H.COOH containing 0.1M-LiCl.

(c) pH 11:- 0.05M-Na_2CO_3 containing 0.1M-LiCl (adjusted to pH 11 with 5M-NaOH).

(d) pH 13:- 0.1M-NaOH $\pm$ 0.1M-LiCl.

4. Analyses

(a) P_t and P_i were measured as previously described (page 31).

(b) Colour due to OM was measured as absorbance at 450 nm (or OD 350 nm for weakly coloured solutions).

(c) Al was measured by the Alizarin Red S method of Bond (1957) and Fe with o-phenanthroline (Bond, 1957). Si was determined with p-methylaminophenol sulphate (Mullin & Riley, 1955).

III. RESULTS AND DISCUSSION

1. Anion exclusion effect

Anionic compounds of low molecular weight behave anomalously on Sephadex gels which are equilibrated and eluted with water. This phenomenon was first reported by Gelotte (1960) and Flodin (1962) and is called 'the Donnan anion exclusion effect' (Davis, 1942). It is due to the interactions between the anionic compounds (mobile phase) and the partially ionised carboxyl groups of the gel beads (stationary phase). The effect produces lower K_d values than expected (Gelotte, 1960) and asymmetric elution profiles (Neddermeyer & Rogers, 1968).

2. Behaviour of standard phosphates

(a) Varying salt concentration -

An eluant salt concentration of 0.01M is required to obtain symmetrical elution profiles when low MW anionic compounds are chromatographed on Sephadex gels (Neddermeyer & Rogers, 1968). Figure 3 shows that the anion exclusion effect may persist after the elution profiles of the anions have become symmetrical. The higher the eluant salt concentration the more the exclusion effect is

FIGURE 3.

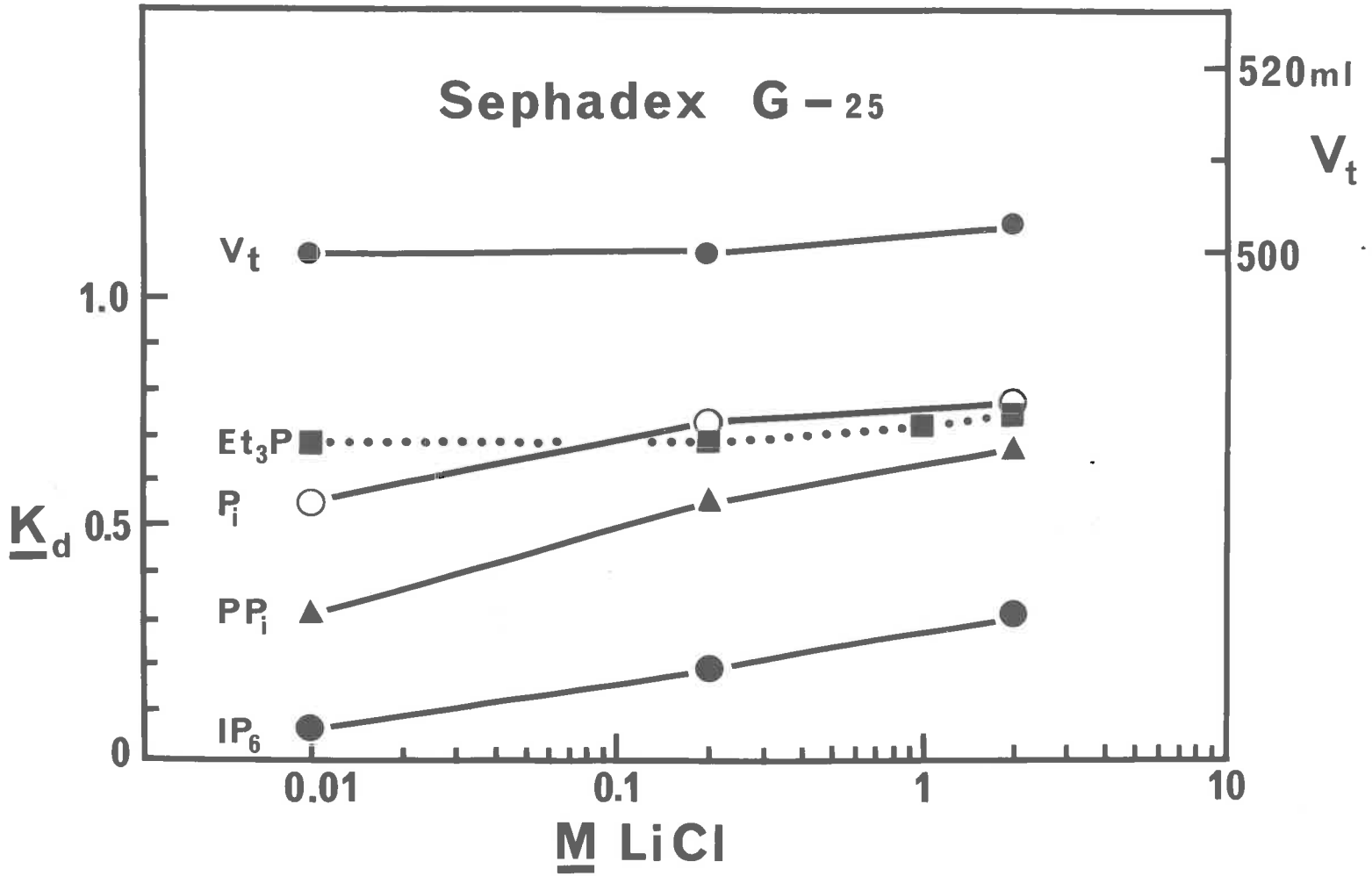
Variation in the gel column and the distribution coefficients of four standard phosphates as a result of changes in the molarity of the eluant electrolyte.

Standard phosphates: triethyl phosphate (Et_3P)
inorganic phosphate (P_i)
inorganic pyrophosphate (PP_i)
myoinositol hexaphosphate (IP_6)

Gel: Sephadex G-25 (100-300 μ).

Column volume: V_t ml.

Eluant: LiCl of various molarities.


Sephadex G-25
520ml
V_t
500
V_t
1.0
Et_3P
P_i
$\underline{K}_d$ 0.5
PP_i
IP_6
0
0.01
0.1
1
10
$\underline{M}$ LiCl

suppressed. By contrast the uncharged triethyl phosphate has an unchanged K_d over the salt concentration range 0.01 to 2.0 M-LiCl.

(b) Varying the pH -

A simple phosphate has the same K_d value within the pH range 3-11 where the ionisation of the carboxyl groups of the gel matrix and the second dissociation of the phosphate groups occurs. Figure 4 shows that high pH produces a marked increase in anion exclusion because of the ionisation of hydroxyl groups in the carbohydrate gel matrix. (A strong salt concentration is needed to substantially repress this effect - Appendix 2).

For any particular compound the exclusion effect at pH 13 was less significant on Sephadex G-50 than on G-25. i.e. the decrease in K_d for each compound going from pH 11 to pH 13 was less on the former than on the latter grade of gel. This reflected the fact that less G-50 gel is required per unit column volume than G-25 gel in the same volume - the water regain of dry G-50 gel is approximately double that of dry G-25 gel.

Similarly the exclusion effect is less on Sephadex G-100 compared with G-50, etc.

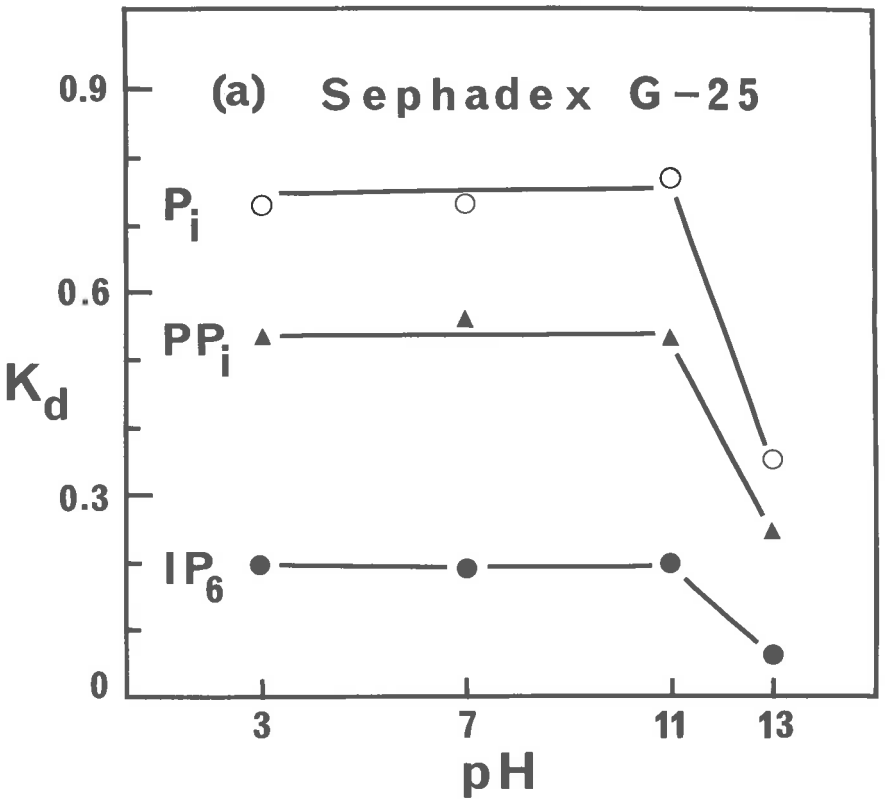
FIGURE 4.

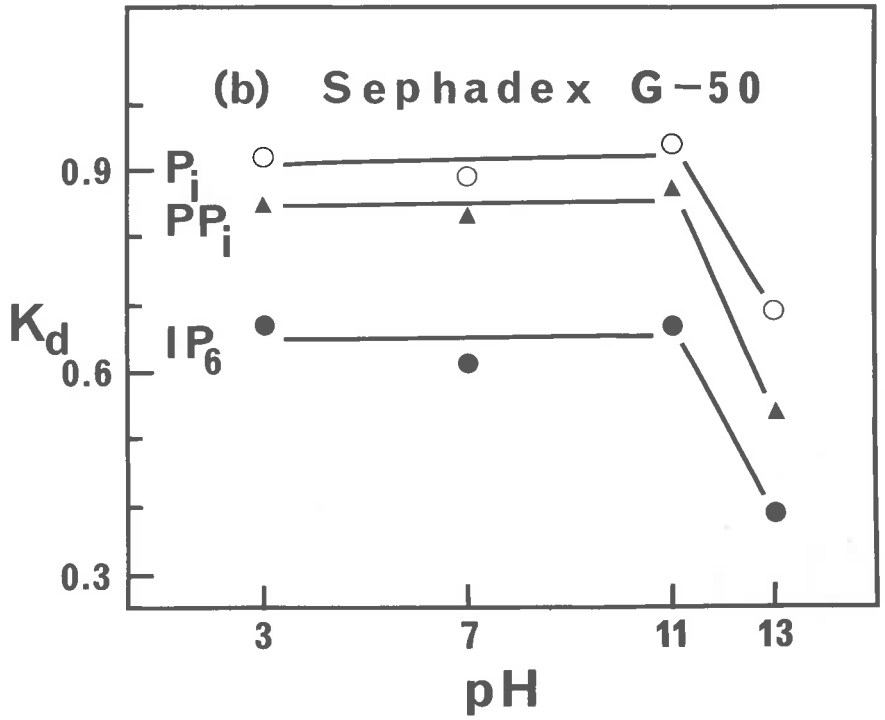
The distribution coefficients of three standard phosphates on two grades of Sephadex at various eluant pH values.

Standard phosphates: inorganic phosphate (P_i)
inorganic pyrophosphate (PP_i)
myoinositol hexaphosphate (IP_6)

Gel grades: (a) Sephadex G-25 (100-300 μ)
(b) Sephadex G-50 (100-300 μ)

Eluant: 0.1M-LiCl adjusted to pH values of 3, 7 11 and 13 (as described in text).


(a) Sephadex G-25
0.9
0.6
0.3
0
K_d
P_i
PP_i
IP_6
3
7
11
13
pH


(b) Sephadex G-50
0.9
0.6
0.3
K_d
P_i
PP_i
IP_6
3
7
11
13
pH

(c) Relationships between the phosphates -

(i) Homologous series --

There is an orderly lowering of K_d values as a homologous series is ascended. Figure 5 shows the inverse relationship between the degree of phosphorylation of the inositol ring and the K_d on two grades of Sephadex which were equilibrated and eluted with 0.1M-LiCl. These results indicate that a mixture of two compounds near each other in the homologous series would not be separated on Sephadex G-25 or G-50.

(ii) Groups of compounds --

Despite the presence of the anion exclusion effect the order of the phosphates on any Sephadex gel grade was always the same for stable phosphates whatever the eluant salt concentration or pH. The consistent relationship between the protein phosvitin, myoinositol hexaphosphate and inorganic phosphate on a range of gels is shown in Figure 6. Similar regularity over a range of pH values has already been demonstrated for phosphates stable to extremes of pH (Fig. 4).

3. Fractionation of crude extracts of soil

(a) Introduction -

For satisfactory fractionation of crude NaOH extracts of soil on Sephadex columns the pH needed to be sufficiently high to prevent the precipitation of humic materials and

Figure 5

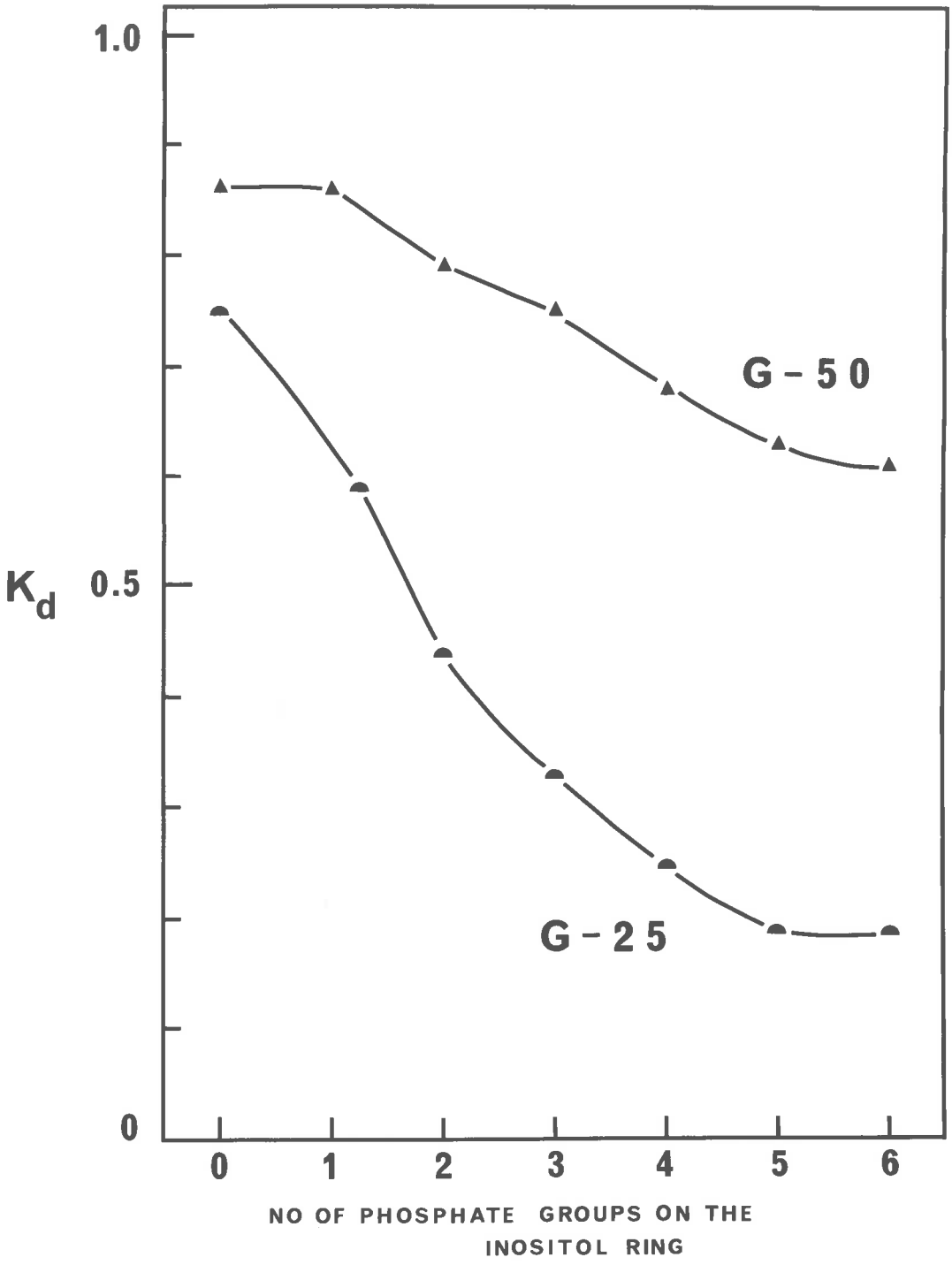

1.0
0.5
0
K_d
G-50
G-25
0
1
2
3
4
5
6
NO OF PHOSPHATE GROUPS ON THE INOSITOL RING

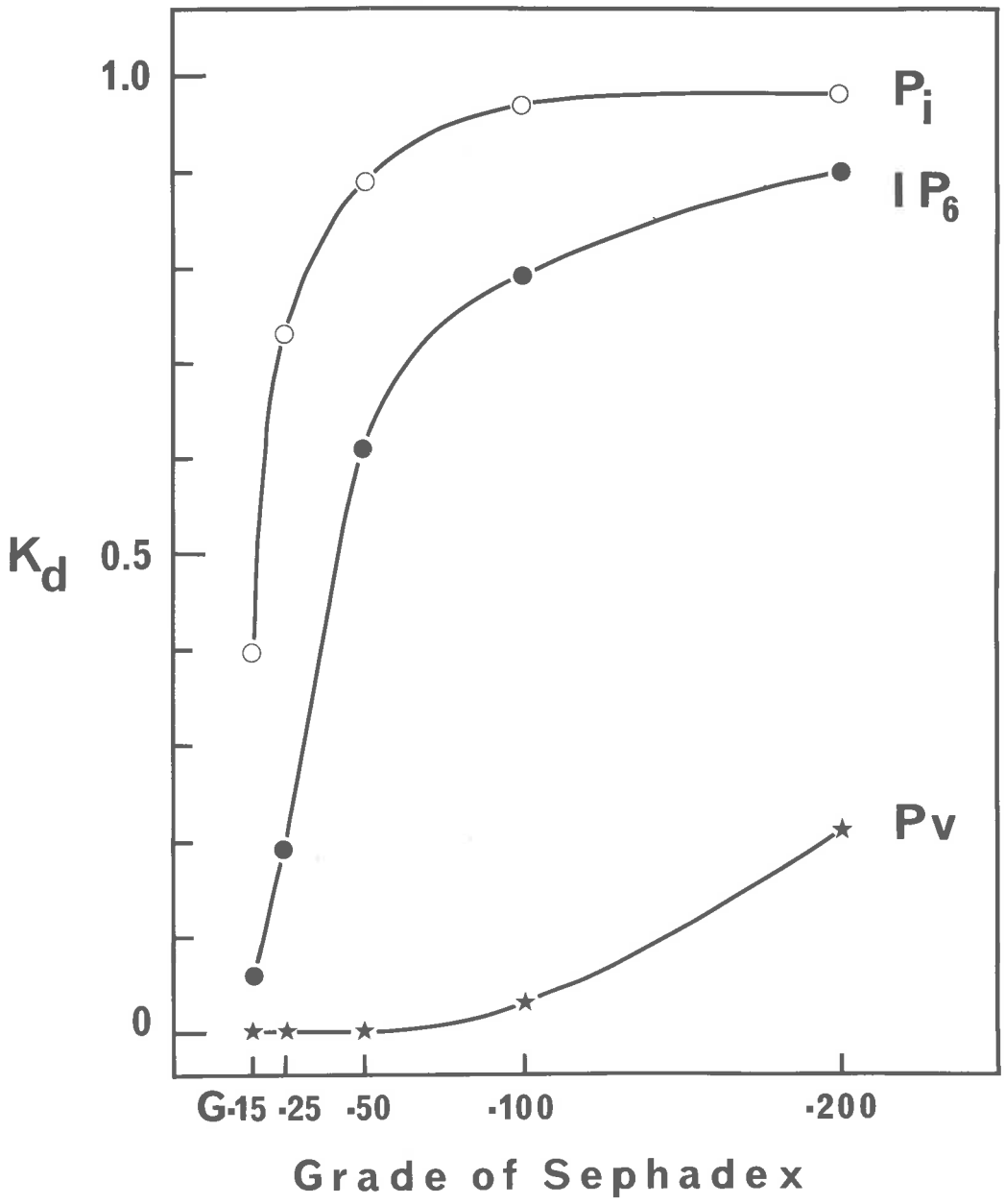
FIGURE 6.

