CHROM. 5446

GEL CHROMATOGRAPHY OF SOIL ORGANIC PHOSPHORUS

J. H. STEWARD AND M. E. TATE
Department of Agricultural Biochemistry and Soil Science, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia (Australia)

(Received May 5th, 1971)

SUMMARY

Fractionation of alkaline extracts of soil on cross-linked dextran gels (Sephadex G-25 and G-50) was achieved by elution with 0.1 *M* NaOH. The inositol polyphosphates gave a distribution coefficient (K_d value) of 0.39 on Sephadex G-50 in alkali and were eluted after an excluded organic phosphate ($K_d = 0$) and before a fraction ($K_d = 0.65$) which consisted mainly of inorganic phosphate.

Chromatography on Sephadex G-50 at high pH gave a rapid measurement of the contribution of each particular fraction to the organic phosphorus extracted from the soil and showed the excluded phosphate to be the major organic phosphorus component in the soils which were examined. The method has been employed to follow the partial degradation by acid hydrolysis of the excluded organic phosphate fraction. A reducing sugar phosphate was observed among the main degradation products of the excluded phosphate and it yielded a characteristic absorption spectrum with the carbazole reagent for uronic acids.

INTRODUCTION

The known organic phosphorus constituents of soil have recently been reviewed by ANDERSON[1], who pointed out that in some soils more than 50% of the organic phosphorus is present as inositol phosphates. However, there is an increasing amount of evidence[2–5] which shows that, in the majority of soils, 60–90% of the organic phosphorus is present in forms other than the inositol polyphosphates (tetra-, penta- and hexaphosphates). In order to study this unidentified organic phosphorus fraction a rapid means for assessing both it and the inositol polyphosphates is essential.

Our earlier study[6] on the gel chromatographic behaviour of inositol polyphosphates, and particularly the dissociation of the chicken blood–haemoglobin–inositol pentaphosphate complex at high pH, led us to examine the gel chromatographic fractionation of alkaline extracts of soil. It has been found that, in contrast to gel chromatographic separations using distilled water[7] and dilute acid[5], when Sephadex G-50 is eluted with 0.1 *M* NaOH, the inositol polyphosphates are resolved from excluded coloured materials. The method provides a rapid and convenient procedure for fractionating and measuring organic phosphorus in extracts from soil.

EXPERIMENTAL

Materials

Sephadex G-25 (100–300 μ) and G-50 (coarse grade: 100–300 μ; medium grade: 50–150 μ) (Pharmacia AB) were preswollen in 0.1 *M* NaOH (24 h).

The soil samples used in this study will be described elsewhere[8].

Methods

Soil extracts. All extracts were prepared by ultrasonic vibration of soil for 3 min in 0.5 *M* NaOH (ref. 8).

For large-scale work, batches of soil (10 g) were extracted in plastic centrifuge tubes (100 ml capacity). Analytical-scale separations were made on aliquots of the large-scale extracts or on extracts prepared by the same procedure using soil (0.5–2.0 g) and 0.5 *M* NaOH (6 ml) in flat-bottom polythene vials (20 ml capacity).

Gel chromatography. Large columns containing preswollen Sephadex G-25 (60 × 5.4 cm diam.) and coarse grade Sephadex G-50 (42.5 × 4.2 cm diam.) were prepared. They were equilibrated and eluted with 0.1 *M* NaOH; 14.5 ml fractions were collected.

For analytical-scale work, a column of 1.6 cm diam. was packed. It consisted of a bottom layer of Sephadex G-50 coarse (17.5 cm) covered by Sephadex 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[9]. The column was equilibrated and eluted with 0.1 *M* NaOH. (Medium and coarse grade Sephadex G-50 gels gave the same K_d values for any particular phosphate). The column effluent was collected in 1.1 ml fractions using a Pleuger Model J drop-counting fraction collector (flow rate 0.275 ml/min).

Chromatography of the soil extracts at high pH resulted in some discolouration of the gel beds, but this did not interfere with subsequent fractionations.

Analytical methods. Distribution coefficients ($K_d = (V_e - V_0)/V_i$, where V_e is the elution volume of the solute, V_0 is the void volume and V_i is the volume of the stationary phase of the gel) were determined for *myo*-inositol hexaphosphate, inorganic phosphate and aluminate on the large Sephadex G-25 and G-50 columns. Tritiated water and Blue Dextran 2000 (Pharmacia AB) were used to determine V_i and V_0, respectively.

Organic phosphorus was determined as the difference between total phosphorus[10], using perchloric acid for the digestions[11] and inorganic phosphorus[12] in the sample. Colour due to organic matter was measured as absorbance at 450 nm (1 cm cell).

