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DETERMINATION OF PHOSPHATASE ACTIVITIES OF SOILS AND ANIMAL WASTES

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Summary—A method is described to determine acid and alkaline phosphatase activities from the rate of decomposition of *p*-nitrophenylphosphate in the presence of large amounts of organic matter, such as occur in the surface layers of soils or in animal wastes. The *p*-nitrophenol formed is separated by high pressure liquid chromatography on a cellulose column from *p*-nitrophenylphosphate and other organic compounds present in the soil or waste extract. After separation, *p*-nitrophenol is measured on-line in a spectrophotometric flow cell at a wavelength of 405 nm. In this way *p*-nitrophenol concentrations down to 0.1 μM can be measured, making it possible to work with substrate concentrations of 1 μM.

The necessity of correcting the phosphatase activity measured in this way for adsorption of enzyme, substrate (*p*-nitrophenylphosphate) and product (*p*-nitrophenol) is discussed.

Acid and alkaline phosphatases are inhibited strongly at phosphate concentrations greater than 0.1 mM, consequently substrate concentrations in the range of 0.01 to 0.1 mM were used.

The method was applied to a number of sandy soils and to pig slurry. Air drying or freeze drying of soils was found to decrease the phosphatase activity. Freeze drying did not affect the phosphatase activity of pig slurry. Michaelis–Menten kinetics were found to apply reasonably well. The resulting kinetic parameters are compared with values from the literature. Phosphatase activities are correlated with organic P and organic matter contents of soils and pig slurry.

INTRODUCTION

Although certain cations (e.g. Ce^{3+}, La^{3+}, Th^{4+}, Zr^{4+}) catalyse the decomposition of phosphate esters (Sir and Rezek, 1966), the phosphatase activity in soils and animal wastes can be said to be mainly a consequence of microbial and plant root activity and thus a measure for both the rate of release and biological fixation of P. Especially when (ab)using soils as filters for large amounts of animal wastes or slurries, the turnover rate of P and thus phosphatase activity in the soil may influence leaching of organic P compounds from these wastes through the soil and consequent pollution of ground and surface water (Hannapel *et al.*, 1963; Gerritse and Zugec, 1977). To study this problem we found it necessary to have a reliable method to determine phosphatase activity.

It seems that *p*-nitrophenylphosphate and β-naththylphosphate are the best substrates for the rapid and sensitive determination of soil phosphatase activity (Ramirez-Martinez and McLaren, 1966a,b; Moss, 1966; Tabatabai and Bremner, 1971; Weetall and Jacobson, 1972; Bartlett and Lewis, 1973; Cervelli *et al*, 1973; Brams and McLaren, 1974; Irving and Cosgrove, 1976). The products formed due to the action of phosphatase enzymes are determined by absorptiometry (*p*-nitrophenol) or fluorimetry (β-naphthol). In both approaches difficulties arise from the strong extinction or quenching by dissolved organic matter. Therefore, to improve the sensitivity it is necessary to separate the product formed from dissolved organic matter, and, in some cases, the substrate.

Other complications are caused by adsorption of substrate, products or enzyme and are not always fully taken into account when interpreting the reaction data (Tabatabai and Bremner, 1971; Cervelli *et al.*, 1973; Brams and McLaren, 1974; Irving and Cosgrove, 1976). Furthermore, both enzyme activation and inhibition can occur by products formed and other compounds, often unknown (Mahler and Cordez, p. 284, 1971; Weetall and Jacobson, 1972). Also more than one type of phosphatase enzyme may be present (Lim and Tate, 1971; Bartlett and Lewis, 1972; Bitar and Reinhold, 1972; Irving and Cosgrove, 1976).

We have developed a method by which, using *p*-nitrophenylphosphate as substrate, the product (nitrophenol) formed can be separated chromatographically from the reaction mixture, and determined without interference. Adsorption isotherms of nitrophenol were measured using the same method after adding known amounts of nitrophenol to the reaction mixture.

MATERIALS AND METHODS

Unless stated otherwise all chemicals were the purest grade available from Merck.

