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USE OF *p*-NITROPHENYL PHOSPHATE FOR ASSAY OF SOIL PHOSPHATASE ACTIVITY

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Summary—A simple method of assaying soil phosphatase activity is described. It involves colorimetric estimation of the *p*-nitrophenol released by phosphatase activity when soil is incubated with buffered (pH 6·5) sodium *p*-nitrophenyl phosphate solution and toluene at 37° C for 1 hr. The method is rapid and precise, and it has significant advantages over methods previously proposed for assay of soil phosphatase activity.

INTRODUCTION

SEVERAL methods have been proposed for estimation of the phosphatase activity of soils (for review, see Skujiņš, 1967). The basic differences in these methods are in the substrate used and in the technique employed to measure hydrolysis of the substrate by phosphatase enzymes. Early workers measured phosphatase activity by estimating the inorganic phosphate released by incubation of soil with organic phosphates, but such methods are unsatisfactory in that they do not allow for fixation of inorganic phosphate by soil constituents (see Skujiņš, 1967). Kroll and Kramer (1955) estimated soil phosphatase activity by determining the phenol released by incubation of soil with phenyl phosphate, and this substrate has been used in several investigations of soil phosphatase activity (e.g. Kramer, 1957; Kramer and Yerdei, 1959; Halstead, 1964). Skujiņš *et al.* (1962) assayed soil phosphatase activity by a procedure in which the amount of glycerophosphate hydrolyzed by incubation of soil with this organic phosphate is estimated by analyses for extractable total and inorganic phosphorus after incubation. This method is tedious and time consuming, and data reported by Skujiņš *et al.* indicate that it has low precision. Ramirez-Martinez and McLaren (1966) recently proposed a method involving fluorimetric assay of the β-naphthol released by incubation of soil with β-naphthylphosphate, but this method is complicated by sorption of β-naphthol by soil constituents and requires that the capacity of each soil analyzed to sorb β-naphthol be determined and allowed for in calculation of results.

We required a method of assaying phosphatase activity for studies of the enzymatic activities of the sand-, silt-, and clay-size fractions of soils and concluded from evaluation of the literature that the phenyl phosphate method proposed by Kramer and Yerdei (1959) was the most satisfactory method available. It was found, however, that the procedure used in this method to extract the phenol released by phosphatase activity did not give quantitative recovery of phenol added to some of our soils and that the colorimetric technique used for estimation of phenol was complicated by instability of the color developed for this analysis. We therefore studied the use of other substrates for estimation of soil phosphatase activity, and these studies showed that the method described here, which involves use of *p*-nitrophenyl phosphate as substrate, permits rapid and precise assay of soil phosphatase activity and has none of the observed defects of previous methods. This new method involves colorimetric estimation of the *p*-nitrophenol released when soil is

incubated with buffered (pH 6·5) sodium *p*-nitrophenyl phosphate solution and toluene at 37° C for 1 hr. The procedure used to extract the *p*-nitrophenol released by phosphatase activity develops the stable color used to estimate this phenol, and it gives quantitative recovery of *p*-nitrophenol added to soils.

MATERIALS

The soils used (Table 1) were surface (0- to 15-cm) samples selected to obtain a wide range in pH and texture. Before use, each sample was air-dried and crushed to pass a 2-mm screen. In the analyses reported in Table 1, pH was determined by a glass electrode (soil: water ratio, 1:2·5), organic carbon by the method of Mebius (1960), and particle-size distribution by pipette analysis (Kilmer and Alexander, 1949) after dispersion by Na-saturated Amberlite IRC-50 resin (Edwards and Bremner, 1965).

TABLE 1. ANALYSES OF SOILS

Soil					
No.	Series	pH	Organic carbon	Clay	Sand
			%	%	%
1	Marshall	5·9	1·85	33	2
2	Lindley	6·0	1·44	19	37
3	Sharpsburg	6·0	2·54	33	2
4	Edina	6·2	1·95	25	1
5	Judson	6·6	2·95	45	1
6	Shelby	6·7	2·46	26	33
7	Grundy	6·8	2·67	30	3
8	Ida	8·0	1·57	27	3

METHOD FOR ASSAY OF PHOSPHATASE ACTIVITY

Reagents

Modified universal buffer (MUB), pH 6·5—Prepare as described by Skujiņš *et al.* (1962).