Gas chromatography of inositol acetates. Inositol polyphosphates were dephosphorylated by prolonged hydrolysis (6 *M* HCl, 40 h, 110°). After concentration to dryness, the residue was acetylated with excess acetic anhydride–sodium acetate (130°/2 h) and chromatographed by the procedure of OADES for alditol acetates[13].

Electrophoresis. Barium salts were converted to the free acids with Dowex 50 (H^+) and were examined by the routine electrophoretic procedure of TATE[14] using 0.1 *M* oxalate buffer at pH 1.5.

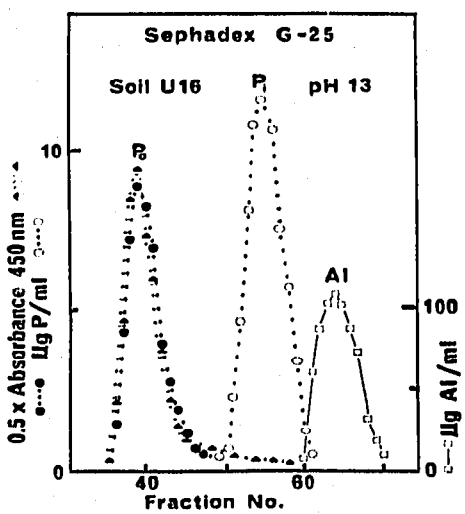
RESULTS

Fractionation with Sephadex G-25

Fig. 1 shows the behaviour of a crude NaOH-extract of the Urrbrae soil U16, using Sephadex G-25 eluted with 0.1 *M* NaOH. The inorganic phosphate (K_d = 0.30) and the aluminate ion (K_d = 0.47) were clearly resolved from the bulk of the organic phosphorus (K_d = 0–0.15) which was mainly excluded from the gel. Very little organic phosphorus was present in the inorganic fraction (tubes 47–62).

Fractionation of the excluded material from Sephadex G-25 with Sephadex G-50

Fig. 2 shows the further fractionation on Sephadex G-50 of the excluded organic



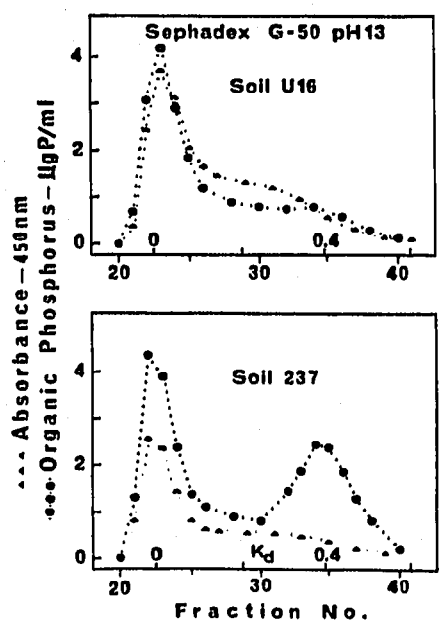


Fig. 1. Separation of an alkaline extract of soil on Sephadex G-25 in 0.1 *M* NaOH into organic phosphorus (P_o), inorganic phosphorus (P_i) and aluminate (Al).

Fig. 2. Sephadex G-50 fractionation in 0.1 *M* NaOH of the excluded organic phosphorus from a G-25 separation. Inositol polyphosphates were isolated from tubes 32–37 in both soils. Soil 237 has a particularly high inositol polyphosphate content.

phosphorus peak from soil U16 and compares it with a soil of high inositol polyphosphate content (237). In both cases, material of K_d = 0.39 (tubes 32–37) coincided with the peak for authentic *myo*-inositol hexaphosphate when chromatographed either alone or in admixture with extracts from soil.

The material in tubes 32–37 of Fig. 2 was isolated as the barium salt and, after conversion to the free acid, was examined by electrophoresis at pH 1.5. The results for the soils, examined to date have been similar and are listed in Table I, which shows that the main components correspond in mobility to *myo*-inositol hexaphosphate, *chiro*-inositol hexaphosphate and *scyllo*-inositol hexaphosphate. Phosphates with the mobilities of inositol penta- and tetraphosphates were also observed and are listed, but material corresponding to lower degrees of phosphorylation were absent.