Variation in the distribution coefficient of three standard phosphates chromatographed on various grades of Sephadex gel.

Standard phosphates: inorganic phosphate (P_i)
myoinositol hexaphosphate (IP_6)
phosvitin (Pv).

Gel grade:		Water regain (g H_2O/g dry gel)
Sephadex G-15	(40-120 μ)	1.5
" G-25	(100-300 μ)	2.5
" G-50	(100-300 μ)	5.0
" G-100	(40-120 μ)	10.0
" G-200	(140-400 μ)	20.0

Eluant: 0.1M-LiCl.


1.0
0.5
0
K_d
P_i
$I P_6$
Pv
G-15
-25
-50
-100
-200
Grade of Sephadex

sesquioxides. Both 0.05M-Na_2CO_3 (pH 11) and 0.1M-NaOH (pH 13) were suitable for this purpose. Because the extracts of soil were already in NaOH all the high pH columns were equilibrated and eluted with 0.1M-NaOH.

(b) Results -

The pattern of elution of the major constituents present in a crude 0.5M-NaOH extract of Soil U16 when chromatographed on Sephadex G-25 is given in Figure 7.

The P_i ($\underline{K}_d$ 0.30) and aluminate ($\underline{K}_d$ 0.47) were well separated from the bulk of the P_o and the humic materials ($\underline{K}_d$ 0.00-0.15). Silicate was present at a consistently high level over the $\underline{K}_d$ range 0-0.5 and no Fe could be detected in any of the fractions.

When tubes 35-47 of Figure 7 were bulked, concentrated and fractionated on Sephadex G-50 at pH 13 the pattern shown in Figure 8a resulted. If sodium phytate was added to the soil sample at some stage prior to this G-50 fractionation step a profile similar to Figure 8b was produced. The free phytate ($\underline{K}_d$ 0.39) was well separated from the rest of the P_o and the bulk of the humic materials ($\underline{K}_d$ 0.00).

FIGURE 7.

Fractionation of a crude 0.5M-NaOH extract of Soil U16 on Sephadex G-25 at pH 13.

Gel grade: Sephadex G-25 coarse (100-300 μ).

Column dimensions: 60 x 5.4 cm diam.

Eluant: 0.1M-NaOH.

Effluent fraction size: 14.5 ml.

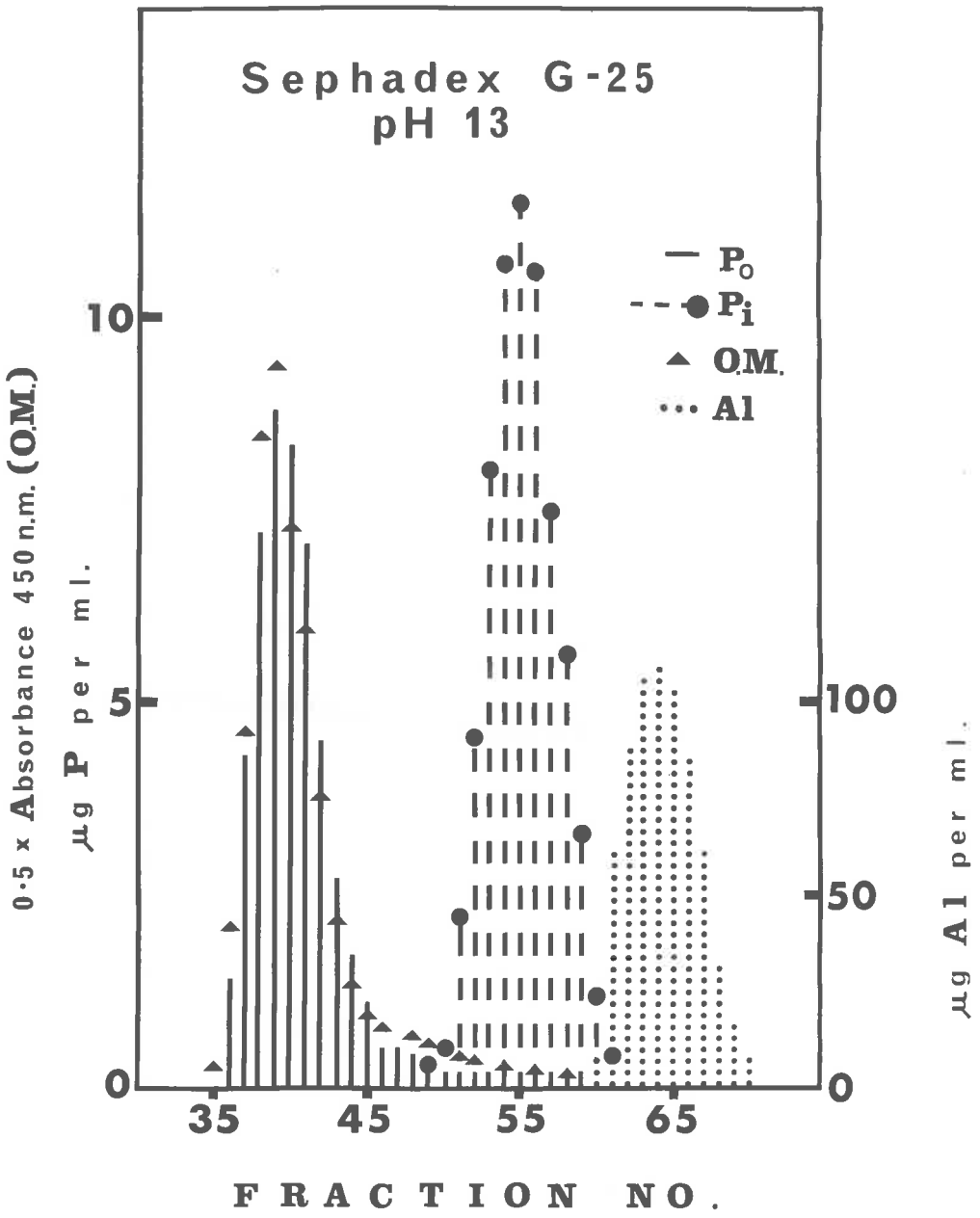

Sephadex G-25
pH 13
— P_o
- - ● P_i
▲ OM.
··· Al
0·5 x Absorbance 450 n.m. (O.M.)
µg P per ml.
µg Al per ml.
10
5
0
100
50
0
35
45
55
65
FRACTION NO.

FIGURE 8.

Fractionation of the P_o excluded from Sephadex G-25 at pH 13 (tubes 35-47 of Fig. 7) on Sephadex G-50 at pH 13.

Gel grade: Sephadex G-50 coarse (100-300 μ).

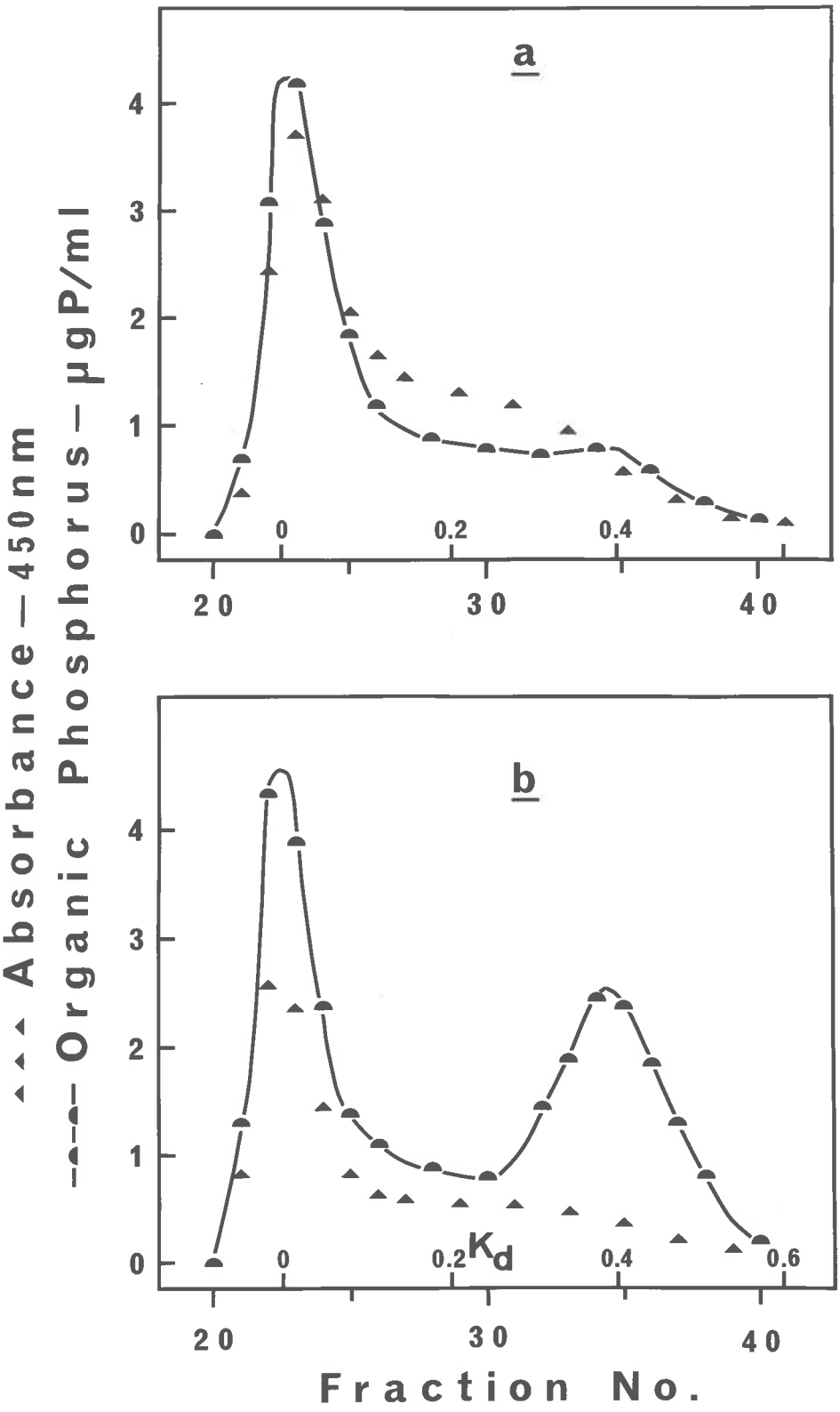
Column dimensions: 42.5 x 4.2 cm diam.

Eluant: 0.1M-NaOH.

Effluent fraction size: 14.5 ml.

(a) Extract of Soil U16 (5 g).

(b) Extract of Soil 237 (1.5 g).


a
b
▲-▲-▲ Absorbance—450nm
●-●-● Organic Phosphorus—μgP/ml
0
1
2
3
4
20
30
40
0
0.2
0.4
0.6
K_d
Fraction No.

The pattern shown in Figure 8b was actually obtained when a NaOH extract of Soil 237 was fractionated on Sephadex G-25 and the excluded material was then chromatographed on Sephadex G-50. The area of the peak bounded by tubes 31 and 38 contained the equivalent of 122 ppm P_o - a value similar to the 118 ppm for the sum of the inositol penta- and hexa- phosphates in this soil (Williams & Anderson, 1968).

The presence of inositol polyphosphates from Soil 237 in this peak of $\underline{K}_d$ 0.39 on Sephadex G-50 was confirmed by isolation of the Ba salt and electrophoresis in oxalate buffer at pH 1.5 (Tate, 1968). [The inositols were also detected by gas-liquid chromatography of the acetates after complete hydrolysis of the polyphosphate fraction in 6M-HCl. See Appendix 3.] During electrophoresis unknown interference at high spotting concentrations slightly diminished the mobility of the inositol phosphates from the extract compared with the corresponding free compounds (Fig. 9) and when the standards were overspotted with the soil inositol polyphosphates the free phytate was also retarded.

(c) Discussion -

A substantially pure sample of inositol polyphosphates can now be obtained from soil with the minimum of chemical

FIGURE 9.

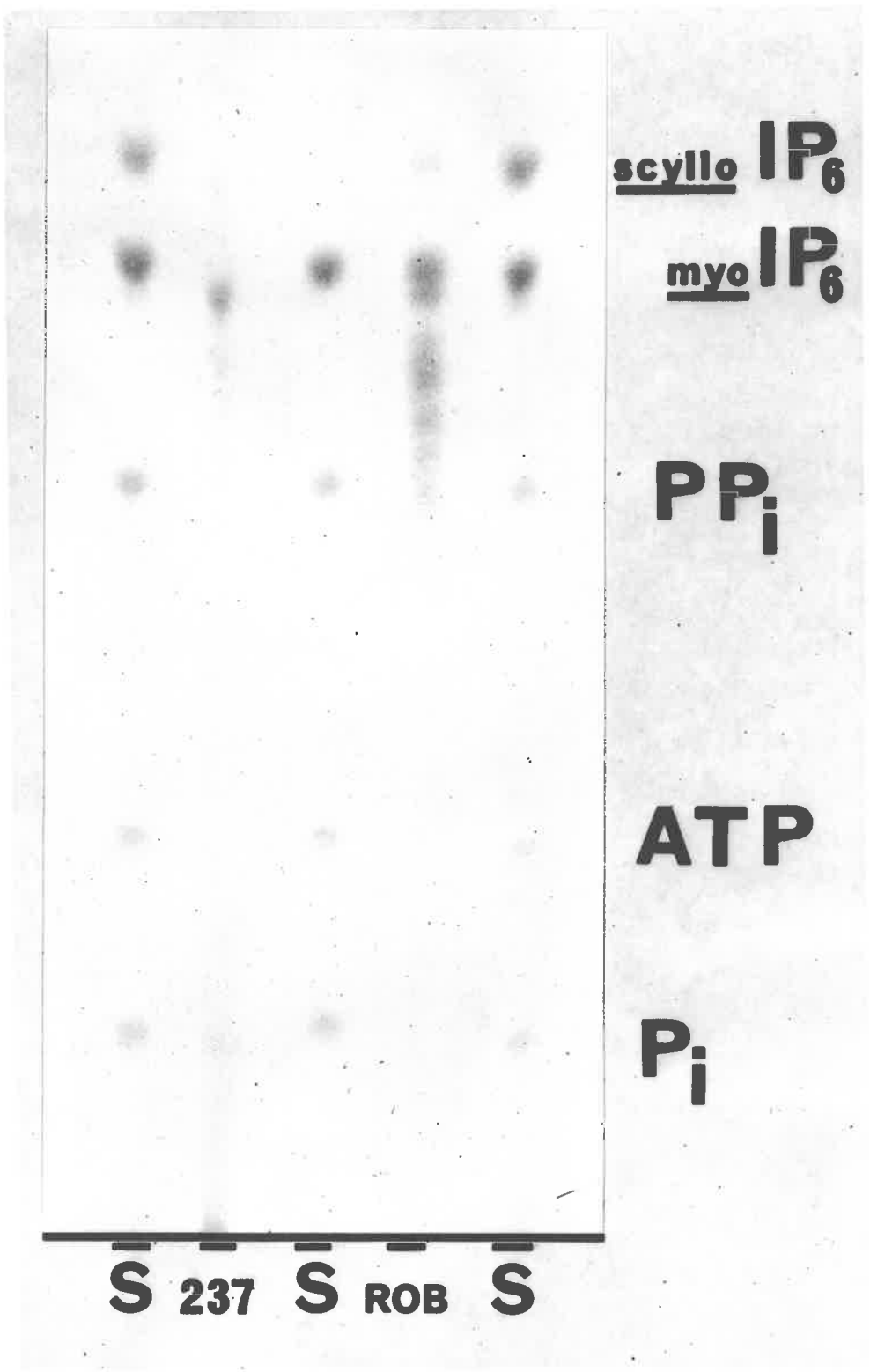
Electrophoresis of the Ba salt of the inositol polyphosphate peak from Sephadex G-50.

Buffer: 0.1M-oxalate, pH 1.5.

Standards: scylloinositol hexaphosphate (scyllo-IP_6)
myoinositol hexaphosphate (myo-IP_6)
inorganic pyrophosphate (PP_i)
adenosine triphosphate (ATP)
inorganic phosphate (P_i)

Soil samples: (a) 237

(b) ROB


scyllo IP6
myo IP6
PPi
ATP
Pi
S
237
S
ROB
S

fractionation or treatment. This material is in a form amenable to detailed examination by paper electrophoresis or anion-exchange chromatography.

Ba precipitation at pH 13 of the phosphates in the peak of K_d 0.39 from Sephadex G-50 was found to remove all of the P_o from solution without co-precipitating the carbohydrate (as determined by the anthrone procedure of Oades, 1967a). Electrophoresis after conversion of the precipitate to the H-salt (with Dowex 50 H^+, -400 mesh) revealed that the hexaphosphates of *scyllo*-, *myo*- and D(*chiro*)- inositols predominated in the sample; no significant amounts of lower inositol phosphates were detected by electrophoresis.

High voltage electrophoresis in oxalate buffer (pH 1.5) is a rapid means of examining the purified free inositol polyphosphate fraction from soil. By developing side-strips it is possible to elute the compounds from the paper (Hariharan, Poole & Johns, 1968) and to determine the P_o and inositol (Appendix 3) content after hydrolysis.

4. Development of a routine chromatographic procedure

(a) Details -

A single Sephadex G-50 column sufficed for the routine analysis of the gross inositol polyphosphate content of soil extracts. At pH 13 the free inositol polyphosphates ($\underline{K}_d$ 0.39) were well separated from P_i ($\underline{K}_d$ 0.65) and the other P_o components ($\underline{K}_d$ 0.00).

Rapid fractionation of 0.25 ml extract was achieved on small columns of 1.6 cm diam, consisting of a bottom layer of G-50 coarse (17.5 cm) covered by G-50 medium (5.5 cm). The medium grade gel gave a compact surface on which the viscous NaOH extract was layered, using a curved-tip pipette (Determann, 1968, p. 46). The column was equilibrated and eluted with 0.1M-NaOH and, under these conditions, there was no difference between the $\underline{K}_d$ value of various phosphate compounds chromatographed on medium and coarse Sephadex G-50.

Column effluent was collected in 1.1 ml fractions using a Pleuger Model J drop-counting fraction collector (8 drops/fraction, 2 drops/min). Fractions were analysed for P_t and P_i. For satisfactory detection of P at least 20 µg P_o/ml of extract was required. Using the adaptations to the extraction procedure as described in the previous

chapter (Section IV, 3(a), p. 46) this made it possible to analyse soils containing > 60 ppm P_o if extracted at a 3:1 solution to soil ratio, or > 240 ppm extractable P_o when using a 12:1 solution to soil ratio (only Soil 250 did not meet these requirements).

(b) Results and discussion -

Some of the patterns obtained with extracts of the soils are shown in Figure 10. The contribution of the inositol polyphosphates to the total P_o extracted is the area of the second peak relative to the sum of the areas of the first two peaks. The third peak was exclusively P_i. Applying the assumptions mentioned previously for concentrated extracts (see pages 33 and 47), the amounts of P_o involved were easily adjusted to a ppm basis.

Features of the patterns obtained were

(i) The similarity of the profiles for all of the soils in the Urrbrae series (Fig. 10(a)-(d); see also page 49, Section V, 2).

(ii) The close agreement that existed between the ppm P_o calculated by this method for the inositol polyphosphate peak of Soils 237, 315 and 350 and the corresponding figure quoted by Williams & Anderson (1968) for the inositol penta- and hexa- phosphate P (Fig. 10(e), (f), (g)).