Toluene—Fisher certified reagent (Fisher Scientific Co., Chicago, Illinois).

p-Nitrophenyl phosphate (PNP) solution, 0·115M—Dissolve 1·927 g of disodium *p*-nitrophenyl phosphate tetrahydrate (Sigma 104, Sigma Chemical Co., St. Louis, Missouri) in MUB, and dilute the solution to 50 ml with MUB. Store the solution in a refrigerator.

Calcium chloride, 0·5M—Dissolve 73·5 g of $CaCl_2.2H_2O$ in water, and dilute the solution to 1 l.

Sodium hydroxide, 0·5M—Dissolve 20 g of NaOH in water, and dilute the solution to 1 l.

Standard *p*-nitrophenol solution—Dissolve 1·0 g of *p*-nitrophenol in water, and dilute the solution to 1 l. Store the solution in a refrigerator.

Procedure

Place 1 g of soil (<2 mm) in a 50-ml Erlenmeyer flask, add 4 ml of MUB, 0·25 ml of toluene and 1 ml of PNP solution, and swirl the flask for a few seconds to mix the contents. Stopper the flask and place it in an incubator at 37° C. After 1 hr, remove the stopper, add 1 ml of 0·5M calcium chloride and 4 ml of 0·5M sodium hydroxide, swirl the flask for a

few seconds, and filter the soil suspension through a Whatman No. 12 folded filter paper. Transfer the filtrate to a Klett–Summerson colorimeter tube and measure its yellow color intensity with a Klett–Summerson photoelectric colorimeter fitted with a blue (No. 42) filter. Calculate the *p*-nitrophenol content of the filtrate by reference to a calibration graph plotted from the results obtained with standards containing 0, 10, 20, 30, 40 and 50 μg of *p*-nitrophenol. To prepare this graph, dilute 1 ml of the standard *p*-nitrophenol solution to 100 ml in a volumetric flask and mix the solution thoroughly. Then pipette 0, 1, 2, 3, 4 and 5-ml aliquots of this diluted standard solution into small flasks, adjust the volumes to 5 ml by addition of water, and proceed as described for *p*-nitrophenol analysis of the incubated soil sample. If the color intensity of the filtrate exceeds that of the 50 μg of *p*-nitrophenol standard, an aliquot of the filtrate should be diluted with water until the colorimeter reading falls within the limits of the calibration graph.

Controls should be performed with each soil analyzed to allow for color not derived from *p*-nitrophenol released by phosphatase activity. To perform controls, follow the procedure described for assay of phosphatase activity but make the addition of 1 ml of PNP solution after the additions of 0·5M $CaCl_2$ and 0·5M NaOH (i.e. immediately before filtration of the soil suspension).

Any colorimeter or spectrophotometer that permits color intensity measurements at 400–420 mμ can be used for colorimetric analysis of the soil filtrate. The maximum absorption of the color measured is at 400 mμ (Bessey *et al.*, 1946), and the color is stable for at least 24 hr. Calibration graphs prepared from standard *p*-nitrophenol solutions as described are highly reproducible and are identical to those obtained when these standards are prepared in MUB instead of water.

RESULTS AND DISCUSSION

Development of method

The colorimetric procedure used for estimation of *p*-nitrophenol in the method described depends upon the fact that alkaline solutions of this phenol have a yellow color (acid solutions of *p*-nitrophenol and acid or alkaline solutions of *p*-nitrophenyl phosphate are colorless). The $CaCl_2$–NaOH treatment adopted for extraction of *p*-nitrophenol after incubation develops the yellow color used to estimate this phenol, and tests with a variety of soils showed that it stopped phosphatase activity and gave quantitative recovery of *p*-nitrophenol (the recovery of 500 ppm of *p*-nitrophenol added to the soils listed in Table 1 ranged from 99·3 to 100·2%). It is necessary to add $CaCl_2$ to prevent dispersion of clay and extraction of soil organic matter during the treatment with NaOH (dispersion of clay complicates filtration, and the dark-colored organic matter extracted by NaOH interferes with colorimetric analysis for *p*-nitrophenol).