TABLE I

ELECTROPHORETIC MOBILITIES ($M(PP_i)$) RELATIVE TO INORGANIC PYROPHOSPHATE OF ORGANIC PHOSPHATES FROM ALKALINE EXTRACTS OF SOIL AND SOME REFERENCE PHOSPHATES IN 0.1 *M* OXALATE BUFFER pH 1.5

	$MPP_i \pm 0.02$
Standards	
Inorganic pyrophosphate	1.00
scyllo-Inositol hexaphosphate	1.41
myo-Inositol hexaphosphate	1.28
D-*chiro*-Inositol hexaphosphate	1.26
neo-Inositol hexaphosphate	1.20
α-Glycerophosphate	0.56
β-Glycerophosphate	0.55
Glucose-6-phosphate	0.47
Inorganic orthophosphate	0.30
Inositol polyphosphates of K_d = 0.39 on Sephadex G-50	
1	1.40[a]
2	1.28[a]
3	1.26[a]
4	1.18
5	1.13
6	1.08
7	1.04
Phosphates in a partial hydrolysate of the G-25 excluded phosphate (K_d = 0) from soil U29	
1 Non-reducing	0.88
2 Non-reducing	0.79
3 Non-reducing	0.71
4 Non-reducing	0.56[a]
5 Reducing	0.47[a]
6 (P_i)	0.28[a]

[a]Major components.

The nature of the inositols in tubes 32–37 was confirmed by gas–liquid chromatography (GLC) after dephosphorylation and acetylation of this fraction[13].

Fractionation with Sephadex G-50 alone, for routine analysis

For preliminary surveys of the organic phosphorus of soils satisfactory separations of the NaOH-extracts were made with as little as 5 μg of organic phosphorus, providing the extract contained at least 20 μg organic phosphorus/ml. A single G-50 column separated the inositol polyphosphate (K_d = 0.39) from the excluded phosphorus (K_d = 0) and the inorganic phosphate fraction (K_d = 0.65). Occasionally the latter fraction contained small amounts of organic phosphates which were less than 2% of the total organic phosphorus in the extract.

The contribution of the inositol polyphosphates to the total organic phosphorus present in the extract was calculated from the phosphorus content of the fractions corresponding to authentic *myo*-inositol hexaphosphate (K_d = 0.39). Results for eight soils are listed in Table II and in the case of two soils (237 and 315) the values are compared with the inositol polyphosphate values obtained by WILLIAMS AND ANDERSON[2] for the same soils. Soils ROB and 350 are compared with results obtained by WILLIAMS AND ANDERSON[2] for soils 531 and 348, respectively, which were obtained

TABLE II

SOIL ORGANIC PHOSPHORUS FRACTIONS ON SEPHADEX G-50 (pH 13)

Soil	*Excluded organic phosphate (K_d = 0)*		*Inositol polyphosphates (K_d = 0.39)*		
			This work		*Literature values*[2]
	% P_o[a]	*p.p.m. soil*	% P_o[a]	*p.p.m. soil*	*p.p.m. soil*
U1	81	57	19	13	—
U16	77	97	23	29	—
U17	77	82	23	25	—
U29	69	126	31	57	—
237	80	480	20	120	118
315	73	797	27	290	356
350	98	559	2	10	2[b]
ROB	75	686	25	230	4[b]

[a] P_o = organic phosphorus.
[b] These figures refer to WILLIAMS AND ANDERSON[2] soils 348 and 531 which were obtained from the same sampling sites as soils 350 and ROB, respectively.

from the same sampling site. In three cases, the agreement was reasonable, but the values obtained by the gel chromatographic procedure for ROB indicated a very much higher inositol polyphosphate content than that reported by WILLIAMS AND ANDERSON for corresponding soil 531. Electrophoresis of the phosphates present confirmed that the peak contained substantial amounts of *myo*- and *scyllo*-inositol polyphosphates and the reason for such differing values for similar samples is not clear.

Nature of the organic phosphorus excluded on Sephadex G-50

Table II shows that the major organic phosphorus component in the soils examined is excluded on Sephadex G-50 in 0.1 *M* NaOH. Studies on the sample from the Urrbrae U16 soil showed that the bulk of the material excluded from Sephadex G-50 was also excluded from Sephadex G-75, G-100 and G-200 when eluted with 0.1 *M* NaOH.

Table II also shows that the maximum amount of the excluded phosphate in the Urrbrae soils was present in the U29 sample (continuous pasture) and extracts from this soil were then used for further studies. Precipitation by acidification to pH 0.2 removed much of the colour[15] ("humic acids") from the excluded material on Sephadex G-25 without significant loss of organic phosphorus from the supernatant ("fulvic fraction"). Neither was there any detectable alteration in K_d value on Sephadex G-50 eluted with 0.1 *M* NaOH, providing the freshly prepared "fulvic acid" was immediately neutralized to pH 7. Very little of the organic phosphorus in this "fulvic fraction" could be adsorbed onto Dowex 1X2 resin after desalting on Sephadex G-10.