A liquid chromatograph was constructed from a high-pressure membrane pump (Orlita DMP 1515), a sample injection valve (Kipp en Zonen LC 7711), a steel column (type 316 stainless-steel precision-bore tubing), Swagelock bottom drilled fittings and a spectrophotometric detector with small-volume flow-through cell (Cecil CE 212). The dimensions of the chromatographic column were: 35 cm × 3 mm i.d. Pump pulsations were eliminated by means of a Bourdon tube and a capillary restrictor connected in series (Huber, 1969). The column was dry-packed with cellulose powder (Schleicher and Schull 142 dg), washed with a solution containing 30% 0.5 M

Table 1. Characteristics of soil horizons used in the phosphatase determination

Number	Type	Depth (cm)	Horizon	pH/KCl	Sand (%)	Organic matter (%)	Fe_2O_3 (%)	Al_2O_3 (%)	P_{total} ($\mu g \cdot g^{-1}$)	$P_{organic}$ ($\mu g \cdot g^{-1}$)
34A	Black	5–14	Al2g	4.6	85	5.1	1.27	0.53	550	280
	"beek" earth	30–55	Cl1g	4.7	93	0.5	0.46	0.31	40	20
	soil	85–100	—	—	—	—	—	—	50	10
34B	"Veld"	5–17	Ap	4.9	88	4.7	0.32	0.70	510	170
	podzol	27–40	B3	4.6	94	0.6	0.32	0.52	50	30
	soil	75–85	Cl1g	4.6	96	0.3	0.38	0.60	50	15
34C-21	Black "enk"	0–22	AanP	3.8	86	5.5	0.45	0.32	690	330
	earth soil on	52–64	Aan3	3.8	88	3.7	0.61	0.34	380	210
	moderpodzol	77–91	B3b	4.5	92	1.2	0.44	1.23	190	60
34C-34	Reclaimed	5–19	Ap	4.2	90	5.3	0.22	0.59	540	210
	"haar" podzol	28–41	B2	4.2	92.5	3.2	0.22	0.76	70	50
	soil	50–60	BC	4.6	96.5	0.8	0.29	0.61	40	20

ammonia and 70% methanol (by volume) at a column pressure of 20 MPa and topped up with cellulose powder until no further shrinkage occurred. A solution of 0.1 M Na_2HPO_4 in water (pH = 7.55 with HCl) was used as eluent for the separation of *p*-nitrophenol at room temperature. The extinction in the flow-through cell was monitored at 405 nm.

The phosphatase activity of the samples was determined after incubating at 30°C for 1 h in a buffer solution containing 50–500 μM *p*-nitrophenylphosphate and 50 mM Na acetate at pH 5 for acid phosphatase or 50 mM Tris(hydroxymethyl)aminomethane at pH 8 for alkaline phosphatase. The amount of sample used was such that not more than about 70% of *p*-nitrophenylphosphate was decomposed in 1 h. This required 0.5–2 g soil or 0.1–0.5 g dried pig slurry. Buffer solution (10 ml) was added and after shaking for 1 h the reaction was stopped by adding 10 ml of an aqueous solution of 10% trichloroacetic acid. The mixture was then centrifuged at 40,000 *g* for 1 h and the supernatant used for the chromatographic determination of *p*-nitrophenol. Blanks were run in the same way without the sample. The *p*-nitrophenol peaks in the chromatograms were integrated automatically and timed (Hewlett Packard 3373 B). The flow-through detector was calibrated by injecting standard solutions of *p*-nitrophenol. The injection volume was 25 μl.

Table 1 gives the characteristics of the sandy soils of which phosphatase activities were determined. Sampling sites were chosen in the neighbourhood of intensive pig-rearing areas. Samples were air dried or freeze-dried and stored in polythene bags in the open air in quantities of 1 kg. Fresh soil samples were stored in the same way at a temperature of 2°C. Freeze-dried pig slurry was stored at −20°C.

Total organic P was determined from the difference in total and inorganic P. Total P was determined after digestion with Fleischmann acid ($HNO_3H_2SO_4$, 1:1 by volume) as a reduced phosphomolybdate complex (Murphy and Riley, 1962). Inorganic P was determined after selective extraction with a solution of HF (40%), HCl (36%) and $TiCl_4$ (10:200:9 by volume) directly in the extract (Tinsley and Özcavasci, 1975; Gerritse and Zugec, 1977). The time necessary to obtain a stable colour of phosphomolybdate increased, however, from the usual 20 min at Ti concentrations up to 10 mM to 4 h at a Ti concentration of 50 mM. Organic P determined in this way was compared with organic P determined with another, more subtle, extraction method (Gerritse and Zugec, 1977). This latter method consists of extracting with 10% aqueous trichloroacetic acid (TCA) followed by an extraction with a solution of 0.3 M NaOH and 0.1 M EDTA (disodium salt). Total organic P is taken as the sum of the differences in total and inorganic P in both extracts. The results obtained for organic P by the "titanium" method and the "TCA/EDTA" method were on average within 10% of each other.