The control analysis is so designed that it allows for the presence of trace amounts of *p*-nitrophenol in some commercial samples of *p*-nitrophenyl phosphate and for extraction of trace amounts of colored soil material by the $CaCl_2$–NaOH treatment used for extraction of *p*-nitrophenol. No chemical hydrolysis of *p*-nitrophenyl phosphate could be detected under the conditions of this treatment.

Tests with soils 4, 5 and 7 showed that the results obtained by the method described were not affected if the soil samples analyzed were treated with 500 ppm of *p*-nitrophenol before incubation.

Choice of buffer pH in the method described was based on studies showing that maximal enzymatic hydrolysis of *p*-nitrophenyl phosphate occurred when pH 6·5 buffer was used (Fig. 1). Previous studies using other substrates (e.g. Rogers, 1942; Skujiņš *et al.*, 1962;

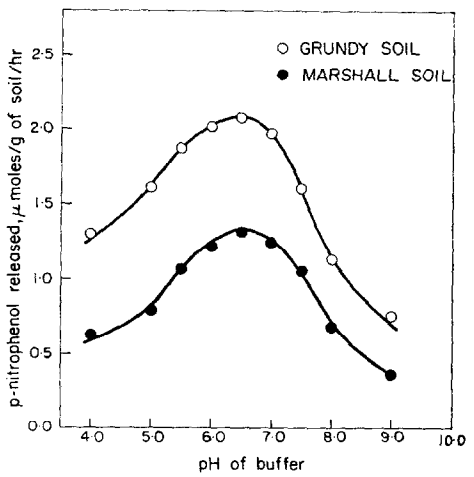

FIG. 1. Effect of pH of buffer on release of *p*-nitrophenol in assay of soil phosphatase activity by method described. Buffer (MUB) was adjusted to desired pH as described by Skujiņš *et al.* (1962). Substrate was 0·0115M sodium *p*-nitrophenyl phosphate.

Halstead, 1964; Ramirez-Martinez and McLaren, 1966) have indicated that most soils exhibit maximal phosphatase activity near neutral pH (6·2–7·0).

Figure 2 shows results obtained in studies of the effect of varying the time of incubation in the method described. The observed linear relationship between time of incubation and amount of *p*-nitrophenol released is evidence that the method proposed measures enzymatic hydrolysis of *p*-nitrophenyl phosphate and that phosphatase assay by this method is not complicated by microbial growth or assimilation of enzymatic reaction products by soil microorganisms. Tests with several soils showed that the release of *p*-nitrophenol in assay of soil phosphatase activity by the method described was a zero-order reaction for more than 12 hr. As Skujiņš (1967) has pointed out, it is advisable in assay of soil enzyme activity to use a procedure that does not require a long incubation time. The sensitivity of the method

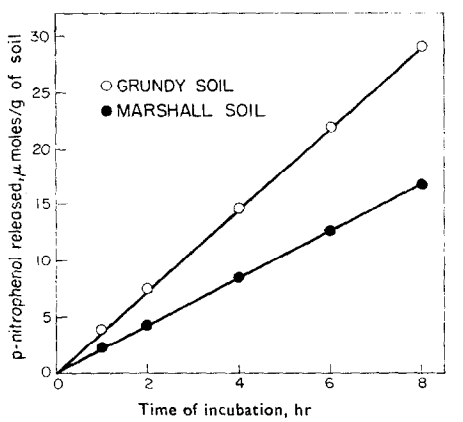

FIG. 2. Effect of time of incubation on release of *p*-nitrophenol in assay of soil phosphatase activity by method described.

described is such that precise results can be obtained even if the short incubation time recommended (1 hr) is reduced to 30 min.

The high precision of the method proposed is illustrated by Table 2, which gives the results of replicate analyses of 8 soils.