Gel chromatography on Sephadex G-50 in 0.1 *M* NaOH was then used to follow the partial acid hydrolysis (1 *M* HCl, 110°) of this excluded "fulvic fraction". Complete hydrolysis to inorganic phosphate was achieved in 7 h under the same conditions.

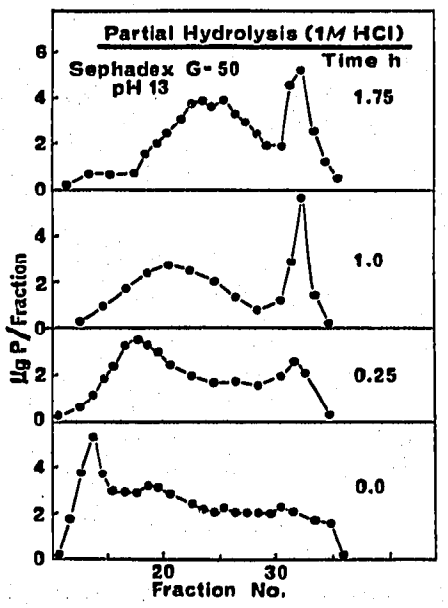


Fig. 3. Sephadex G-50 fractionations of stages during the partial acid hydrolysis (1 *M* HCl/100°) of the excluded organic phosphorus from Sephadex G-25 (soil U29). The final peak which gradually appears at tube 33 is predominantly inorganic phosphate.

Fig. 3 shows the gradual increase in K_d value and the concomitant release of inorganic phosphate (P_i) in the early stages of the hydrolysis. After 1¾ h hydrolysis (30% P_i release) the bulk of the material was partly included in the gel ($K_d = 0.4$). At this stage the hydrolysis mixture was neutralised and desalted on Sephadex G-10, prior to adsorption of the acidic components on Dowex 1X2 (Cl^-) resin. Analysis showed that all of the phosphate was now retained by the resin. Neutral carbohydrates were eluted with water and the acidic components were then desorbed by elution with 1 *M* HCl. The organic phosphorus content of the eluate, equivalent to 40% of the organic phosphorus in the original extract, was then examined by electrophoresis. The detectable phosphates are listed in Table I.

The phosphate of $M(PP_i) = 0.47$ gave a pinkish brown spot with the *p*-anisidine·HCl reagent[16] for reducing sugars and a light blue spot with the naphthoresorcinol dip for uronic acids[17]. Although the latter reagent is not specific for uronic acids, elution of this phosphate ($M(PP_i) = 0.47$) and reaction with the carbazole reagent of BITTER AND MUIR[18] gave a characteristic spectrum with absorption maxima at 530, 570 and 700 nm. The glucuronic acid standard showed absorption maxima at 490, 530 and 560 nm.

The remaining products of the partial hydrolysis consisted of inorganic phosphate and series of non-reducing phosphates which have not been positively identified. The mobility of the major non-reducing phosphate ($M(PP_i) = 0.56$) is similar to the mobilities of α and β glycerophosphates ($M(PP_i) = 0.58, 0.55$).

DISCUSSION

symmetrical peaks are still obtained using 0.1 *M* alkali. The increasing pore size and concomitant reduction of the anion exclusion effect with the more loosely cross-linked Sephadex G-50 gel results in the partial inclusion of the inositol polyphosphates, as shown in Fig. 2.

Sephadex G-50 chromatography under alkaline conditions clearly offers a useful alternative to humic acid precipitation and hypobromite oxidation for the removal of extraneous organic matter[1,15] during the isolation of inositol polyphosphates. Direct desalting of the Sephadex G-50 fractions containing the inositol polyphosphate peak gave material containing much carbohydrate and was unsuitable for electrophoresis. However, precipitation of the phosphates as barium salts removed much of the interfering material. The free acids from these barium salts gave satisfactory electrophoretograms. For preparative studies of inositol polyphosphates and the excluded phosphate, it is desirable to remove the inorganic phosphate and aluminate using Sephadex G-25, as in Fig. 1, prior to chromatography with G–50.

For routine surveys rapid measurements of the excluded phosphate and of the inositol polyphosphate fraction can be made with a single G-50 column. Any inorganic phosphate which may be present occurs as a peak which follows the inositol polyphosphates. The degree of resolution obtained is dependent to some extent on the sample size to column ratio, and the loading technique[9] becomes more important with the smaller columns.