(iii) The disparity between the results for Soil ROB [Fig. 10(h)] and the value obtained by Williams & Anderson (1968) for the inositol penta- and hexa- phosphates in the corresponding sample (531). The inositol polyphosphate peak on Sephadex G-50 increased greatly after an acid pretreatment (pattern not shown) so that it accounted for 70% (480 ppm) of the extracted P_o. Electrophoresis of the Ba salt confirmed that inositol polyphosphates were the major component in this fraction.

FIGURE 10

The fractionation of crude 0.5M-NaOH extracts of soil on a small Sephadex G-50 column.

Gel grades: Sephadex G-50 coarse (100-300 μ)
Bottom 17.5 cms.
Sephadex G-50 medium (50-150 μ)
Top 5.5 cms.

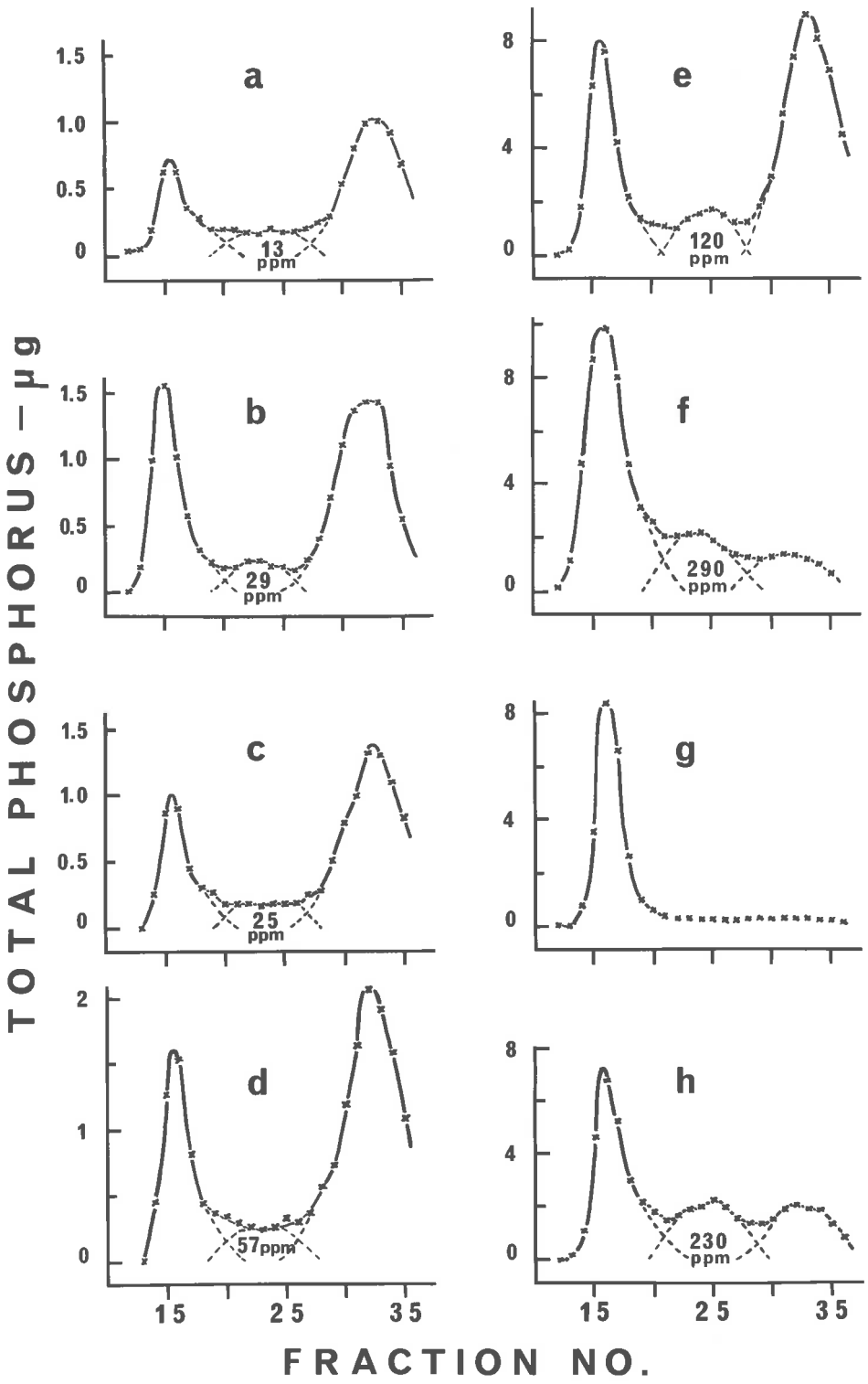
Column dimensions: 23 cm x 1.6 cm diam.

Eluant: 0.1M-NaOH.

Effluent fraction size: 1.1 ml.

Flow rate: 0.3 ml/min.

Samples:
(a) U1
(b) U16
(c) U17
(d) U29
(e) 237
(f) 315
(g) 350
(h) ROB


a
13 ppm
b
29 ppm
c
25 ppm
d
57ppm
e
120 ppm
f
290 ppm
g
h
230 ppm
TOTAL PHOSPHORUS – µg
FRACTION NO.
15
25
35

IV. OTHER APPLICATIONS OF GEL CHROMATOGRAPHY

1. The unidentified soil organic phosphorus

The material which was completely excluded from Sephadex G-50 at pH 13 (tubes 20-26, Fig. 8a) was concentrated and divided into two equal fractions. One half was chromatographed on Sephadex G-100, the remainder was fractionated on Sephadex G-200 (pH 13). The indefinite fractionation in both cases (Fig. 11) led to the conclusion that the phosphorus was present in polydisperse macromolecules and little benefit was obtained by further fractionation on the higher grades of Sephadex.

2. Examination of materials containing haemoglobin

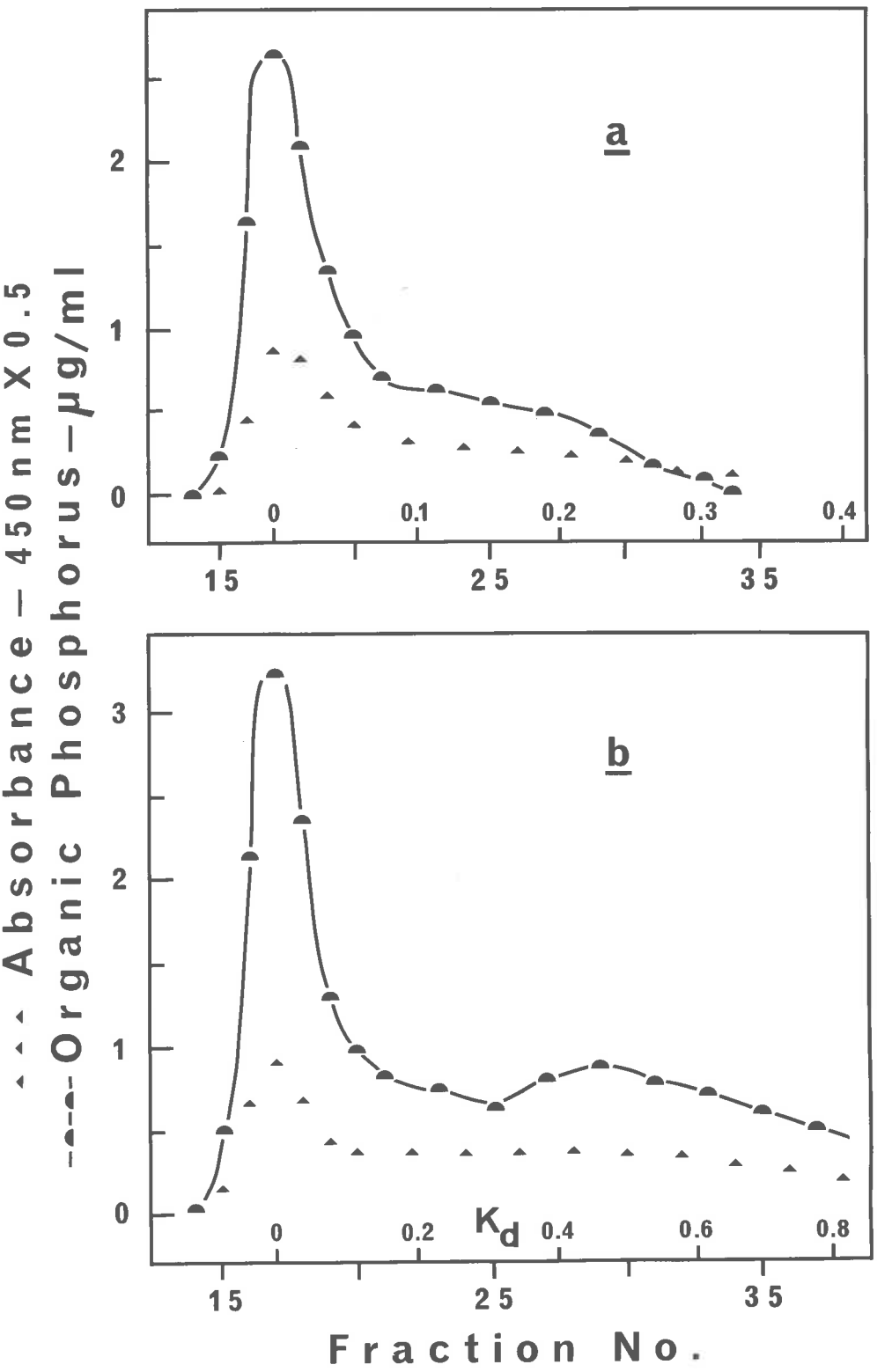
The application of gel chromatography to the elucidation of the nature of the avian haemoglobin-inositol pentaphosphate complex is described in Appendix 2.

Association of an organic phosphate with mammalian haemoglobin (Benesch & Benesch, 1969) as well as the avian haemoglobin-P_o complex led to the suggestion (by Dr. D.J. Greenland) that a leghaemoglobin-P_o association may exist in root nodules. Evidence in the literature

FIGURE 11.

Fractionation at pH 13 on higher grades of Sephadex of the material which was excluded from Sephadex G-50 (tubes 20-26, Fig 8a).

	(a)	(b)
Gel:	Sephadex G-100 (40-120 μ)	Sephadex G-200 (40-120 μ)
Column dimensions:	30 cm x 5.6 cm diam.	42 cm x 4.2 cm diam.
Eluant:	0.1M-NaOH	0.1M-NaOH
Effluant fraction size:	14 ml	14 ml
Flow rate:	3 ml/min	0.7 ml/min


a
b
Absorbance – 450nm X 0.5
Organic Phosphorus–µg/ml
Fraction No.
K_d
0
0.1
0.2
0.3
0.4
0.6
0.8
15
25
35

that phosphate application in soils enhances legume growth appeared to support this hypothesis (Sekhon, Kanwar & Bhumbla, 1966; De Mooy & Pesek, 1966; Khare & Rai, 1968).

Two sources of root nodules were obtained from bean plants: Vicia faba, cv. tick-bean, growing in the field; and Glycine max (L.) Merr (Soybean) which was inoculated with Rhizobium japonicum and grown in the glasshouse by the method of Cartwright & Thomas (1968). (The soybeans, inoculant and pots were kindly supplied by Mr. Gordon Grandison, CSIRO Division of Horticultural Research, Glen Osmond.)

Plants were harvested at the time of maximum nodule development (just prior to flowering), the freshly harvested nodules were frozen in liquid nitrogen and extracted by grinding in water. After centrifuging the extracts the clear, pink haemoglobin was fractionated on Sephadex G-50, equilibrated and eluted with 0.1M-LiCl.

The extract from the tick-bean nodules resulted in the fractionation shown in Figure 12. Although considerable leghaemoglobin was obtained from both sources no detectable amounts of any P_o compound were associated with the leghaemoglobin peak. It was concluded that a leghaemoglobin-P_o association does not occur in the root nodules of tick-bean and soybean plants.

FIGURE 12.

Gel chromatography of an aqueous leghaemoglobin extract of root nodules.

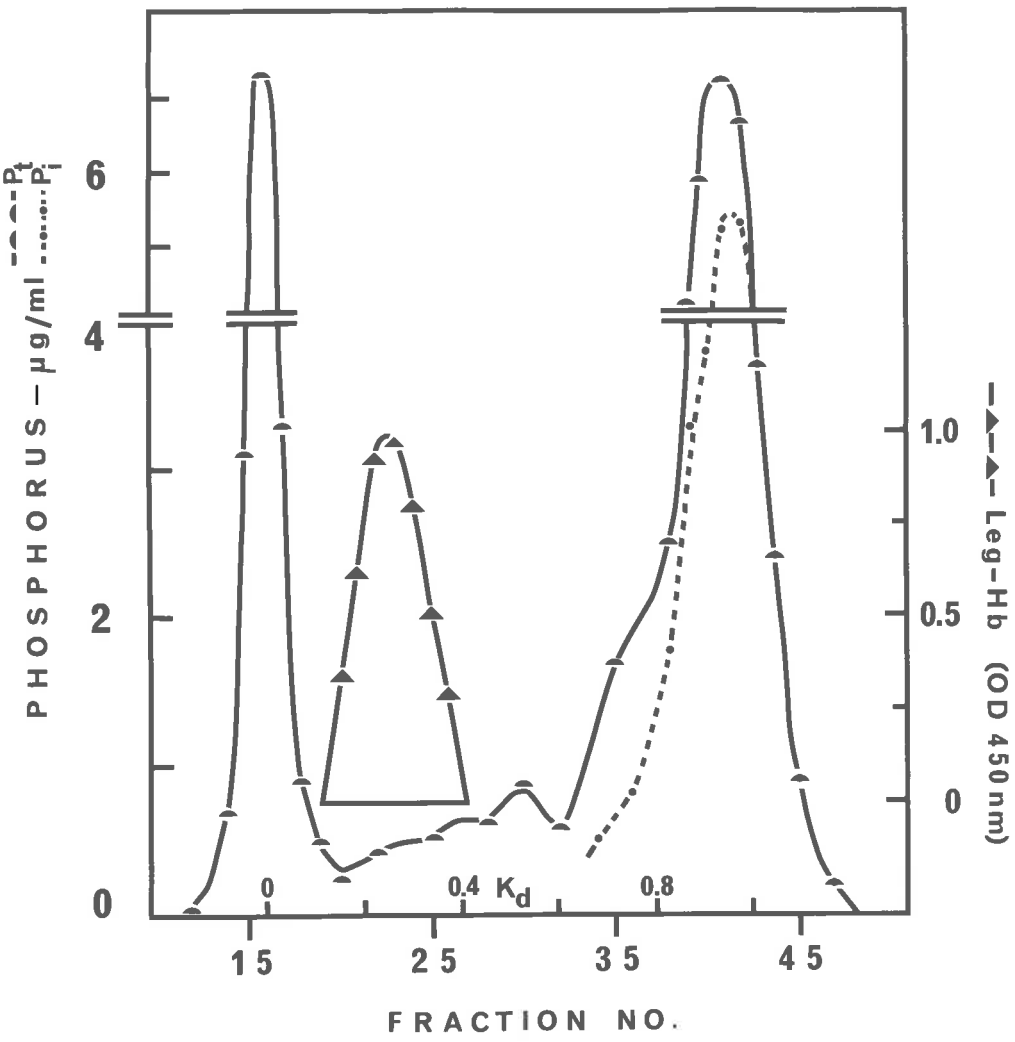
Gel: Sephadex G-50 coarse (100-300 μ).

Column dimensions: 58 x 4 cm diam.

Eluant: 0.1M-LiCl.

Effluent fraction size: 16.5 ml.

Source: Nodules of *Vicia faba* cv. tick-bean.


PHOSPHORUS – µg/ml ----Pt .....Pi
6
4
2
0
1.0
0.5
0
Leg-Hb (OD 450 nm)
0
0.4 Kd
0.8
15
25
35
45
FRACTION NO.

3. Extracts of microorganisms

Because of the likely involvement of microorganisms from soil in the synthesis of inositol polyphosphates (see page 18), extracts of a soil microorganism that had been grown in pure culture on a synthetic medium were examined by gel chromatography.

A strain of *Actinomyces* sp., known as 4A, which had been isolated from the Urrbrae red brown earth was obtained from Dr. J.H. Warcup. This microorganism was chosen for examination because the fungal culture used by Cosgrove (1964) was actinomycetes-free; also the predominant fungi which are active in the Urrbrae soils during the summer months are the actinomycetes (J.H. Warcup - personal communication).

Actinomycete hyphae were grown by inoculating a lactate-glucose enriched inorganic medium (pH 6.5) and incubating at 27° for a fortnight. The hyphae were harvested by centrifugation and dried in an oven.

Spores were obtained by growing the culture for six weeks in the dark at 27° on a shallow orthophosphate-buffered lactate-glucose-peptone agar (pH 7.0). The spores were loosened by rubbing the surface of the agar with a soft brush; the dark particles were then harvested by flooding the plate with dist H_2O.

Each sample was thrice extracted by vibrating ultrasonically in 0.1M-NaOH. After centrifuging and neutralizing the supernatant the extracts were fractionated on Sephadex G-50 which was equilibrated and eluted with 0.1M-LiCl. The results are shown in Figure 13.

Most of the P extracted from the hyphae was P_i, inorganic polyphosphate or ATP. (P_{30} in the figure refers to the inorganic P detected after 30 mins hydrolysis in a boiling water-bath with 1M-$HClO_4$). The extracts of the spores contained little P_o and no inositol polyphosphates were detected.

The patterns obtained indicated however that Sephadex G-50 is a useful grade for fractionating the P in extracts of microorganisms.

FIGURE 13.

The fractionation by gel chromatography of extracts from a pure culture of Actinomyces sp.

Gel grade: Sephadex G-50 (100-300 μ).

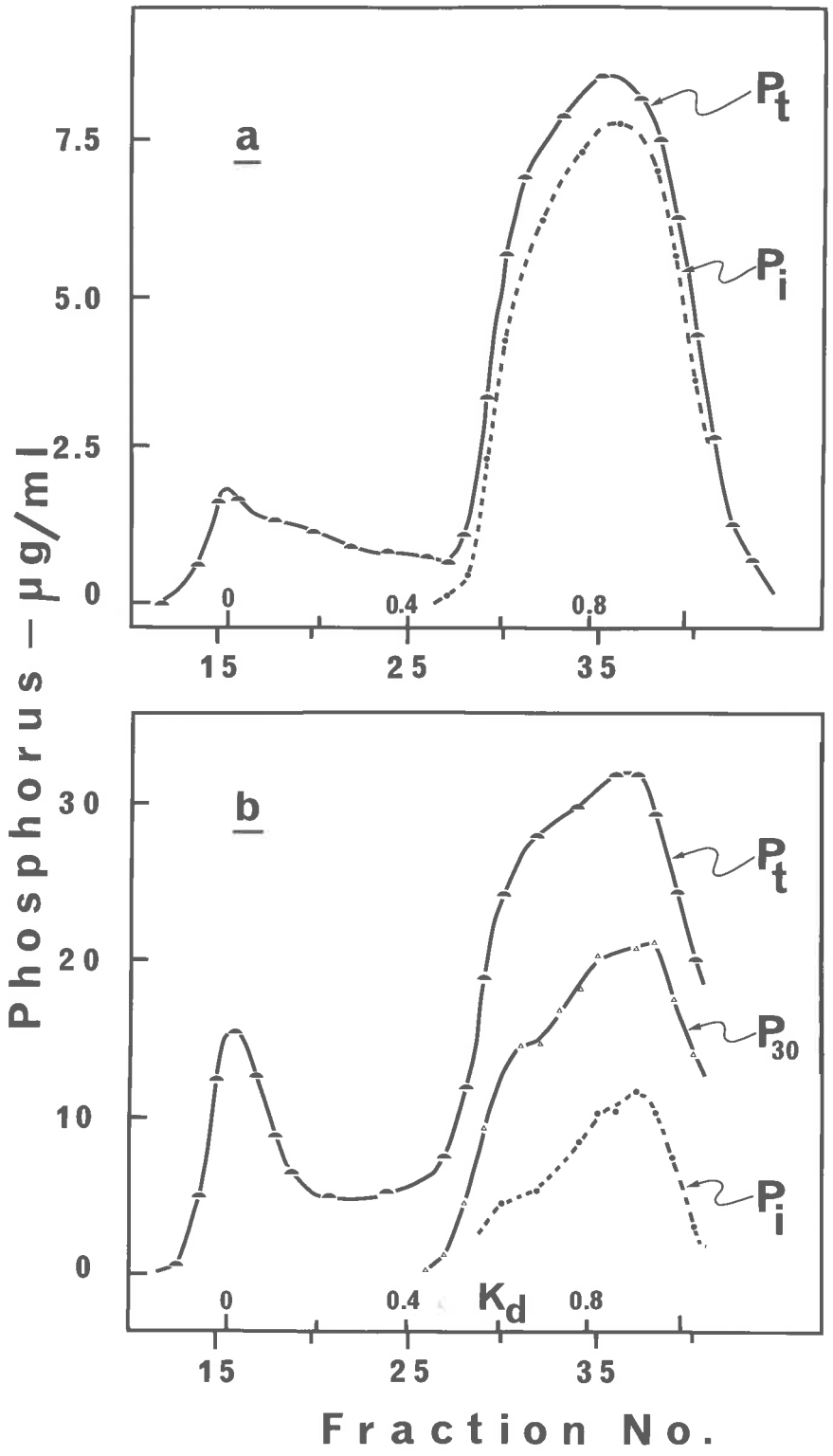
Column dimensions: 45 x 3.8 cm diam.