TABLE 2. PRECISION OF METHOD

Soil No.	μMoles of *p*-nitrophenol released/g of soil/hr*		
	Range	Mean	S.D.†
1	2·78–2·82	2·80	0·04
2	2·15–2·26	2·21	0·03
3	4·60–4·71	4·65	0·05
4	4·14–4·24	4·19	0·04
5	5·83–5·97	5·90	0·03
6	4·02–4·15	4·10	0·04
7	4·75–4·82	4·79	0·03
8	1·62–1·73	1·65	0·03

* Results of 8 analyses of each soil.
† Standard deviation.

Comparison of p*-nitrophenyl phosphate and phenyl phosphate methods*

Table 3 shows results obtained when the phosphatase activities of 8 soils were assayed by the *p*-nitrophenyl phosphate method proposed (method A), by a modification of this method involving use of water instead of MUB (method B), by a phenyl phosphate method (D) essentially identical to that used by Kramer and Yerdei (1959), and by a modification of the Kramer–Yerdei method involving use of pH 6·5 MUB instead of water (method C). Tests showed that modification of the Kramer–Yerdei method by use of pH 6·5 MUB instead of water did not affect the colorimetric analysis performed in this method to determine the phenol released by enzymatic hydrolysis of phenyl phosphate.

TABLE 3. COMPARISON OF *p*-NITROPHENYL PHOSPHATE (PNP) AND PHENYL PHOSPHATE (PP) METHODS

Soil No.	PNP method*		PP method†	
	A	B	C	D
	μMoles of PN or P released/g of soil/hr‡			
1	2·81	2·78	1·99	2·76
2	2·20	2·27	1·51	2·18
3	4·60	4·43	4·01	4·39
4	4·08	4·10	3·21	3·94
5	5·95	5·90	5·05	5·55
6	4·15	4·16	3·85	4·09
7	4·78	4·72	4·10	4·66
8	1·70	1·95	0·70	1·11

* A—method proposed; B—method proposed modified by use of 4 ml of water instead of 4 ml of MUB.
† C—soil (1 g) was treated with 4 ml of MUB (pH 6·5), 0·25 ml of toluene and 1 ml of 0·115M sodium phenyl phosphate, and the phenol released by incubation of this mixture for 1 hr was extracted with 0·3% potassium aluminum sulfate solution (20 ml) and determined colorimetrically as described by Kramer and Yerdei (1959); D—as in C, but used 4 ml of water instead of 4 ml of MUB.
‡ PN, *p*-nitrophenol; P, phenol.

The data in Table 3 show that the phosphatase activity values obtained by the *p*-nitrophenyl phosphate method proposed (A) were similar to, but usually higher than, those obtained by the Kramer–Yerdei method (D) and that the values obtained by both methods decreased in the order (soil no.) 5 > 7 > 3 > 6 > 4 > 1 > 2 > 8. The values obtained by the *p*-nitrophenyl phosphate method were not significantly affected when water was used instead of MUB, but the values obtained by the phenyl phosphate method were markedly lower when pH 6·5 MUB was used instead of water. No explanation of the latter finding is apparent. The possibility that recovery of phenol after incubation was affected by the presence of MUB was investigated, but the results showed that, whereas the recovery by the phenol extraction procedure used in the Kramer–Yerdei method of 200 ppm of phenol added to the 8 soils employed ranged from 90·2 to 97·9% in the absence of MUB, the recovery was practically quantitative (99·1 to 99.8%) when this method was modified by use of pH 6·5 MUB instead of water.