Results obtained by the G-50 survey procedure are listed in Table II and these emphasize the important contribution made to the organic phosphorus by phosphates other than the inositol polyphosphates, a point which has also been mentioned by workers using other methods[5,7,19–22].

Attention is drawn to the fact that the phosphorus pattern obtained on G-50 is markedly dependent on the completeness with which the organic phosphorus is extracted from soil. For example, Fig. 2 shows the percentage of phytate *in the extract* of soil 237 (soil–solution ratio, 5:1) as approximately 38%. However, the more effective extraction procedure (solution–soil ratio, 12:1) used for soil 237 in Table II gave a figure of 20%. It is clear that the excluded material is less easily extracted than the inositol polyphosphates.

Although exclusion from a gel is not always a consequence of size and may also be due to a charge exclusion, the failure of the excluded phosphate from the Sephadex G-50 fractionation to be adsorbed onto an anion-exchange resin suggested that it was a phosphorylated macromolecule whose size prevented penetration to the adsorption sites of the resin. This hypothesis was verified by partial degradation of the phosphate with acid (Fig. 3), so that the sample was obtained in depolymerized form suitable for adsorption on anion-exchange resins.

The phosphorylated fragments from the partial hydrolysis represented 40% of the original organic phosphorus in the extract. Electrophoresis revealed that the two major components were a non-reducing phosphate ($M(PP_i) = 0.56$) and a reducing sugar phosphate ($M(PP_i) = 0.47$), whose properties suggest a monophosphorylated uronic acid, uronolactone or a related compound.

Both of these phosphates ($M(PP_i) = 0.56$ and 0.47) have been detected in partial hydrolysates of soils U29, U16, 273 and 350, and the presence of phosphorus in a polysaccharide fraction containing uronic acids has been mentioned by McKERCHER[22]. A closer examination of these compounds and other partial hydrolysis

fragments may give an insight into the nature of the excluded organic phosphate from alkaline extracts of soil.

ACKNOWLEDGMENTS

Financial support of this work by the Commonwealth Wheat Industry Research Council is gratefully acknowledged. One of us (J.H.S.) has held a Commonwealth Post-Graduate Scholarship.

Thanks are due to Dr. D. J. COSGROVE and C. H. WILLIAMS of the C.S.I.R.O. Division of Plant Industry, Canberra, for reference samples of *scyllo-*, *neo-* and *chiro-*inositol hexaphosphates and the four eastern Australian soil samples (237, 315, 350 and ROB).

REFERENCES

1 G. ANDERSON, in A. DOUGLAS MCLAREN AND G. H. PETERSON (Editors), *Soil Biochemistry*, Edward Arnold, London, 1967, p. 67.
2 C. H. WILLIAMS AND G. ANDERSON, *Austr. J. Soil Res.*, 6 (1968) 121.
3 R. B. MCKERCHER AND G. ANDERSON, *Can. J. Soil Sci.*, 19 (1968) 302.
4 J. K. MARTIN, *N.Z. J. Agr. Res.*, 13 (1970) 522.
5 T. I. OMOTOSO AND A. WILD, *J. Soil Sci.*, 21 (1970) 224.
6 J. H. STEWARD AND M. E. TATE, *J. Chromatrogr.*, 45 (1969) 400.
7 J. R. MOYER AND R. L. THOMAS, *Soil Sci. Soc. Amer. Proc.*, 34 (1970) 80.
8 J. H. STEWARD AND J. M. OADES, personal communication.
9 H. DETERMANN, *Gel Chromatography*, 2nd Edn., Springer-Verlag, Berlin-Heidelberg-New York, 1969, p. 46.
10 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
11 D. A. USHER, *J. Chromatogr.*, 12 (1963) 262.
12 M. K. JOHN, *Soil Sci.*, 109 (1970) 214.
13 J. M. OADES, *Austr. J. Soil Res.*, 5 (1967) 103.
14 M. E. TATE, *Anal. Biochem.*, 23 (1968) 141.
15 G. ANDERSON, *J. Soil Sci.*, 12 (1961) 276.
16 L. HOUGH AND J. K. W. JONES, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 28.
17 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann, London, 1960.
18 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.
19 R. L. THOMAS AND D. L. LYNCH, *Can. J. Soil Sci.*, 40 (1960) 113.
20 J. F. DORMAAR, *Can. J. Soil Sci.*, 43 (1963) 235.
21 J. K. MARTIN, *N.Z. J. Agr. Res.*, 7 (1964) 723.
22 R. B. MCKERCHER, in J. M. HOLMES (Editor), *9th Int. Congress Soil Sci. Trans., Adelaide, South Australia, 1968*, Elsevier, New York, N.Y., 3 (196 547.