Eluant: 0.1M-LiCl.

Effluent fraction size: 16.5 ml.

(a) Spores.

(b) Hyphae.


a
b
P_t
P_i
P_{30}
K_d
0
0.4
0.8
15
25
35
7.5
5.0
2.5
0
30
20
10
Phosphorus – µg/ml
Fraction No.

THE UNIDENTIFIED FRACTION

OF

SOIL ORGANIC PHOSPHORUS

I. INTRODUCTION

Much of the P_o extracted by NaOH from soils has yet to be identified. The preceding sections have shown the ease with which this unknown P_o can be separated from other important constituents in crude soil extracts, viz polyvalent cations (in particular Al), P_i, free inositol polyphosphates and much of the silica.

The fractions completely excluded from Sephadex G-50 at pH 13 contained humic materials, carbohydrate and some silica, as well as the P_o. This P_o was acid labile (95% hydrolysis after an overnight reflux in 5M-HCl) but it appeared to be alkali-stable (no hydrolysis after 1 hr in 0.1M-NaOH at 110°). These observations eliminated the presence of substantial amounts of phosphonates (Quin, 1967) or phosphoproteins (Perlmann, 1955). A lack of bases and the absence of apurinic acid after the acid hydrolysis indicated that very little DNA-phosphorus was present. Extraction of Soil U16 (55 g) with 80% Dioxan (Schoch, 1938) in a Soxhlet apparatus for 66 hr yielded the phospholipid products lecithin and lysolecithin, but they accounted for only 2% of the P_o (cf. Anderson, 1967).

Further attempts were therefore made to fractionate and purify the unidentified P_o in the Urrbrae soil. This work is described in the following sections.

II. ELECTROPHORESIS OF MATERIAL EXCLUDED BY SEPHADEX G-50

1. Preliminary

Three different buffer systems of high pH were used:

(a) pH 9, 0.05M-sodium borate. This has been used to separate the coloured components of humic materials by Ferrari & Dell'agnola (1963) and Dorado & Del Rio (1969).

(b) pH 13, 0.3M-NaOH and 0.2M-Kgluconate is a buffer which sequesters polyvalent cations (so that a sample of Fe phytate electrophoreses as freely as its H salt) thus increasing the possibility of separating P_o from the coloured materials.

(c) pH 9, Tris buffer containing 6M-urea. Any H-bonding between components would be destroyed by this buffer.

Electrophoresis in buffers (a) and (b) was performed on Whatman 3MM cellulose paper with the equipment described by Tate (1968). To avoid immobilisation (by adsorbtion) of colour (Ferrari & Dell'agnola, 1963) and P_o on the origin it proved essential to spot the material onto the paper after it had been moistened with buffer.

Electrophoresis in buffer (c) was performed using cellulose acetate strips in a Gelman No. 51101 electrophoresis chamber.

2. Material as such

When the material excluded on Sephadex G-50 at pH 13 from Soil U16 (see Fig. 8a, tubes 20-26) was electrophoresed in the three buffers the coloured materials were separated into three areas. In order of decreasing mobility (towards the anode) these areas were coloured yellow-orange (diffuse), dark brown (band) and light grey-brown (diffuse area extending between brown band and the origin).

Perchloric acid digestion of 1 cm strips cut at right angles to the direction of movement allowed the P_o distribution to be determined. In each instance most of the P_o was associated with the mobile coloured materials (cf. Dormaar, 1963). In Figure 14 the result for the borate electrophoresis is shown.

3. After fractionation on Sephadex G-100

Sections (i) (tubes 15-21) and (ii) (tubes 22-30), from the G-100 fractionation described in conjunction with Figure 11a, were concentrated and electrophoresed in buffers (a) and (b). The distribution of colour after $1\frac{1}{4}$ hr was recorded and 1 cm strips were then digested for P_o determination.

FIGURE 14.

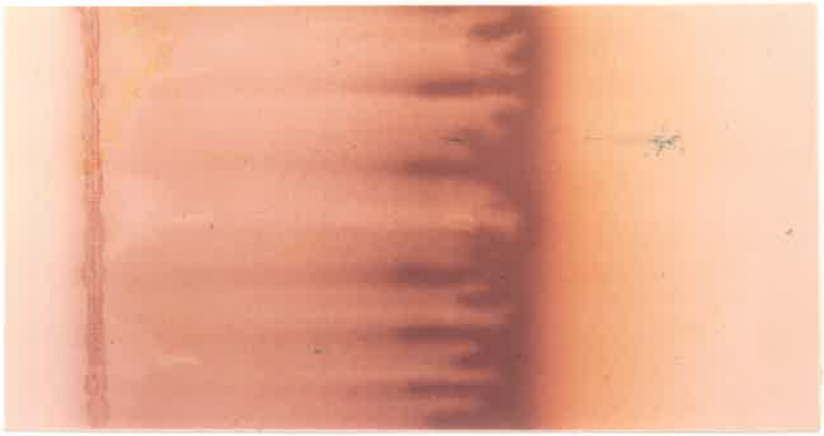
Paper electrophoresis (pH 9) of material from Soil U16 which was excluded from Sephadex G-50 (tubes 20-26, Fig. 8 a).

Electrophoresis conditions: 75 mins, 1500 volts.

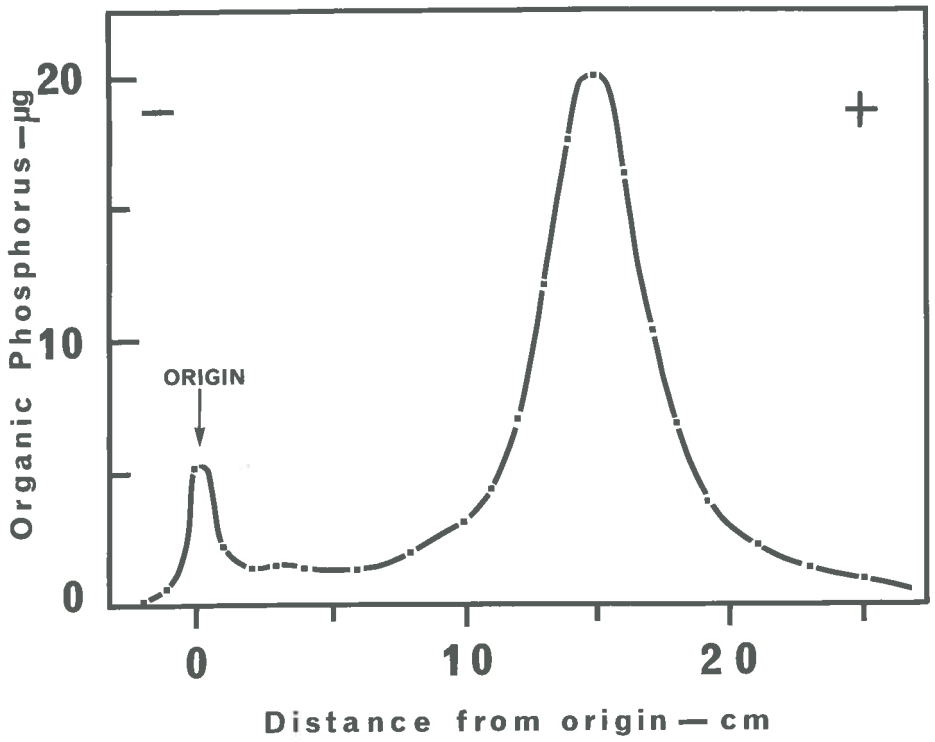
(a) Electrophoretogram

(b) P_o-profile

a



b


Organic Phosphorus—µg
20
10
0
−
+
ORIGIN
0
10
20
Distance from origin — cm

The results, in Figure 15, show that the G-100 column separated two dissimilar, coloured fractions containing P_o. As the differences in mobility between the two fractions may have been due to the effect of the coloured components rather than P_o compounds no conclusion could be drawn about the nature of the latter. This fractionation of coloured components was similar to that obtained by Dorado (1969) and Del Rio (1969) using lower grades of Sephadex and eluting with dist H_2O.

FIGURE 15.

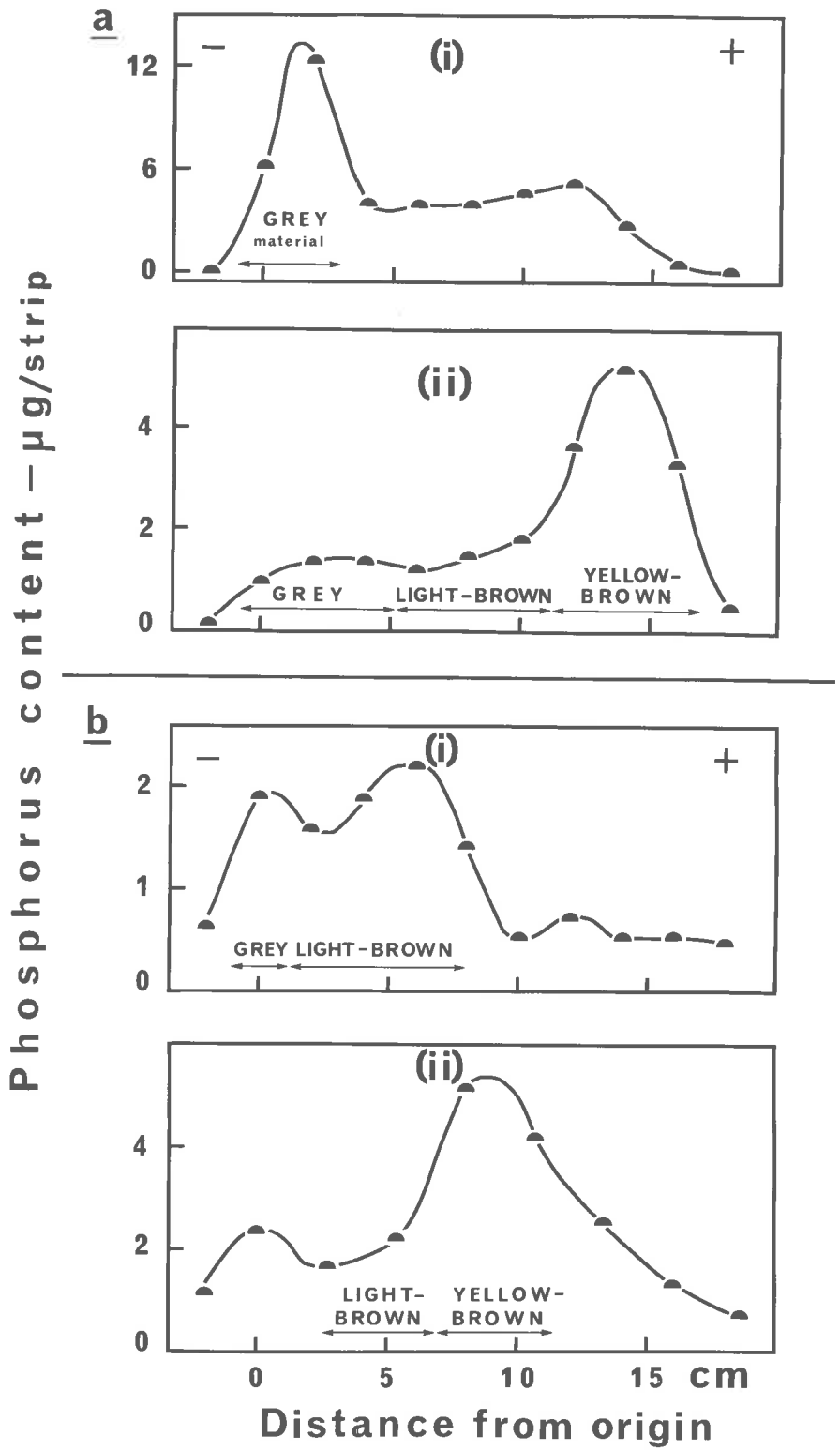
Electrophoresis of fractions from Soil U16 after chromatography on Sephadex G-100 (see Fig. 11 a).

Buffer: (a) 0.05M-Borate, pH 9.

(b) 0.1M-NaOH, pH 13.

Fractions: (i) Tubes 15-21 of Fig. 11 a.

(ii) Tubes 22-30 of Fig. 11 a.


a
(i)
–
+
12
6
0
GREY
material
(ii)
4
2
0
GREY
LIGHT-BROWN
YELLOW-
BROWN
b
(i)
–
+
2
1
0
GREY LIGHT-BROWN
(ii)
4
2
0
LIGHT-
BROWN
YELLOW-
BROWN
0
5
10
15 cm
Distance from origin
Phosphorus content – µg/strip

III. DIALYSIS OF A CRUDE NaOH EXTRACT OF SOIL

1. Introduction

All of the results on higher grades of Sephadex had indicated that much of the P_o extracted by NaOH from soils existed in association with humic materials as a macromolecular complex. Dialysis against distilled water appeared to be a method of obtaining large quantities of salt-free material for identification studies. It was also another possible means of investigating the P_o-humic acid association (by following the release of P_t and P_i during dialysis).

2. Experimental details

Dialysis tubing which allowed diffusion of free myo-inositol hexaphosphate was selected for the experiment. A 0.5M-NaOH extract (60 mls) of Soil U16 (10 g) was dialysed in this tubing against continuously-stirred dist H_2O (600 ml) over a period of 95 hr. The diffusate was replaced with dist H_2O every 10 hr (approx.). In each diffusate the P_t, P_i, S_i, Al and pH was determined. At the end of the time the non-diffusable material was similarly analysed.

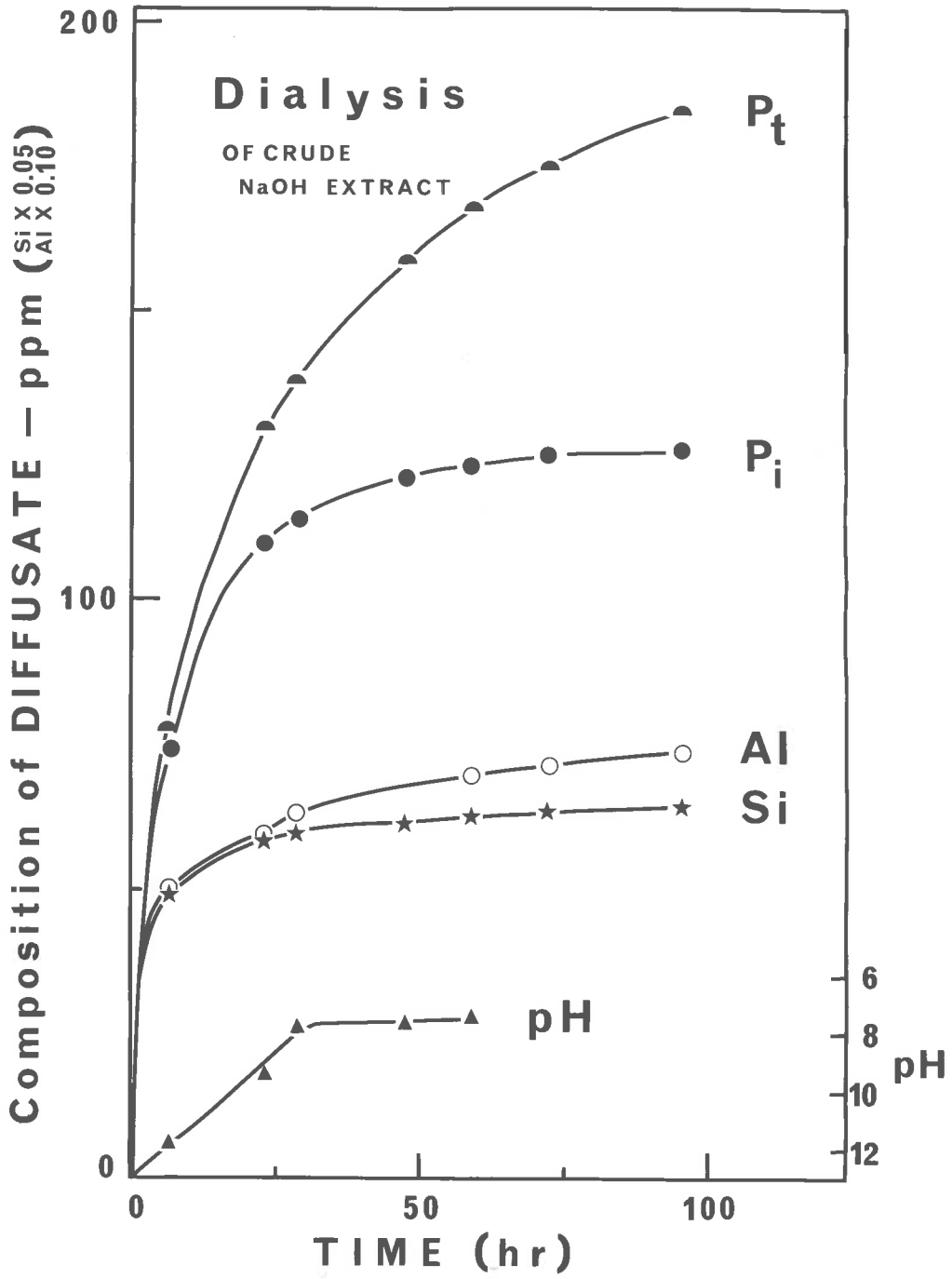
3. Results and discussion

The accumulation with time of these compounds in the diffusate is recorded in Figure 16. Diffusion of the inorganic components was almost complete after 30 hr. The amount of P_o which had diffused in this time (30 ppm) accounted for the inositol polyphosphates in the extract (as determined by gel chromatography on Sephadex G-50, see Fig. 10).

During subsequent stirring and dialysis further release of P_o occurred - this P_o must have been released from association with humic materials either as a result of precipitation of sesquioxides with decreasing pH, or as a result of the rupture of bonds linking P_o to humic acids, or both. (Diffusates at all stages contained light yellow-coloured materials.) Figure 16 indicated that the diffusate from 30 hr onwards may be a source of salt-free, low MW organic phosphates which were formerly associated with humic materials in the crude extract. This aspect is worthy of further study.

At the end of 95 hr the non-diffusable materials were removed from the tubing and centrifuged. (Perchloric acid digestion of the tubing, after a short rinse with dist H_2O, showed that no P had adhered to its surface.) The precipitate accounted for 5 ppm P_t and the supernatant

Figure 16


Dialysis
OF CRUDE
NaOH EXTRACT
Pt
Pi
Al
Si
pH
Composition of DIFFUSATE — ppm (Si X 0.05, Al X 0.10)
200
100
0
0
50
100
TIME (hr)
6
8
10
12
pH

contained 131 ppm Al, 9 ppm Si and 47 ppm P_t. There was no detectable free Fe in the supernatant but on addition of NaOH a precipitate, which appeared to be ferric hydroxide, formed and some P_i was released (cf. Weir & Soper, 1963; Levesque, 1969).