In the phenyl phosphate methods used by Kramer and Yerdei (1959) and other workers for estimation of soil phosphatase activity, the amount of phenol released by incubation is determined by measuring the intensity of the blue color formed on treatment of phenol with the Gibbs reagent (2, 6-dibromoquinone-chloroimide). Kramer and Yerdei (1959) measured the intensity of this color 30 min after addition of the Gibbs reagent (see also Halstead, 1964) and reported that the color developed after 30 min was stable for 24 hr. We found, however, that, under the conditions used by these workers, the intensity of the blue color obtained reached a maximum after about 2·5 hr, remained almost constant for about 1·5 hr after 2·5 hr, and thereafter decreased steadily with increase in the time of color development. We therefore adopted a 2·5 hr development time for colorimetric determination of phenol in the phenyl phosphate methods described in Table 3. Some results obtained in studies of the stabilities of the colors developed in the *p*-nitrophenyl phosphate and phenyl phosphate methods are reported in Table 4. They show that, whereas the color developed in the *p*-nitrophenyl phosphate method proposed is stable for 24 hr, the color developed in the phenyl phosphate method undergoes significant changes in intensity during this time. Besides having this advantage, the colorimetric analysis procedure used in the *p*-nitrophenyl phosphate method is superior to that used in the phenyl phosphate method in that it does not involve use of unstable reagents (the Gibbs reagent is unstable) and does not require that the temperature and pH of color development be strictly controlled. Several investigations have shown that, besides being affected by the time of color development, the intensity of the color obtained by treatment of phenol with the Gibbs reagent is markedly affected by the pH and temperature of color development (e.g. Gibbs, 1927; Ettinger and Ruchhoft, 1948; Mohler and Jacob, 1957).

TABLE 4. STABILITIES OF COLORS DEVELOPED IN *p*-NITROPHENYL PHOSPHATE (PNP) AND PHENYL PHOSPHATE (PP) METHODS

Method	Time after development of color (hr)										
	0	0·5	1	1·5	2	2·5	3	4	6	12	24
	Klett–Summerson reading										
PNP*	130	130	130	130	130	131	130	130	131	130	130
PP†	60	65	70	73	75	77	77	76	75	71	67

* Color obtained with 40 μg of *p*-nitrophenol by colorimetric procedure used in PNP method described.
† Color obtained with 40 μg of phenol by colorimetric procedure used in PP method of Kramer and Yerdei (1959).

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REFERENCES

BESSEY O. A., LOWRY O. H. and BROCK M. J. (1946) A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J. biol. Chem.* **164,** 321–329.

EDWARDS A. P. and BREMNER J. M. (1965) Dispersion of mineral colloids in soils using cation exchange resins. *Nature, Lond.* **205,** 208–209.

ETTINGER M. B. and RUCHHOFT C. C. (1948) Determination of phenol and structurally related compounds by the Gibbs method. *Analyt. Chem.* **20,** 1191–1196.

GIBBS H. D. (1927) Phenol tests. III. The indophenol test. *J. biol. Chem.* **72,** 649–664.

HALSTEAD R. L. (1964) Phosphatase activity of soils as influenced by lime and other treatments. *Can. J. Soil Sci.* **44,** 137–144.

KILMER V. J. and ALEXANDER L. T. (1949) Methods of making mechanical analyses of soils. *Soil Sci.* **68,** 15–24.

KRAMER M. (1957) Phosphatase–Enzym–Aktivität als Anzeiger des biologisch nutzbaren Phosphors im Boden. *Naturwissenschaften* **44,** 13.

KRAMER M. and YERDEI G. (1959) Application of the method of phosphatase activity determination in agricultural chemistry. *Soviet Soil Sci.* **9,** 1100–1103.

KROLL L. and KRAMER M. (1955) Der Einfluss der Tonmineralien auf die Enzym-Aktivität der Bodenphosphatase. *Naturwissenschaften* **42,** 157–158.

MEBIUS L. J. (1960) A rapid method for the determination of organic carbon in soil. *Analytica chim. Acta* **22,** 120–124.

MOHLER E. F. and JACOB L. N. (1957) Determination of phenolic-type compounds in water and industrial waste waters. *Analyt. Chem.* **29,** 1369–1374.

RAMIREZ-MARTINEZ J. R. and MCLAREN A. D. (1966) Determination of soil phosphatase activity by a fluorimetric technique. *Enzymologia* **30,** 243–253.

ROGERS H. T. (1942) Dephosphorylation of organic phosphorus compounds by soil catalysts. *Soil Sci.* **54,** 439–445.

SKUJIŅŠ J. J. (1967) Enzymes in soil. In *Soil Biochemistry* (A. D. McLaren and G. H. Peterson, Eds), pp. 371–414, Marcel Dekker, New York.

SKUJIŅŠ J. J., BRAAL L. and MCLAREN A. D. (1962) Characterization of phosphatase in a terrestial soil sterilized with an electron beam. *Enzymologia* **25,** 125–133.