RESULTS

An example of a chromatogram of a soil extract after incubation with *p*-nitrophenylphosphate is shown in Fig. 1. Using high pressure liquid chromatography (HPLC) with a cellulose column it is thus possible to separate *p*-nitrophenol from dissolved

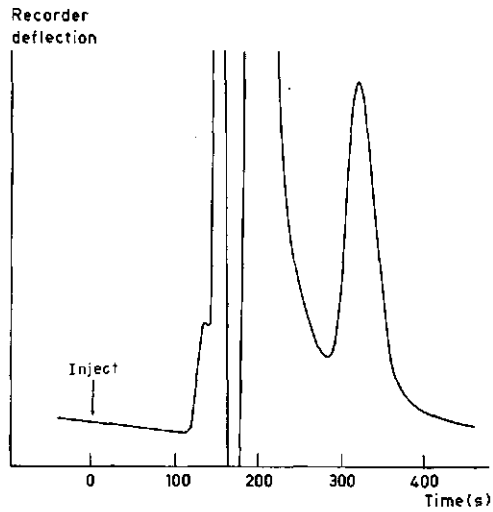

Fig. 1. Example of a chromatogram of a soil extract after incubation with 250 μmoles l^{-1} *p*-nitrophenylphosphate at pH 8. The retention time of *p*-nitrophenol is 324 s. The other peaks are from organic compounds in the soil extract. The *p*-nitrophenol concentration in the extract, calculated from the peak area, is 11 μmoles l^{-1}.

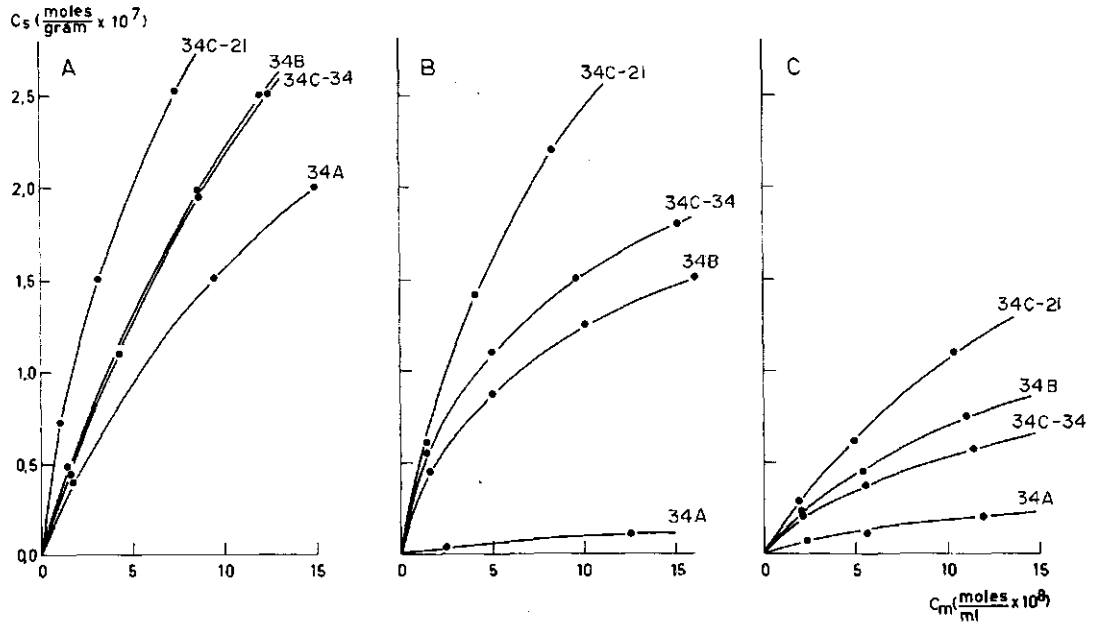

Fig. 2. Adsorption isotherms for *p*-nitrophenol in soils, determined after addition of trichloroacetic acid to the incubated soil and buffer mixture. Use of tris or acetate as buffer gave identical results. The index numbers in the figures correspond to the soil numbers in Table 1. A. Isotherms determined in the top layer of the soils. B. Isotherms determined in the third layer. C. Isotherms determined in the fifth layer.

organic matter and *p*-nitrophenylphosphate. With the system used (wavelength 405 nm) as little as 0.1 μmole l^{-1} *p*-nitrophenol could be measured, making it possible to use substrate concentrations of 1 μmole l^{-1}.