The pH of the final supernatant was 6.4. When lyophilised it yielded a chocolate powder, containing 1% P, which was mostly water-soluble. Dell'agnola & Ferrari (1969) reported a similar preparation which contained 2.7% P after dialysing humic materials which had been excluded from Sephadex G-25.

The powder was dissolved in 0.1M-NaOH and centrifuged. When the supernatant was fractionated on Sephadex G-50 at pH 13, 90% of the phosphorus and the colour were excluded. The included phosphorus was mostly the P_i that had been released when the pH was raised.

It was concluded that this product of prolonged dialysis closely resembled the material which was excluded by Sephadex G-50 at pH 13 from crude NaOH extracts of soil. Gel chromatography however was much quicker than dialysis for obtaining such a preparation, from extracts of up to 300 g of soil, and no further use of dialysis was made in this work.

IV. REMOVAL OF COLOURED MATERIALS

1. General

The failure of electrophoresis and dialysis to separate the majority of the P_o from humic materials led to the use of precipitation of humic acids at low pH. Anderson (1961) found that when the pH was less than 1, the humic acids which were precipitated from NaOH extracts of four soils contained "a small and constant proportion of the P_o". Later Dormaar (1968) showed that 30% of the P_o in some soils may be coprecipitated at low pH values. Other workers have also indicated that most of the P_o is separated from humic acids by acidification (Caldwell & Black, 1958; Cosgrove, 1963; Martin, 1964; Omotoso & Wild, 1970a).

2. Precipitation of humic acids at low pH

Crude 0.5M-NaOH extracts of Soil 29 (extracted at 6:1 solution to soil ratio) were acidified to pH 0.2 with conc HCl. After standing at room temp for 1 hr the sample was centrifuged. The residue was washed twice with 0.1M-HCl and the washings were added to the supernatant which was then analysed.

Providing the soil had received an acid pretreatment (1 hr shake with 0.1M- or 1M- HCl) recovery of P_o in the

acid-soluble (fulvic) fraction was 95%. This figure did not alter when conc H_2SO_4 was used to lower the pH. However if the sample was allowed to stand overnight at pH 0.2 (Anderson, 1961) the P_o recovered represented 75% of the P_o extracted, although the recovery of P_t was the same. Thus approximately 20% of the P_o had hydrolysed during the overnight standing in acid - indicating the possible nature of the P_o compounds.

Analysis showed that the fulvic fraction from a crude NaOH extract of Soil U29 contained all of the P_i, Si and carbohydrate and most of the polyvalent cations, as well as the P_o that had been extracted. However when the pH 0.2, 1 hr precipitation was performed on material excluded from Sephadex G-25 at pH 13 (Fig. 7, tubes 35-47) the ensuing fulvic fraction was substantially free of polyvalent cations and P_i and had a greatly diminished silica content. (Such a result was only achieved when the NaOH concentration was first raised from 0.1M- to 0.5M-, by addition of solid NaOH or by a five-fold concentration. The high salt concentration allowed effective precipitation of humic acids - see Theng, Wake & Posner, 1968 - without coprecipitation of P_o.)

In all subsequent work described in this thesis the material excluded from Sephadex G-25 at pH 13 was concentrated eight-to-ten-fold before precipitation of the humic acids at pH 0.2 with conc HCl. The orange-coloured fulvic fraction contained 95% of the extracted P_o of which 5% hydrolysed in the course of the concentration and precipitation steps. The concentrated solution contained 100 µg P_t/ml (for material extracted at 6:1 from U29) and considerable amounts of carbohydrate (by anthrone - Oades, 1967a).

3. Removal of colour from the fulvic fraction

In similar fulvic preparations Swincer et al. (1968a) found that Polyclar AT (Antara Chemicals, GAF New York) effectively retained most of the colour when the pH was less than 2, without significant losses of polysaccharide.

Complete recovery of P_o was also found in the light yellow effluent of the fulvic fraction after passage through acid-washed Polyclar AT. After elution the effluent was immediately neutralised to pH 7 with solid NaOH and retained for further analysis. (Effective and rapid removal of the colour was later achieved by stirring 40 ml of the fulvic fraction with 2 g of Polyclar for 2 min and then centrifuging or filtering to remove the Polyclar.)

V. FRACTIONATION BY ANION-EXCHANGE CHROMATOGRAPHY AND EXAMINATION OF THE PRODUCTS

1. Preparation of the sample

To transfer all of the P in the neutralised fulvic fraction to an anion-exchange resin it was necessary to first remove the silica that was present, then to partly degrade by acid hydrolysis and finally to desalt the sample.

(a) Precipitation of silica - from the neutralised fulvic fraction occurred in the course of concentration on a rotary film evaporator at 35°. Silica coagulated in the presence of large amounts of salt at neutral pH (Allen & Matijevic, 1969, 1970; De Passe & Watillon, 1970) and could be removed by centrifugation without losses of phosphorus or carbohydrate from the supernatant.

(b) If the sample were then desalted on Sephadex G-10 (Fig. 17) and the P-containing fractions (tubes 17-29) passed through Dowex AG-1(x2), Cl^- resin less than 50% of the P_o was retained by the resin. The remainder was found in the carbohydrate-rich effluent. [This result was reminiscent of the fractions described by McKercher & Moyer, see page 19.]

FIGURE 17.

Desalting of a fulvic acid solution by gel chromatography.

Gel grade: Sephadex G-10 (40-120 μ).

Column size: 46 x 3.5 cm.

Eluant: dist H_2O.

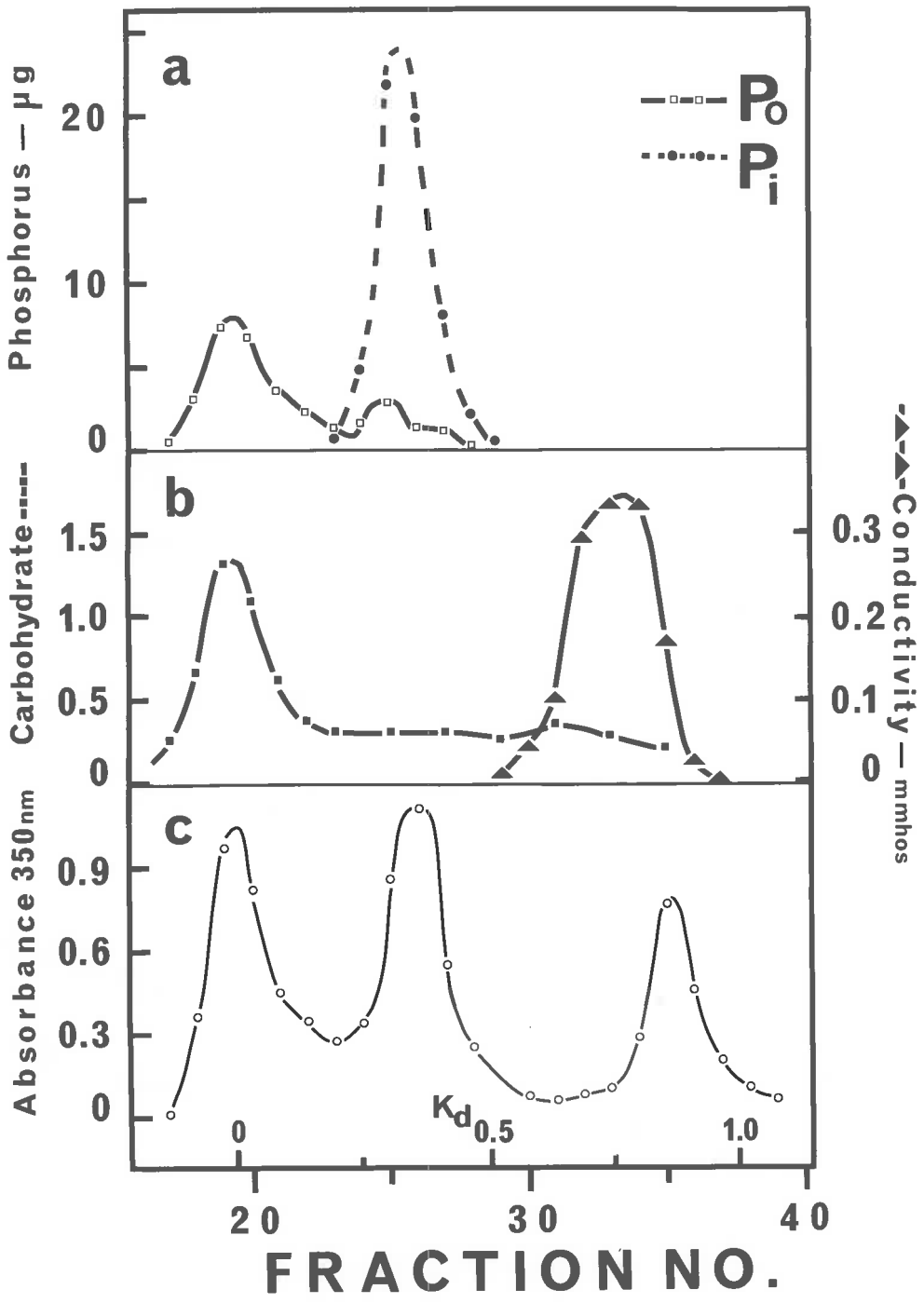
Effluent fraction size: 10 ml.

Components analysed:

(a) P_o and P_i

(b) Carbohydrate and Salt

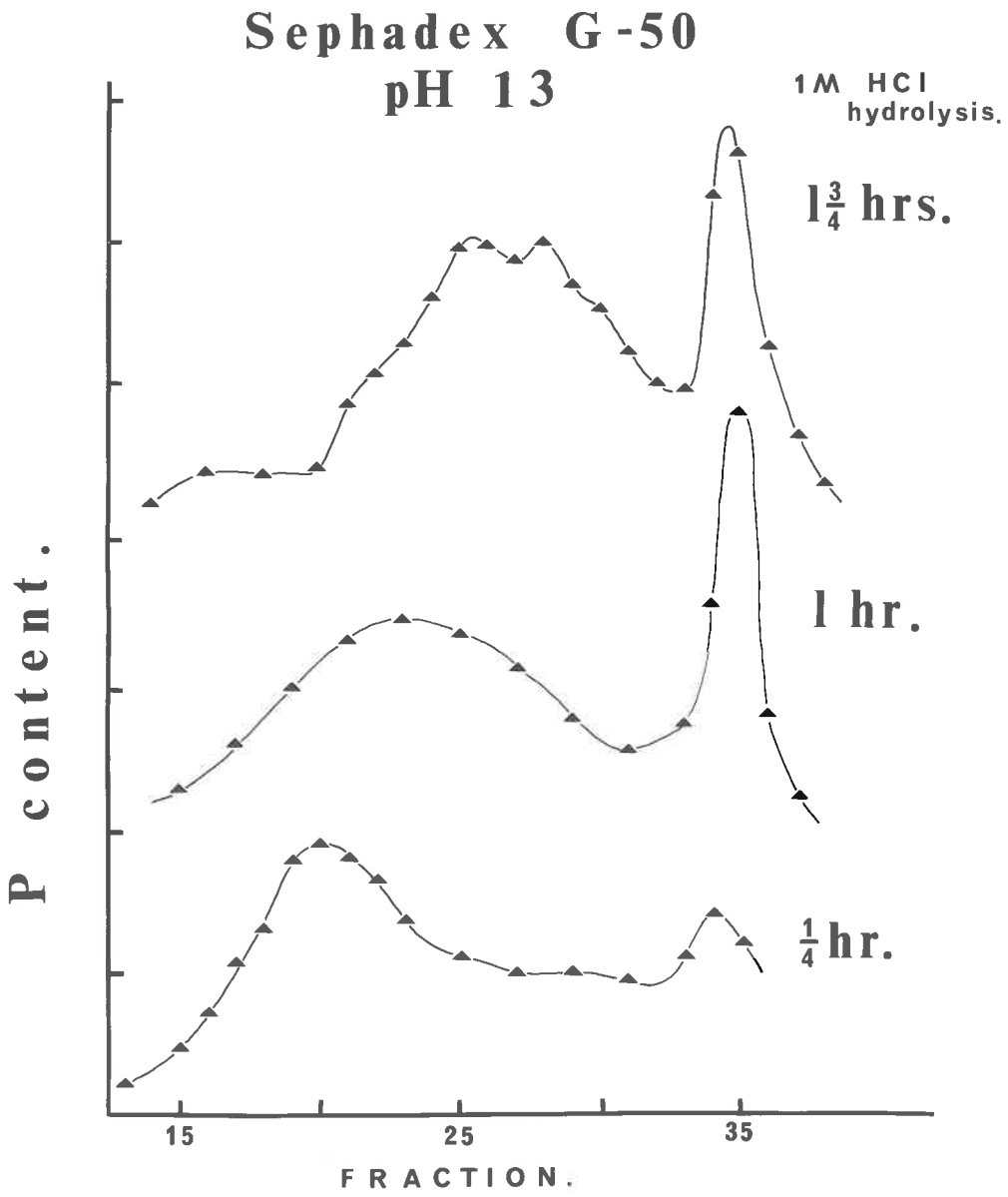
(c) Colour


a
b
c
Po
Pi
Phosphorus — µg
Carbohydrate
Absorbance 350nm
Conductivity — mmhos
20
10
0
1.5
1.0
0.5
0.3
0.9
0.6
0.3
0
0.3
0.2
0.1
0
Kd0.5
0
1.0
20
30
40
FRACTION NO.

When the corresponding fulvic acid fraction from an overnight pH 0.2 precipitation was similarly treated, 70% of the P_o was retained on the resin. Degradation of the P_o component(s) during overnight standing was thought to explain this result (see pages 73, 81). Such a conclusion was verified by refluxing the fulvic fraction in HCl (made to 1M) and following the distribution of the P_o with time on Sephadex G-50 at pH 13.

Figure 18 shows that there was a progressive increase in the K_d of the major P_o component(s) with time of hydrolysis; and there was a concomitant increase in P_i (tubes 31-37). It was concluded that this change in K_d reflected a decrease in size of the constituent molecules and not an increase in adsorption during chromatography. [Reversible adsorption of some coloured materials from soil extracts invariably occurs during fractionation on the lower grades of Sephadex gels in the presence of salt. The coloured material in tubes 34-37 of Fig. 17 is a good example of this (cf. Flodin, 1962; Lindqvist, 1967). Similarly the coloured material in the fulvic acid hydrolyses eluted, behind the salt peak, in tubes 31-36 (K_d 1.10) of Fig. 18. An effect not hitherto experienced during the gel chromatographic investigations for this thesis was the movement of the P_i in apparent association

Figure 18


Sephadex G-50
pH 13
1M HCl
hydrolysis.
1 3/4 hrs.
1 hr.
1/4 hr.
P content.
15
25
35
FRACTION.

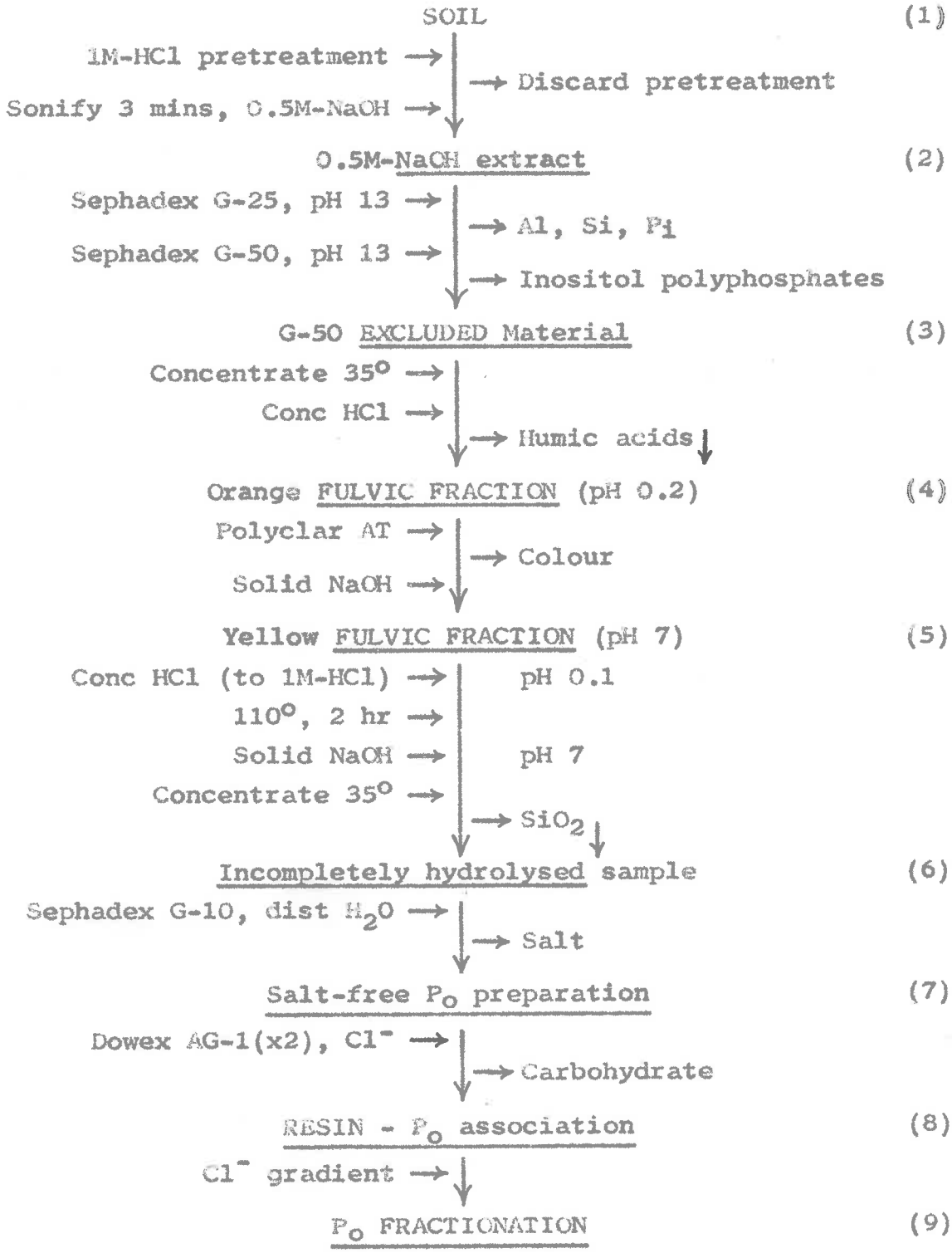
with the retarded colour (K_d 1.10). The reason for this interaction in the presence of NaOH was not known. When aliquots of the hydrolysate were chromatographed on the same gel equilibrated and eluted with 0.1M-LiCl the K_d of the colour was still 1.10 but the K_d of the P_i had decreased to the expected value of 0.60.]

After 2 hr of reflux the K_d of the main P_o peak exceeded the K_d of free inositol hexaphosphate (Fig. 18) indicating that the product was of fairly small dimensions. At this stage about 30% of the P_o in the sample had been completely hydrolysed.

(c) Following desalting of the neutralised sample on Sephadex G-10 all of the P_o was retained by the Dowex resin, whereas most of the carbohydrate passed through in the effluent.

The experimental steps which enabled the unknown P_o in soil to be fractionated have been summarised in a flow sheet (next page). For soils containing low amounts of inositol polyphosphates the Sephadex G-50 fractionation can be omitted before proceeding from step (3). The procedure developed in steps (1) to (5) for the phosphorylated macromolecule was, in many aspects, similar to the procedure used by Swincer et al. (1968a) for the isolation of polysaccharide material from similar soils.

STEPS LEADING TO THE FRACTIONATION OF THE UNIDENTIFIED ORGANIC PHOSPHORUS EXTRACTED FROM SOIL BY NaOH



2. Electrophoresis of standard phosphates

The P_o in the fractions from anion-exchange chromatography was examined by electrophoresis, using the apparatus described by Tate (1968). Three buffers were used:

(a) 0.1M-Oxalate, pH 1.5 (Sieffert & Agranoff, 1965).

(b) 0.05M-Borate, adjusted to pH 10.0 with NaOH (Foster, 1957; Frahn & Mills, 1959).

(c) 0.1M-Citrate, pH 5.0.

The mobilities of a number of standard compounds were obtained for comparison with the unknown phosphates. Mobilities in buffers (a) and (b) were calculated relative to PP_i using fructose as the non-migrating marker. In the absence of a suitable non-migrating marker for buffer (c) the mobilities were calculated relative to glucose using rhamnose (M_G 0.52) as the internal reference (Garegg & Lindberg, 1961).

Compounds were detected with the following dip reagents: phosphomolybdate (Harrap, 1960); silver nitrate (Trevelyan, Procter & Harrison, 1950) including pentaerythritol in the case of buffer (c) (Frahn & Mills, 1959); benzidine-periodate (Gordon, Thornburg & Werum, 1956); p-anisidine HCl (Hough & Jones, 1962); naphthoresorcinol (Smith, 1960) and ninhydrin (Toennies & Kolb, 1951).

The mobilities of the standards and the unknown compounds (see next sections) are listed in Table 16.

TABLE 16.

Mobility of reference compounds and organic phosphates from Soil U29 in three buffer systems

Reference Compound	BUFFER		
	(a) OXALATE (Marker Fructose, mobility relative to pyrophosphate)	(b) CITRATE (Marker Fructose, mobility relative to pyrophosphate)	(c) BORATE (Internal reference Rhamnose, mobility relative to glucose)
	M_{PP_i}	M_{PP_i}	M_G
P_i	0.31	0.72	1.64 (elongated)
Glucose-1-Phosphate	0.49	0.40	1.10
Glucose-6-Phosphate	0.45	0.40	1.30
Fructose-6-Phosphate	0.48	0.42	1.28
Ribose-5-Phosphate	0.51	0.44	1.35
Fructose 1,6 di-Phosphate	0.76	0.65	1.45
ATP	0.55	0.64	-
α-Glycerophosphate	0.58	0.55	1.33
β-Glycerophosphate	0.55	0.56	1.35
Glucuronic acid	0.00	0.48	1.26
Galacturonic acid	0.00	0.45	1.14
AMP	-0.09	0.27	1.13
Unknown Compounds			
A	0.56	-	-
B	0.47	0.41	1.24/1.17
C	0.33	-	-
D	0.00	-	1.24/1.10

3. Anion-exchange chromatography

(a) Preliminary experiment -

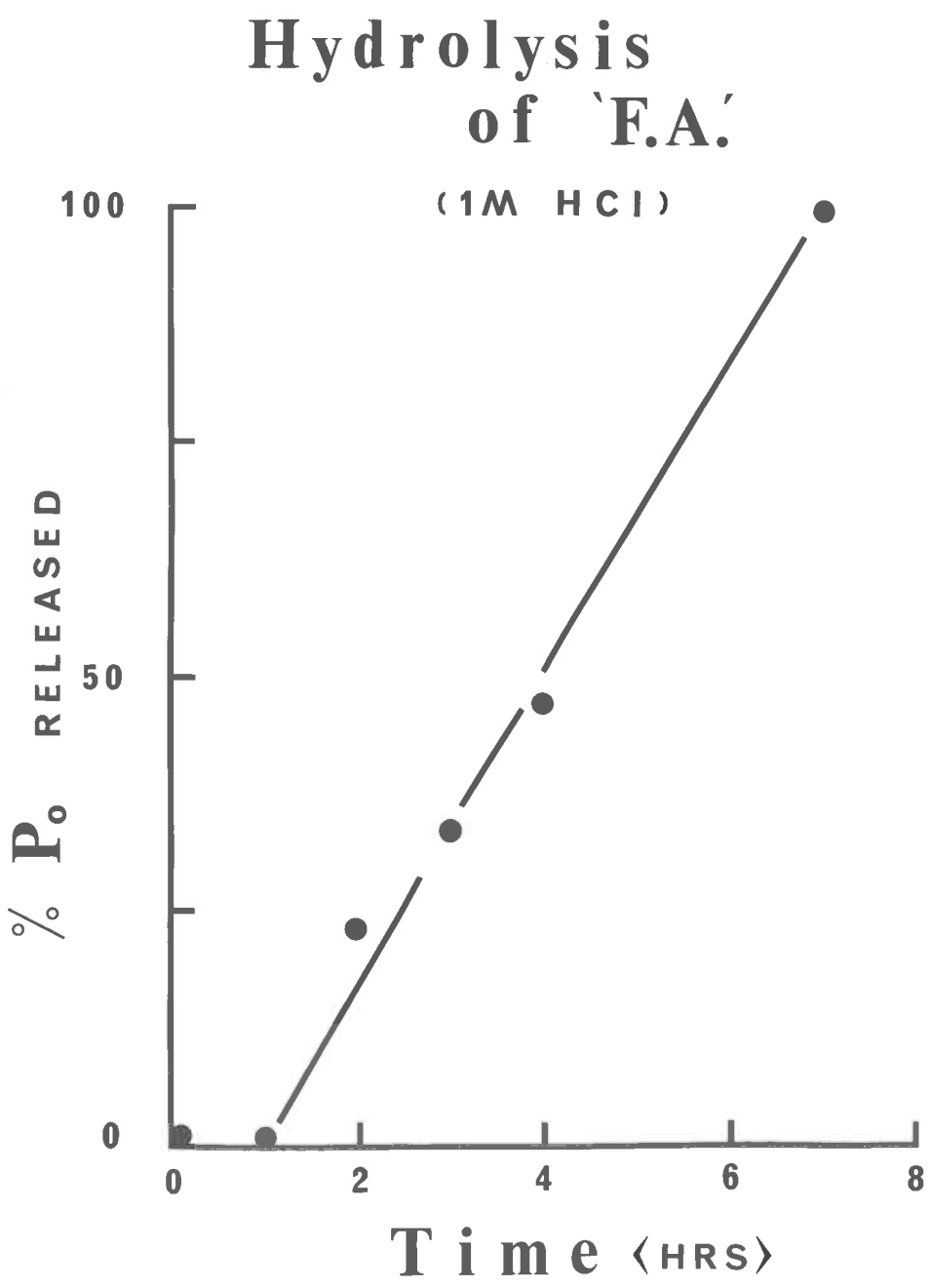
The salt-free P_o preparation (step (7) in the flow sheet) was loaded onto Dowex Cl^- resin. Two phosphate peaks were eluted and the phosphorus in each peak accounted for one-third of the P_o originally extracted from Soil U29 by NaOH (i.e. the total eluted P accounted for two-thirds of the P_o).

(i) The first peak --

was very loosely bound to the resin (eluted by approx. 0.2M-LiCl). This fraction contained large amounts of uronic acids (by the carbazole method of Bitter & Muir, 1962) as well as P_o and P_i. After desalting on Sephadex G-10 an aliquot was electrophoresed in buffer (a). Two major phosphate spots were detected - one was P_i (M_{PP_i} 0.31), the other was an organic phosphate (M_{PP_i} 0.47) which had the property of a reducing-sugar compound (light-brown spot with p-anisidine HCl, dark-brown spot with $AgNO_3$).

The rest of the desalted material was made 1M- with respect to HCl and refluxed at 110°. Complete release of P_i was effected in 7 hr (Fig. 19). Apart from P_i there were no recognisable products in the residue after hydrolysis.

Figure 19



(ii) The second peak --

was more strongly bound to the exchange resin (eluted with approx. 0.35M-LiCl). It gave a purple colour with the carbazole reaction, rather than the pink produced by known uronic acids. Electrophoresis in buffer (a) of the desalted material indicated the presence of four organic phosphates which were not reactive to p-anisidine HCl (M_{PP_i} 0.72, 0.83, 1.00, 1.20) and one phosphate (M_{PP_i} 0.00) which gave a light-red spot with p-anisidine HCl.

(b) Detailed fractionation -

A salt-free P_o preparation from Soil U29 (60 g) was passed through Dowex AG-1(x2), Cl^- resin (14 cm x 2.1 cm diam). The column was eluted with an acid gradient (1 l. dist H_2O - mixing vessel; 1 l. 1.5M-HCl - reservoir) and 13.5 ml fractions were collected. Figure 20(a) shows the elution pattern of the P_o and P_i. Aliquots of tubes 14-25 gave the aforementioned purple colour with the carbazole reaction; this effect was associated with shifts in the peaks of the absorbtion spectra of the complex, compared with those peaks resulting from D-glucuronic acid [Fig. 20(b)]. Such organic phosphates may have accounted for the extra acidity of a uronic acid fraction of a peat polysaccharide preparation which was investigated by Barker, Hayes, Simmonds & Stacey (1967).

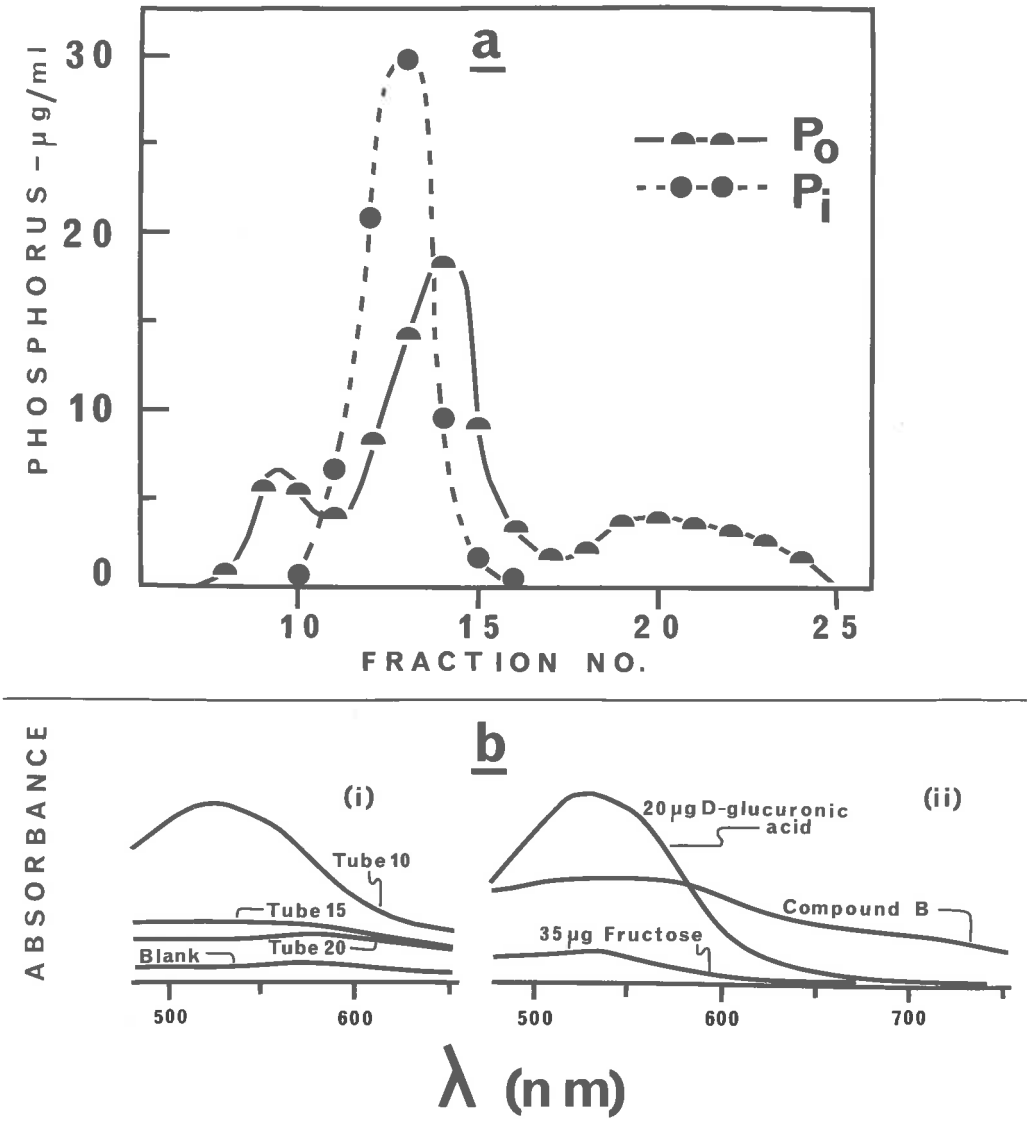
FIGURE 20.

(a) Fractionation of unknown P_o on Dowex Cl^- during elution with HCl gradient.

(b) Spectra produced by reaction of the samples containing unknown P_o with the carbazole reagent.

(i) Fractions from (a).

(ii) Spectra of compound B.


a
PHOSPHORUS – µg/ml
30
20
10
0
10
15
20
25
FRACTION NO.
Po
Pi
b
ABSORBANCE
(i)
Tube 10
Tube 15
Tube 20
Blank
500
600
(ii)
20 µg D-glucuronic acid
Compound B
35 µg Fructose
500
600
700
λ (nm)

The material in tubes 11-17 of Figure 20(a) was concentrated and electrophoresed in buffer (a); side strips were developed and the areas on the undeveloped paper which corresponded to the phosphate spots (Fig. 21) were eluted with water (Hariharan et al., 1968). Some of the properties of the components are listed in Table 17. It was concluded that compound C was P_i, and that compound B was the major organic phosphate in this sample.

By contrast to the discrete separation of the four spots in oxalate, borate electrophoresis of fractions 11-17 [Fig. 20(a)] showed a streak of P_o and reducing substances ($AgNO_3$ dip) from M_G 0.46 to M_G 1.27.

4. Examination of the major organic phosphorus component

Compound B, the major unidentified organic phosphate, was further examined. It did not react with the diphenylamine reagent for deoxy sugars (Dische, 1962) but did produce the purple colour with carbazole. Figure 20(b)(ii) shows a bump at 700 nm that had been missed in examining previous samples. As oxalate anions were the only other component in the solution of compound B the purple colour was probably not caused by interfering ions (Bitter & Muir, 1962).

FIGURE 21.

Oxalate electrophoresis of the major unidentified P_o compounds from Soils 237 and U29.

Standards: myoinositol hexaphosphate (IP_6)
inorganic pyrophosphate (PP_i)
adenosine triphosphate (ATP)
inorganic phosphate (P_i)

Unknown compounds: A, B, C, D.

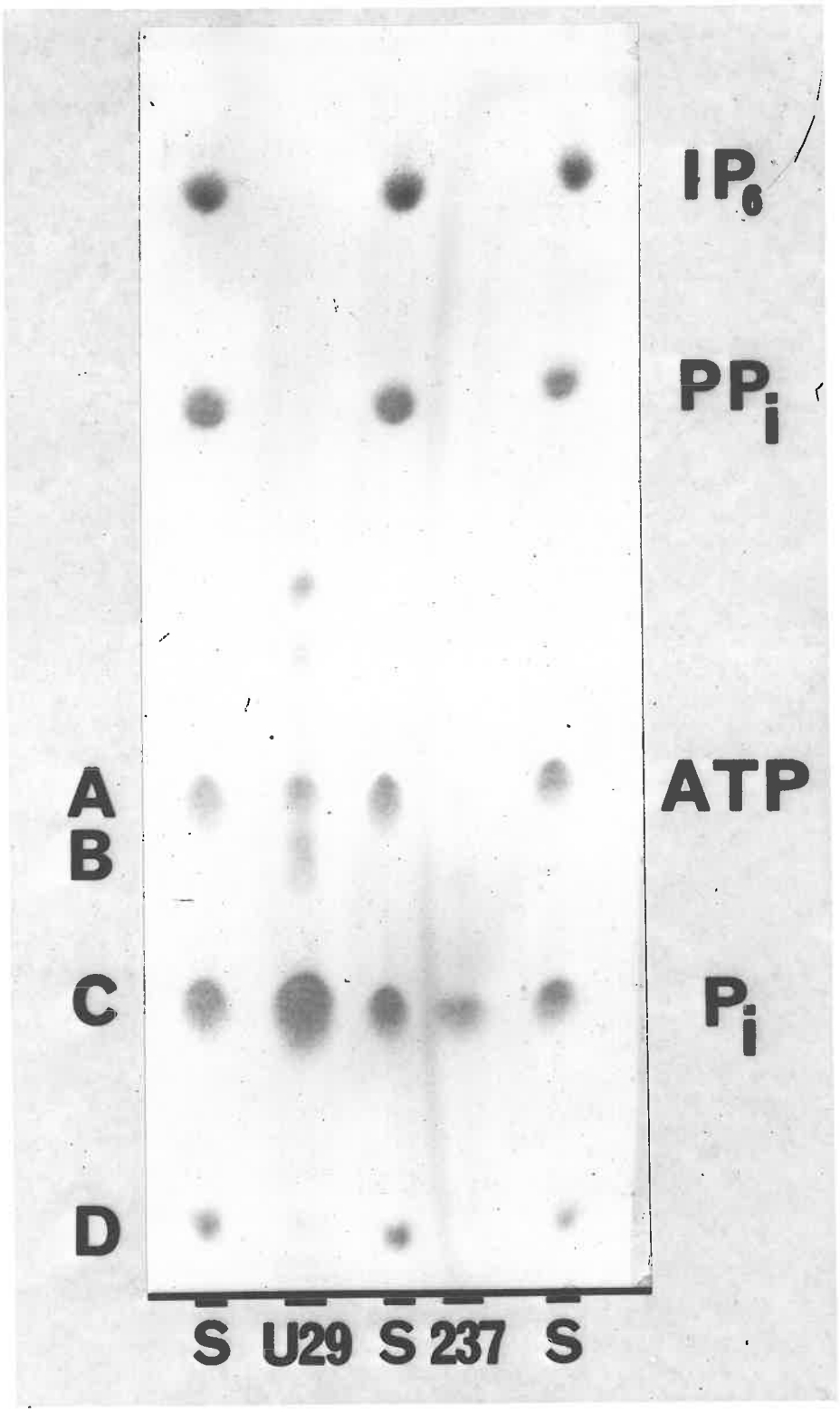

IP6
PPi
ATP
Pi
A
B
C
D
S
U29
S
237
S

TABLE 17.

Separation of the phosphate compounds in tubes 11-17 [Fig. 20(a)] by electrophoresis at pH 1.5

Phosphate Spot	M_{PP_i}	P_t (μg)	% of P_{in} A-D	Reaction to		
				p-anisidine HCl	Naphthoresorcinol	Ninhydrin
A	0.55	37	16	-	-	-
B	0.45	54	23	Light brown	Weak	-
C	0.31	140	60	-	-	-
D	0.00	3	1	Cherry	Strong	Weak

Electrophoresis of fraction B in buffer (c) produced two components: B1 (M_G 1.24) and B2 (M_G 1.17). They both were strongly reducing to $AgNO_3$ (in the presence of pentaerythritol).

In buffer (b) the mobility of B was M_{PP_i} 0.41. This showed the absence of a free carboxyl group and ruled out the possibility that B was a phosphorylated uronic acid or a triose phosphate (Dische & Borenfreund, 1951). The possibility remains that compounds B1 and B2 are phosphorylated lactones. (The weak reaction of compound B with naphthoresorcinol - Table 17 - is not conclusive evidence of a uronic acid or uronolactone - Bartlett, 1959b).

The reducing power of B was completely destroyed after overnight hydrolysis (110°) at pH 5 or in 1M-HCl, but wheat germ acid phosphatase (pH 5) had no effect after an overnight incubation (room temp).

Borohydride reduction of B did not alter its mobility in buffer (a) or (of B1 and B2) in (c). The sensitivity of reduced B to $AgNO_3$ was greatly diminished however, and reduced B had no reaction with p-anisidine HCl. Overnight hydrolysis of reduced B (110°, pH 5) decreased its mobility in buffer (a) (M_{PP_i} 0.00, detected by benzidine-periodate). Three hydrolysis products were noted after electrophoresis in (c) (M_G 1.11, 0.84, 0.70 - major component) and four

products were detected after electrophoresing in (b) (Mpp_i 0.89, 0.36, 0.15 - major component; -0.03).