The data obtained from phosphatase reactions in soils and pig slurry must be corrected for adsorption. It is difficult, however, to evaluate how to correct for the adsorption of *p*-nitrophenylphosphate under reaction conditions as the phosphatase reaction may take place mainly at the soil surface owing to adsorption of both enzyme and substrate. In view of this, data were only corrected for adsorption of *p*-nitrophenol. Adsorption isotherms of *p*-nitrophenol for soil and pig slurry are shown in Figs 2 and 3. The adsorption of *p*-nitrophenol to soils is related to organic matter content, as can be seen by comparing the adsorption isotherms of Fig. 2 with the corresponding data for organic matter in Table 1. An exact function is difficult to establish without a knowledge of the structure and thus effective specific surface area of the organic matter in different soil samples. The adsorption has thus to be determined for each sample separately.

In Table 2 results of acid phosphatase measurements in a number of soils, described in Table 1, are given. A first order reaction was assumed and a substrate concentration range of 50–500 μmoles l^{-1} was used. The concentrations of *p*-nitrophenol found after incubation were corrected for adsorption. The data were fitted to the integrated Michaelis-Menten equation (Mahler and Cordez, 1971, pp. 274–278):

$$V_{max} \cdot t = a \cdot S_o - K_m \cdot \ln(1 - a) \qquad (1)$$

where V_{max} = the maximum decomposition rate of *p*-nitrophenylphosphate (a linear function of the enzyme concentration), t = time, a = phosphatase activity expressed as the fraction of *p*-nitrophenylphosphate (= S_o) that has decomposed, S_o = initial concentration of *p*-nitrophenylphosphate, and K_m = a function of reaction rates and a constant at a given temperature.

The inhibitory effect of phosphate was measured (Fig. 4) for acid (pH 5) and alkaline (pH 8) phospha-

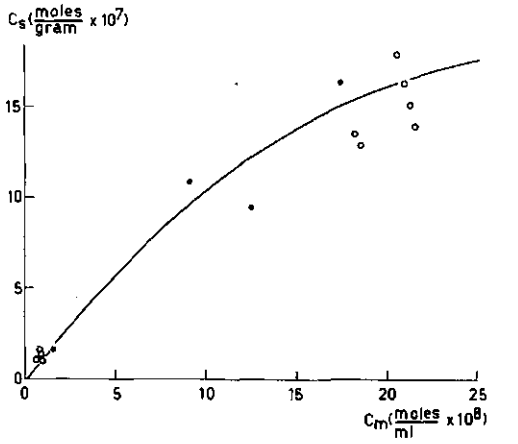

Fig. 3. Adsorption isotherm of *p*-nitrophenol in freeze-dried pig slurry, determined after addition of trichloroacetic acid to the incubated slurry and buffer mixture. Use of tris (○) or acetate (●) as buffer gave identical results.

Table 2. Data calculated from acid phosphatase activity measurements in the soil layers described in Table 1

Soil number	Depth (cm)	K_m (μmoles l^{-1})	C.V. of K_m (%)	r	V_{max} (μmoles g^{-1} h^{-1})	n
34A	5–14	90	—	—	70	2
	30–55	70	50	0.80	7	8
	85–100	*120*	*30*	*0.98*	*2*	*8*
34B	5–17	100	50	0.81	50	8
	27–40	230	50	0.90	5	8
	75–85	150	20	0.96	2	8
34C-21	0–22	90	25	0.93	65	8
	52–64	300	20	0.97	60	8
	77–91	100	25	0.94	6	8
34C-34	5–19	120	25	0.93	120	8
	29–41	100	15	0.98	25	8
	50–60	190	20	0.96	5	8

K_m, V_{max} = Kinetic parameters as given in equation 1. C.V. = coefficient of variation of K_m. r = correlation coefficient between $a \cdot S_o$ and $ln(1 - a)$ in equation 1. n = number of analyses.

tase. If the results are expressed as a function of the $H_2PO_4^-$ concentration instead of total inorganic P then curves 1 and 2 in Fig. 4 coincide, indicating that $H_2PO_4^-$ is the inhibiting ion. It can be said that to prevent inhibition by phosphate, the $H_2PO_4^-$ concentration should not exceed about 100 μmoles l^{-1}. The possible inhibition or activation by *p*-nitrophenol and buffer anions (e.g. acetate or maleate) was not investigated.