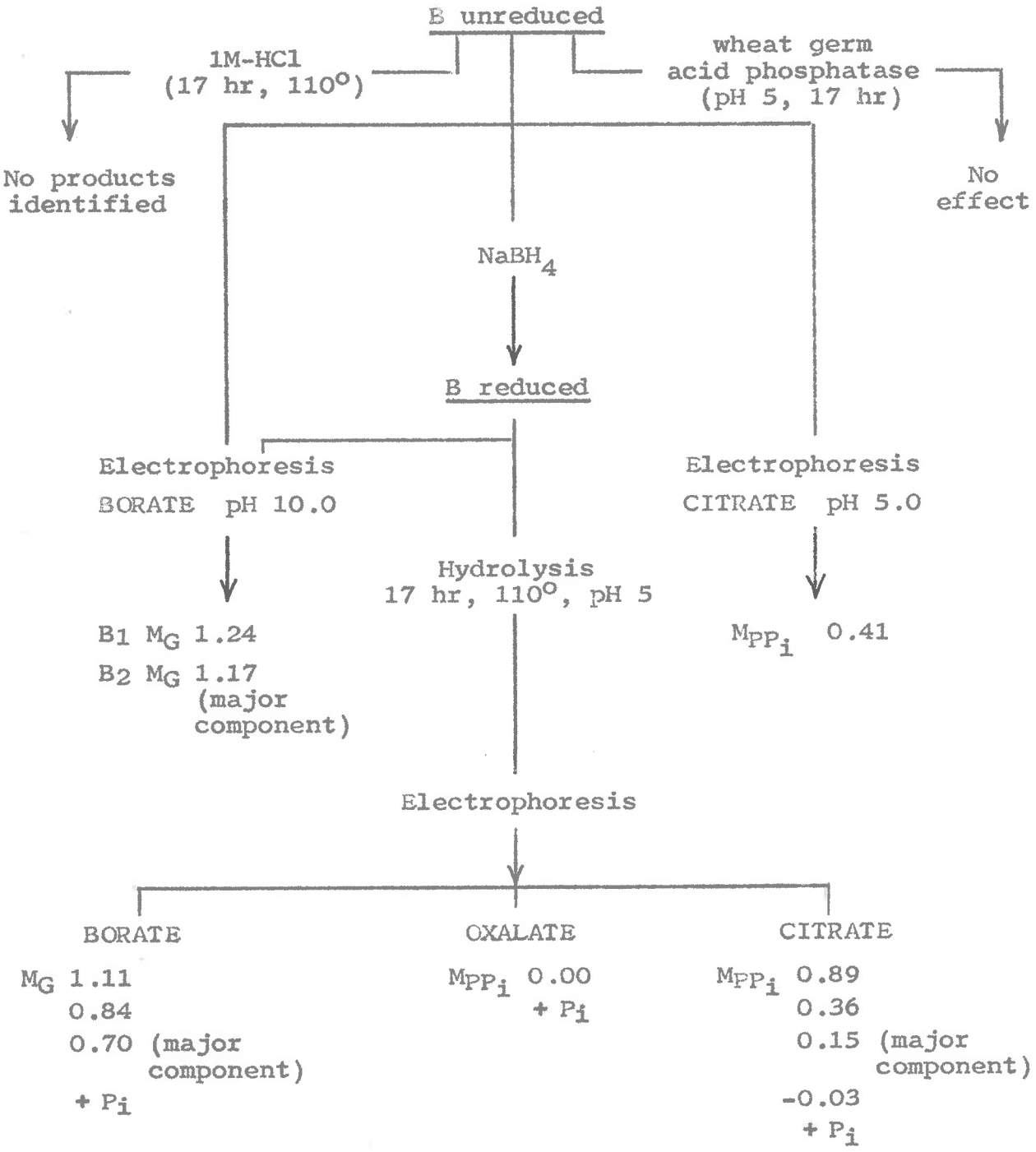
The investigations made on compound B have been outlined on the following page. Lack of material prevented further experiments, but future studies by electrophoresis, paper chromatography and gas-liquid chromatography should lead to an identification of compound B.

Compounds A and B (Table 17) may be similar to the two unidentified organic phosphates reported by Halstead & Anderson (1970) and Omotoso & Wild (1970b). The stability of compound B to acid phosphatase recalled a similar result of McKercher (1968) with alkaline phosphatase. When NaOH extracts of Eastern Australian Soils 237 and 350 were treated with the methods in the flow sheet (page 86), A and B were again the major organic phosphates (see Fig. 21 for the sample from Soil 237).

These results suggest that the techniques developed in this thesis on the Urrbrae soil will enable the isolation of a major proportion of the P_o in the form of monomeric phosphates from a variety of soil types. Positive identification of these phosphorylated fragments is necessary before the macromolecules containing the P_o can be examined in detail. The results that have been obtained are consistent with the hypothesis that a phosphorylated polysaccharide is a major component of soil organic phosphorus.

Summary of: The Examination of compound B

(M_{PP_i} 0.45 in oxalate buffer)



APPENDICES

APPENDIX 1

Detailed figures of the P extracted by the methods of Mehta et al., 1954; Harrap, 1963; Saunders & Williams, 1955

TABLE 18

Extraction of P(ppm) from 1 g of the Urrbrae and Eastern Australia soils by the method of Mehta et al. (1954)

Soil		EXTRACTION STEP											
		(1) Conc HCl			(2) Cold NaOH			(3) Hot NaOH			Total		
		P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o
URRBRAE Series	1	211	178	33	29	12	17	15	7	8	255	197	58
	5	262	227	35	36	14	22	16	8	8	314	249	65
	15	328	254	74	48	20	28	26	5	21	402	279	123
	16	305	238	67	57	20	37	27	5	22	389	263	126
	17	284	236	48	44	17	27	20	5	15	348	258	90
	29	403	323	80	59	26	33	31	0	31	493	349	144
EASTERN AUSTRALIA													
250		138	116	22	21	12	9	24	2	22	183	130	53
CH		171	120	51	33	8	25	19	3	16	223	131	92
237		1860	1798	62	488	311	177	205	148	57	2553	2257	296
315		810	473	337	289	90	199	126	4	122	1225	567	658
253		1473	1342	131	429	218	211	162	76	86	2064	1636	428
ROB		1026	769	257	471	152	319	222	64	158	1719	985	734
350		6893	6240	653	96	15	81	49	2	47	7038	6257	781

TABLE 19

Extraction of P (ppm) from 1 g of the Urrbrae and Eastern Australia soils by the method of Harrap (1963)

Soil		EXTRACTION STEP											
		(1) 5% EDTA			(2) Cold NaOH			(3) Hot NaOH			Total		
		P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o	P_t	P_i	P_o
URRBRAE Series	1	25	21	4	101	58	43	66	51	15	192	130	62
	5	32	30	2	134	79	55	76	68	8	242	177	65
	15	45	42	3	176	91	85	106	69	37	327	202	125
	16	33	28	5	183	94	89	102	73	29	318	195	123
	17	35	31	4	179	98	81	89	64	25	303	193	110
	29	77	66	11	234	127	107	130	69	61	441	262	179
EASTERN AUSTRALIA													
250		24	18	6	35	15	20	45	23	22	104	56	48
CH		84	53	31	102	50	52	44	16	28	230	119	111
237		23	16	7	1036	669	367	436	303	133	1495	988	507
315		95	32	63	927	205	722	340	133	207	1362	370	992
253		23	16	7	511	259	252	504	328	176	1038	603	435
ROB		13	5	8	782	289	493	590	287	303	1385	581	804
350		1498	1370	128	43	18	25	413	102	311	1954	1490	464

TABLE 20.

Figures for the extraction of P_o from the Eastern Australia soils by the method of Saunders & Williams (1955) - taken from Williams & Anderson (1968)

SOIL	250	P_o	51
	CH		123
	237		564
	315		929
	253		580
	ROB		955
	350		340*

* Determined by J.H.S., Oct. 1970.

APPENDIX 2

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GEL CHROMATOGRAPHY OF INOSITOL POLYPHOSPHATES AND THE AVIAN HAEMOGLOBIN–INOSITOL PENTAPHOSPHATE COMPLEX

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(Received September 30th, 1969)

SUMMARY

Gel chromatography of inositol polyphosphates has shown that a substantial anion exclusion effect exists which is diminished, but not necessarily eliminated, by concentrations of eluant electrolyte up to 2 *M*. Under the appropriate conditions of ionic strength and pH, gel chromatography provides a useful adjunct to the established fractionation procedures for inositol phosphates. It has been successfully used to demonstrate that myoinositol 1,3,4,5,6-pentaphosphate forms a strong ionic association with pigeon and chicken haemoglobins.

INTRODUCTION

Established methods for the fractionation of inositol polyphosphates include anion-exchange chromatography, paper chromatography, paper electrophoresis and fractional precipitation. These procedures have been reviewed by COSGROVE[1]. In addition moving paper electrophoresis[2] and thin-layer chromatography[3] have now been described.

Separations of a series of compounds by chromatography on a cross-linked dextran gel (Sephadex) have been reported for inorganic polyphosphates[4] and polynucleotides[5]. The present study records the distribution coefficients[6] ($K_d = (V_e - V_0)/V_t$, where V_e is the elution volume of the solute, V_0 is the void volume and V_t the volume of the stationary phase) of various inositol polyphosphates and some other reference compounds on a number of Sephadex gels. The variation of these K_d values with ionic strength and pH is examined. The application of gel chromatography to the elucidation of the ionic nature of the pigeon and chicken haemoglobin-inositol pentaphosphate complex is described.

EXPERIMENTAL

Materials

The Sephadex (Pharmacia AB) gels and columns used are shown in Table I.

TABLE I

SEPHADEX GELS AND COLUMNS

Sephadex grade	*Mesh size* (μ)	*Column parameters*[a]				*Sample* (*ml*)	*Fraction size* (*ml*)
		Length (*cm*)	V_t (*ml*)	V_0 (*ml*)	V_i (*ml*)		
G-200	140–400	36	1020	290	700	10	16.5
G-100	40–120	78	770	260	470	8	14.0
G-50	100–300	58	710	270	410	6	14.0
G-25	100–300	52	500	210	250	5	16.5
G-15	40–120	46	300	120	130	4	7.0

[a] For pH < 13 and eluant salt concentrations $\leqslant 2$ *M*.

The reference compounds, their methods of preparation or source and the symbols used in this paper are: myoinositol hexaphosphate[7], IP_6; myoinositol tripyrophosphate[7], $\pi_3\emptyset$; chicken blood myoinositol pentaphosphate[7], $IP_5(CB)$; alkaline hydrolysis myoinositol pentaphosphate[2], $IP_5(OH)$; myoinositol tetraphosphate[8], IP_4; myoinositol triphosphate[8], IP_3; myoinositol diphosphate[8], IP_2; myoinositol monophosphate[9], IP_1; glycerol phosphoryl myoinositol[10], GPI; myoinositol*, I; orthophosphate*, P_1; inorganic pyrophosphate*, PP_1; adenosine monophosphate*, AMP; adenosine triphosphate*, ATP; phosvitin[11], Pv; triethyl phosphate*, Et_3P; and fructose*, Fr.

Washed red blood cells were lysed and the haemolysates obtained by the DRABKIN procedure[12].

Methods

Columns were prepared and packed after equilibration with eluant, in accordance with manufacturers' directions. Lithium chloride was used as the eluant electrolyte to facilitate recovery of the ethanol-insoluble lithium phosphates[13].

The sample (200–300 μg P) was layered direct onto the filter paper or gauze disc that covered the gel surface and suitable fractions were collected. All runs were made at room temperature (20–27°). K_d values were calculated by use of Blue Dextran 2000 (Pharmacia AB) and tritiated water to determine V_0 and V_i, respectively.

Aliquots from each fraction were analysed for total phosphorus[14]. Inorganic phosphorus was determined by the ascorbic acid method[15], fructose was measured by the anthrone method[16] and inositol by periodate oxidation[17]. Haemoglobin absorbance was measured at 577 nm.

The location of the inositol pentaphosphate in haemolysate fractions was determined by precipitation of the acid-soluble phosphorus as the barium salt with subsequent electrophoresis of the anions in 0.1 *M* oxalate at pH 1.5 as previously described[7].

RESULTS AND DISCUSSION

Anion exclusion

The anomalous behaviour of ionic compounds of low molecular weight eluted

* Commercial products.

with water on Sephadex gels has been appreciated since the initial study of GELOTTE[6]. The nature of this effect has been greatly clarified by the work of NEDDERMEYER AND ROGERS[18], who found that irregular elution profiles of anions became symmetrical at 0.01 *M* eluant salt concentration and showed that the asymmetric profiles in distilled water could be attributed to a Donnan anion exclusion effect.

We have investigated the effect of eluant salt concentrations above that which is necessary to produce symmetrical profiles. Fig. 1 shows the results for the eluant salt molarities in the range 0.01 *M*–5 *M* for inorganic phosphate, pyrophosphate, myoinositol hexaphosphate and triethyl phosphate on Sephadex G-25.

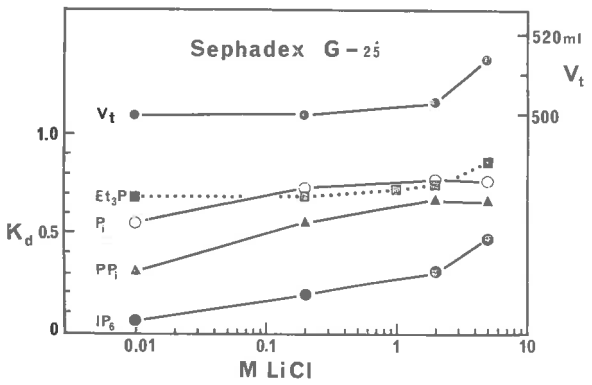

Fig. 1. Variation of the gel column (V_t) and distribution coefficients (K_d) of triethyl phosphate (Et_3P), inorganic phosphate (P_i), inorganic pyrophosphate (PP_i) and myoinositol hexaphosphate (IP_6) with the molarity of the eluant electrolyte (lithium chloride).

The uncharged molecules triethyl phosphate (Fig. 1), fructose (Table II) and myoinositol (Table III) have virtually constant K_d values at eluant electrolyte concentrations of 0.01–0.2 *M*. By contrast the anions of Fig. 1 each show a marked increase in K_d in this range. Iodide and sulphate anions[19] as well as polythymidylates[5] show analogous behaviour. Such results suggest the continued existence of a significant anion exclusion effect above the 0.01 *M* limit suggested by NEDDERMEYER AND ROGERS[18].

With eluant electrolyte concentrations above 2 *M* the situation becomes more complex. Both inorganic phosphate and pyrophosphate have constant K_d values, but the myoinositol hexaphosphate and triethyl phosphate show increased K_d values. It is not possible to decide between the anion exclusion and adsorption mechanisms in the case of the highly charged hexaphosphate but adsorption appears to be the only feasible explanation for the behaviour of the uncharged triethyl phosphate. Interpretation of the results is further complicated by an unexpected swelling of the gel matrix (V_t) above 2 *M* (Fig. 1).

The results from a variety of reference compounds on a range of Sephadex gels are listed in Table II and from this table it is again apparent that, for anionic compounds which are detectably excluded from the gel pores ($K_d < 0.8$) at 0.01 *M* eluant salt concentration, there is invariably an increase in K_d at higher salt concentrations.

Fig. 2 shows the effect of pH on K_d values in the presence of 0.1 *M* eluant salt. In the pH range 3–11 where the ionisation of carboxyl groups of the gel matrix and

TABLE II

DISTRIBUTION COEFFICIENTS OF REFERENCE COMPOUNDS ON SEPHADEX GELS

Grade	*Eluant molarity*	*Distribution coefficients* (K_d) ± 0.05						
		Pv	IP_6	PP_i	*ATP*	P_i	*Fr*	*AMP*
G-15	0.01	0.00	0.00	0.04	0.16	0.26	0.63	0.74
	0.20	0.00	0.06	0.25	0.35	0.40	0.65	1.00
G-25	0.01	0.00	0.06	0.31	0.60	0.56	0.75	0.91
	0.20	0.00	0.19	0.56	0.69	0.73	0.75	1.06
	2.00	0.00	0.30	0.67	0.93	0.77	0.81	1.30
	5.00	0.00	0.47	0.65	0.88	0.76	0.79	1.15
G-50	0.01	0.00	0.45	0.81	0.91	0.91	0.89	1.14
	0.10	0.00	0.61	0.83	0.93	0.89	0.93	1.14
G-100	0.01	0.03	0.79	0.97	—	0.97	0.97	1.07
G-200	0.01	0.21	0.90	—	—	0.98	0.98	—

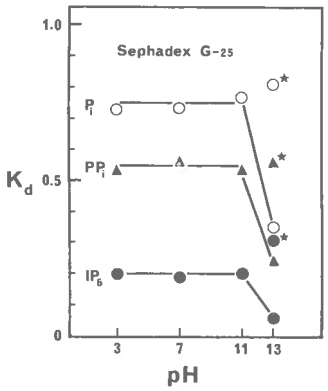

Fig. 2. Variation of the distribution coefficient (K_d) of inorganic phosphate (P_i), inorganic pyrophosphate (PP_i) and myoinositol hexaphosphate (IP_6) with the pH of the eluant electrolyte (0.1 *M* lithium chloride). Values with asterisks at pH 13 refer to an electrolyte concentration of 2 *M*.

the second dissociation of phosphate groups occurs, the incorporation of 0.1 *M* eluant salt is sufficient to suppress the expected fall in K_d values caused by the repulsion of these groups.

However, under strongly alkaline conditions (pH 13), where the ionisation of hydroxyl groups in the carbohydrate gel matrix is significant, a marked anion exclusion effect becomes apparent with a striking reduction in K_d values for the phosphates. If the concentration of lithium chloride in the eluant at pH 13 is now increased to 2 *M*, the K_d values marked by asterisks in Fig. 2 are obtained and the anion exclusion is again substantially repressed.

Molecular size

DETERMANN[20] has comprehensively reviewed the various empirical relationships between K_d values and molecular size parameters. In general there is an orderly

lowering of K_d values as a homologous series is ascended. Table III shows such an inverse relationship between the degree of phosphorylation of the inositol ring and the K_d value on two grades of Sephadex at two eluant salt concentrations. Similar results have been obtained by HOHN AND SCHALLER[5] for oligonucleotides and by OHASHI *et al.*[4] for inorganic phosphates.

TABLE III

DISTRIBUTION COEFFICIENTS OF MYOINOSITOL POLYPHOSPHATES ON SEPHADEX G-50 AND G-25

Grade	*Eluant molarity*	*Distribution coefficients* (K_d) ± 0.05									
		IP_6	$\pi_3\phi$	$IP_5(OH)$	$IP_5(CB)$	IP_4	IP_3	IP_2	IP_1	GPI	I
G-25	0.01	0.06	0.06	0.06	0.06	0.12	0.19	0.31	0.44	0.53	0.75
	0.20	0.19	0.19	0.19	0.19	0.25	0.33	0.44	0.59	0.56	0.75
G-50	0.01	0.45	0.48	0.56	0.54	0.57	0.64	0.70	0.86	0.97	0.83
	0.10	0.61	0.61	0.62	0.64	0.68	0.75	0.79	0.86	0.97	0.86

Because both anion exclusion effects and molecular size contribute to the observed K_d values in Table III no attempt has been made to establish a simple logarithmic relationship between K_d and a molecular size parameter alone. Nevertheless by using a suitably calibrated column with appropriate eluant salt concentrations an unknown member of the series could be identified.

On the other hand Table III also indicates that Sephadex gel chromatography is unlikely to be of much value for separation of mixtures of closely related members of the inositol polyphosphate series which are more satisfactorily resolved by ion-exchange[1,21] and electrophoretic procedures[2].

Application

The major inositol polyphosphate which can be isolated from chicken blood by acidic protein precipitants is now known to be myoinositol 1,3,4,5,6-pentaphosphate[7]. Unsuccessful attempts to isolate the pentaphosphate by ultrafiltration of haemolysed red blood cells suggested that covalent or ionic linkages to some macromolecule may be present. A similar observation has been made for diphosphoglyceric acid in mammalian red blood cell haemolysates by SOLOMON *et al.*[22].

In order to examine whether or not the inositol polyphosphate was covalently or ionically linked to a macromolecule, both pigeon and chicken haemolysates were examined by gel chromatography.

The results for chicken blood haemolysate on Sephadex G-50 are shown in Fig. 3. A similar pattern was obtained for pigeon haemolysate. At 0.1 *M* eluant salt concentration and pH 7 (Fig. 3a), conditions of ionic concentration and pH which approximate to those of the red blood cell, there is a strong association of the inositol pentaphosphate with the pigeon and chicken haemoglobins. At high pH (Fig. 3b) or high eluant salt concentrations (Fig. 3c) the complex is dissociated, which indicates a strong ionic association is present under the conditions of Fig. 3a.

On Sephadex G-50 with 0.1 *M* eluant and pH 7 (Fig. 3a) the haemoglobin–inositol pentaphosphate complex is coincident with the void volume. To confirm that

the polyphosphate was associated with the haemoglobin and not some other protein, the haemolysate was examined on Sephadex G-100 under the same elution conditions. The inositol pentaphosphate and haemoglobin peaks were found to be coincident with $K_d = 0.31$.

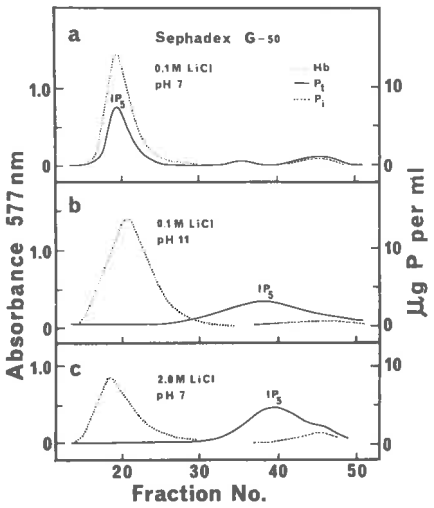

Fig. 3. (a) Assocation of the inositol pentaphosphate and chicken haemoglobin peaks at pH 7 and 0.1 *M* eluant electrolyte concentration. (b) Dissociation of the inositol pentaphosphate and haemoglobin peaks at high pH. (c) Dissociation of the complex by 2 *M* eluant electrolyte at neutral pH.

BENESCH AND BENESCH[23] have shown that the association of organic polyphosphates with both mammalian and avian haemoglobins has a considerable effect upon the oxygen affinity of the haemoglobin. In the case of diphosphoglyceric acid it is only the deoxyhaemoglobin which binds strongly to the phosphate under approximately physiological conditions. However the spectrum of the chicken and pigeon haemoglobin–polyphosphate complexes with pronounced bands at 538 and 577 nm indicates clearly that the inositol pentaphosphate binds strongly to the oxyhaemoglobin, and the binding of oxygen and inositol pentaphosphate to haemoglobin is not a mutually exclusive process as in the case of diphosphoglyceric acid[23].

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APPENDIX 3

GAS-LIQUID CHROMATOGRAPHY OF INOSITOL ACETATES

1. Introduction

One of the steps in a detailed analysis of inositol phosphates is the determination of the quantity and type of the inositol isomer(s) in the sample. The quantity of free inositol can be measured either indirectly, by determining the products from chemical oxidation of free inositol, or directly by microbiological assay. Separation of the inositols by paper or column chromatography is generally necessary to ascertain which isomers are present (Posternak, 1965).

Determinations of inositol by gas-liquid chromatography (GLC) of volatile trimethylsilyl (Sweeley, Bentley, Makita & Wells, 1963; Lee & Ballou, 1965; Ueno, Kurihara & Nakajima, 1967; Rumpf, 1969) or hexa-O-acetyl (Krzeminski & Angyal, 1962) derivatives are well known. However application of the technique to the detection of inositols from inositol polyphosphate fractions of soil extracts are rare (Cosgrove & Tate, 1963; Halstead & Anderson, 1970).

2. Methods

In this work suitable adaptations of the techniques developed by Oades (1967b; Oades, Kirkman & Wagner, 1970) were applied to the determination of inositol in hydrolysates of soil samples which contained inositol polyphosphates.

(a) Hydrolysis of samples -

Limited information was available on the best conditions to release inositol from its polyphosphate esters. Different conditions were used by Fleury (1946), Smith & Clark (1952) and Cosgrove (1963). A 40 hr hydrolysis in 6M-HCl at 110° appeared to be most commonly favoured (Cosgrove, 1963; Martin & Wicken, 1966; Halstead & Anderson, 1970).

To confirm the suitability of the above-mentioned conditions samples of highly purified Na and Ferric myoinositol hexaphosphate were hydrolysed in sealed-tubes with 6M-HCl (110°) ~~and~~ or 50% (v/v) NH_4OH (135°) over periods of 0 to 50 hr. (Myoinositol was also treated in the same way.) These hydrolysates were acetylated by heating for 10 min at 60° with acetic anhydride in the presence of conc H_2SO_4. The acetates were extracted with chloroform and the recovery of myoinositol was recorded by GLC of the acetates using sorbitol as the internal standard.

Figure 22 shows that strongly acidic conditions were best for hydrolysis. Although release of myoinositol was complete after 30 hr of hydrolysis with 6M-HCl in the case of sodium phytate (Fig. 22a) the optimum time for ferric phytate was 40 hr (Fig. 22b). The interaction between release and degradation of myoinositol became important after 40 hr of hydrolysis for the Fe salt (Fig. 22b and c). Oxalate electrophoresis of the H^+ form of myoinositol hexaphosphate which was prepared from the Fe phytate before and after 40 hr hydrolysis in 6M-HCl at 110°, confirmed that there had been complete release of P_i from ferric myoinositol hexaphosphate (IP_6, Fig. 23).

It was assumed that the conditions suitable for the complete release of myoinositol by hydrolysis of myo-inositol hexaphosphate would also apply to the polyphosphates of the other inositol isomers (cf. Cosgrove, 1966b for enzymic dephosphorylation). Soil samples (10 g) were thus refluxed with 6M-HCl (150 ml) at 110° for 40 hr.

(b) Preparation of inositol acetates -

The hydrolysates were cooled and an internal standard was added. The samples were centrifuged in plastic tubes and the HCl in the supernatant was removed by rotary film evaporation. The hydrolysates were acetylated either by

Figure 22

INOSITOL CONTENT DURING HYDROLYSIS.

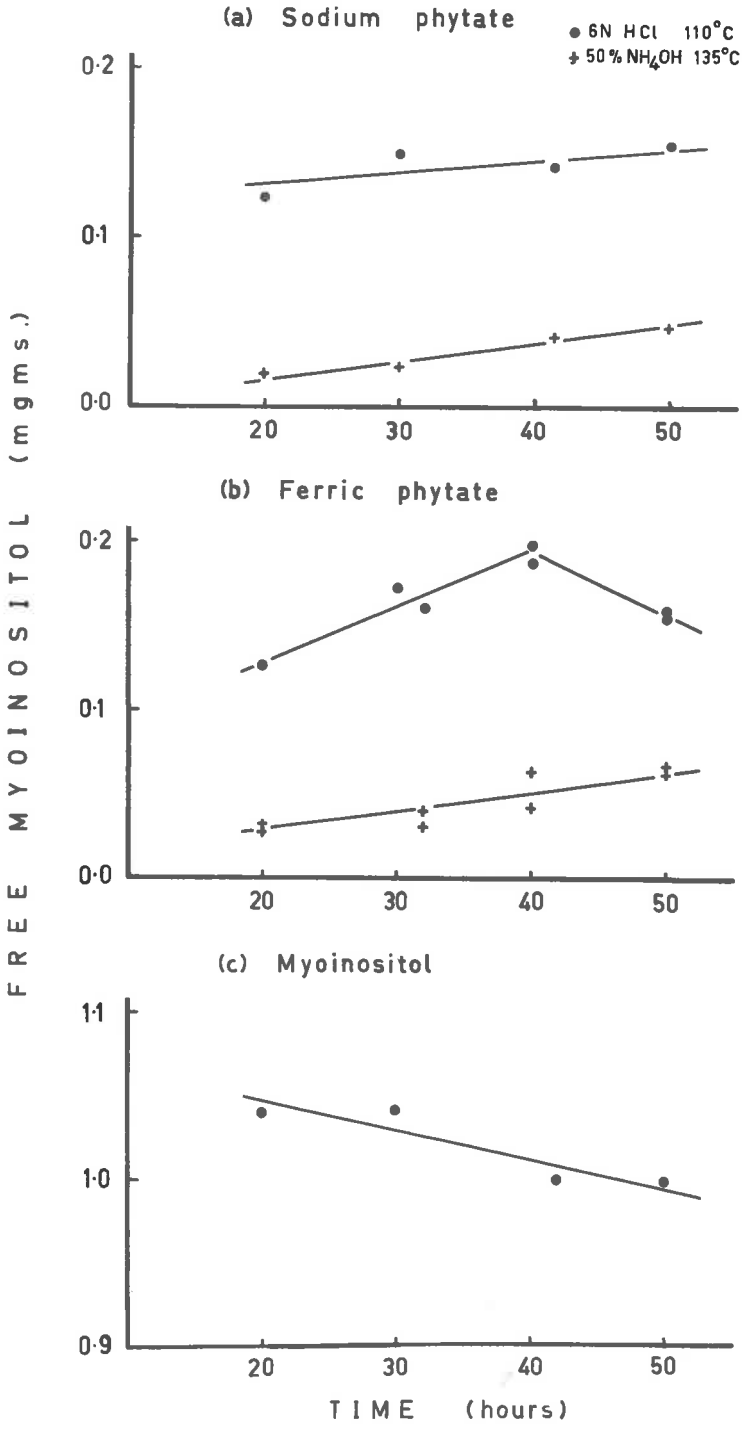


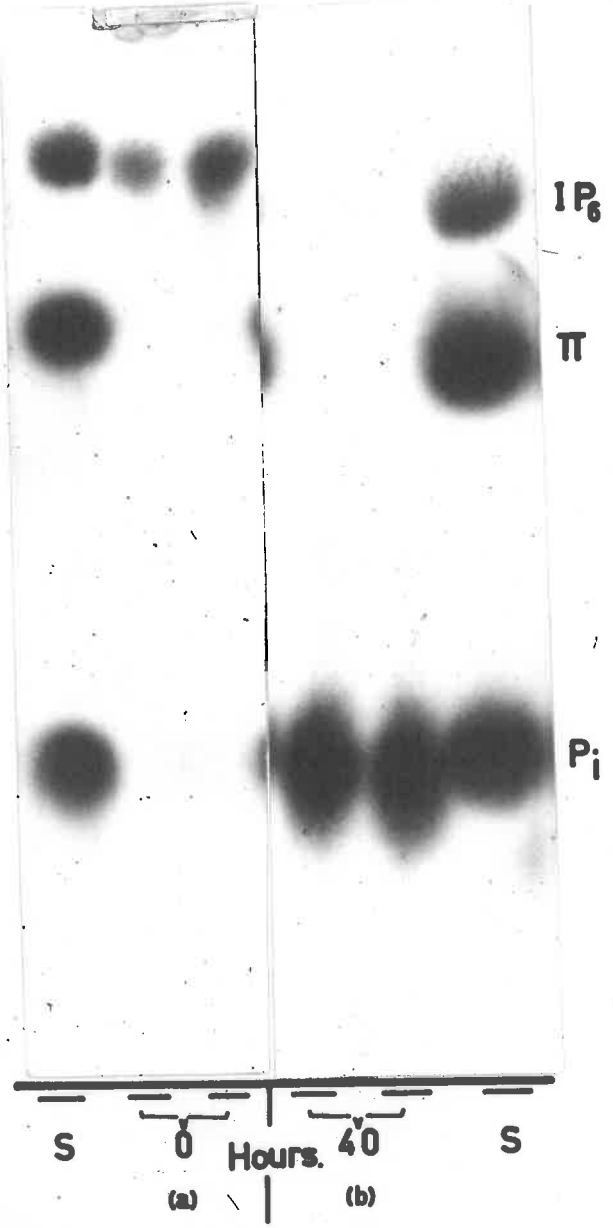
FIGURE 23.

The degradation of ferric phytate (IP_6) after hydrolysis for 40 hr in 6M-HCl (110°).

Fig 23.

ELECTROPHORESIS OF IP_6.

(a) Before (b) After HYDROLYSIS



S = Standards.

π = Pyrophosphate.

standing overnight in acetic anhydride - redistilled, dry pyridine (1:1 v/v, 70 ml) or by transferring the sample to 12.5 x 1.6 cm Kimax screw-top test tubes, drying in an air stream at 60° and then heating for 2 hr at 130° with 10 ml acetic anhydride and 5 mg anhydrous Na acetate.

After acetylation the mixtures were dried by evaporation with a stream of air whilst simultaneously heating the samples in a water-bath at 60°. The inositol acetates extracted from the reaction mixture by chloroform were filtered under pressure through a small column (5 x 20 mm) of silica gel G using chloroform as eluant (Oades et al., 1970). The chloroform filtrates were evaporated to dryness.

(c) Gas chromatography -

The inositol acetates were dissolved in chloroform and injected into a Perkin Elmer 801 gas chromatograph with dual flame-ionisation detector. The glass columns (1.84 m x 3.2 mm internal diam) were packed with 10% (w/w) ECNSS-M on Embacel (60-100 mesh). The temp was programmed from 140° to 220° @ 1.7°/min, carrier gas flow-rate was 40 ml N_2/min and the temp of the detector and injection port was 250° and 200° respectively.

Identification of the inositols was based on comparison with the retention times on the gas chromatograph of known inositol acetates. Positive identification of the inositols could be made by collecting each peak from the column and examining the acetates (Tate & Bishop, 1962) or the de-acetylated material (Angyal, McHugh & Gilham, 1957).

3. Results

GLC of hexa-O-acetyl derivatives of the standard inositols showed that neither the *neo*- and D(*chiro*)-isomers, nor the *scyllo*- and *epi*- isomers separated from each other under the particular conditions of operation (Fig. 24(a) and (b), cf. Krzeminski & Angyal, 1962).

The traces shown in Figure 24(c), (d) and (e) included sorbitol acetate as the internal standard. Use of quebrachitol as an internal standard (Oades *et al*., 1970) was avoided because when heated in the presence of HCl (Section (b), page 102) it was demethylated to D(*chiro*)-inositol. (Xylitol had a retention time of 30 min and would have been a suitable alternative to sorbitol.) The sorbitol peaks represent 100 ppm in Figure 24 and, assuming a 1:1 response for equal amounts of sorbitol and the inositols, the inositol content of each soil is shown in Table 21.

FIGURE 24.

Gas-liquid chromatography of alditol acetates, and the quantitation of inositols released after hydrolysis in the whole soil.

Trace: (a), (b) Standard compounds

(c) Soil U29

(d) Soil U17

(e) Soil U1

Standard acetates:

1. Glycerol
2. neoinositol
3. D(chiro)-inositol
4. Sorbitol (internal standard)
5. myoinositol
6. scylloinositol
7. epiinositol

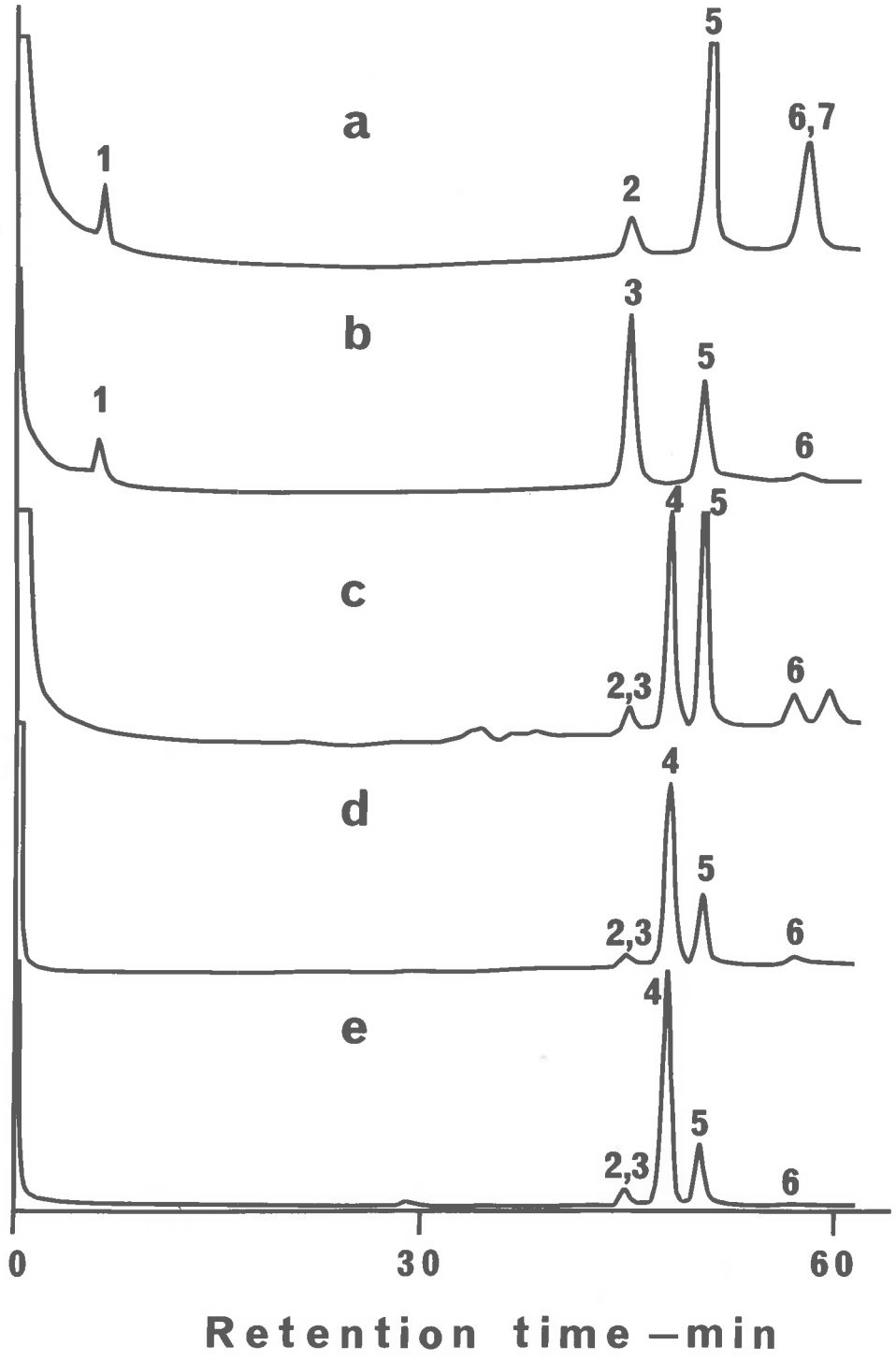

a
1
2
5
6,7
b
1
3
5
6
c
2,3
4
5
6
d
2,3
4
5
6
e
2,3
4
5
6
0
30
60
Retention time –min

TABLE 21.

The inositol contents of four Urrbrae soils, determined by gas-liquid chromatography

Soil	Inositol content (ppm)				Inositol polyphosphates
	D(*chiro*)- + *neo*-	*myo*-	*scyllo*-	Total	(ppm P)*
U 1	6.0	25.3	1.6	32.9	13
16	8.3	61.0	7.9	77.2	29
17	5.4	35.4	3.7	44.5	25
29	8.3	140.6	20.3	169.2	57

* From Figure 10.

Since inositol and phosphorus make similar contributions to the molecular weight of an inositol hexaphosphate (180.2 and 185.9 respectively) the results of Table 21 indicated that considerable amounts of inositol in these soils were not phosphorylated. (Electrophoresis of the inositol polyphosphates from Urrbrae soils had not revealed any lower inositol polyphosphates). Free *myo*-inositol is ubiquitous (Posternak, 1965), therefore these results were not surprising.

It was concluded that the GLC of the inositol present after hydrolysis of whole soils may not be a true indication of the *myo*inositol polyphosphate status of the soil; on the other hand the method is probably a reliable indication of the polyphosphates of the other inositol

isomers which do not exist widely as free compounds. The method can be applied to purified fractions of inositol polyphosphates or to any materials for which information about the inositol content is required (see page 15).

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