From Table 2 it follows that phosphatase concentration decreases markedly with depth in the soil.

To investigate the effect of drying and time of storage on the phosphatase activity of soil samples, the top layers of the soils (Table 1) were sampled again (about a year after the first sampling). Part of the soil samples were stored at 2°C immediately after sampling in closed polythene bags, part was air-dried and part was freeze-dried. The acid and alkaline phosphatase activities of these soil samples were monitored for 3 months. The values of V_{max} (Table 3) and K_m (Table 4) for each sample were found by fitting the phosphatase activities at three different substrate concentrations to equation 1. The concentrations used were 50, 100 and 250 μmoles l^{-1}. Analyses were done in duplicate. As can be seen in Tables 3 and 4, large fluctuations can occur. Drying of the soils appears to decrease phosphatase concentration ($= V_{max}$) by a factor of 2–3. K_m values appear to be increased slightly by drying. Except for soil 34C-21 soil phosphatase concentrations (V_{max}) determined at pH 5 and pH 8 do not differ much. It can thus be said that probably only one type of enzyme is present with K_m values of about 100 μmoles l^{-1} at pH 5 and 700 μmoles l^{-1} at pH 8.

Table 3. The phosphatase concentration in the top layers of the soils mentioned in Table 1 as a function of time and storage conditions, measured at pH = 5 (acid phosphatase) or pH = 8 (alkaline phosphatase)

Age (days)	V_{max}(μmoles $g^{-1} \cdot h^{-1}$) Soil 34A Wet	Soil 34A Dried	Soil 34B Wet	Soil 34B Dried	Soil 34C-21 Wet	Soil 34C-21 Dried	Soil 34C-34 Wet	Soil 34C-34 Dried	
1	250	n.d.	100	n.d.	250	n.d.	120	n.d.	5
3	450	n.d.	120	n.d.	450	n.d.	150	n.d.	5
10	400	100	90	55	350	170	130	65	5
30	200	100	100	75	150	170	70	65	5
45	250	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
65	n.d.	n.d.	100	n.d.	n.d.	n.d.	n.d.	n.d.	5
75*	n.d.	90	n.d.	55	n.d.	150	n.d.	50	5
90	350	100	90	55	250	150	150	40	5
30	100	120	120	75	100	65	75	65	8
80*	n.d.	60	n.d.	50	n.d.	50	n.d.	40	8
90	400	150	150	80	80	60	70	65	8
Moisture: (%)	15		4.8		7.1		14.5		

The values given are for V_{max} (equation 1) in μmoles g^{-1} dry soil h^{-1}.

The C.V. in each determination was about 10–20%.

The "wet" soils were stored at 2°C in closed polythene bags. The dried soils were air dried and kept at room temperature.

* These samples were freeze-dried and kept at room temperature. n.d. = not determined.

Table 4. Phosphatase reaction rate constants (K_m in equation 1) in the top layers of the soils mentioned in Table 1 as a function of time and storage conditions, measured at pH = 5 (acid phosphatase) and pH = 8 (alkaline phosphatase)

Age (days)	K_m (μmoles l^{-1}) Soil 34A Wet	Soil 34A Dried	Soil 34B Wet	Soil 34B Dried	Soil 34C-21 Wet	Soil 34C-21 Dried	Soil 34C-34 Wet	Soil 34C-34 Dried	pH
1	140	n.d.	90	n.d.	130	n.d.	110	n.d.	5
10	n.d.	50	n.d.	120	n.d.	70	n.d.	110	5
30	40	80	50	130	70	90	90	100	5
45	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
65	n.d.	n.d.	70	n.d.	n.d.	n.d.	n.d.	n.d.	5
75*	n.d.	160	n.d.	230	n.d.	100	n.d.	200	5
90	90	90	70	90	30	80	70	250	5
30	60	450	800	900	1000	800	700	900	8
80*	n.d.	600	n.d.	1000	n.d.	1000	n.d.	800	8
90	400	600	1000	800	700	700	500	900	8
Moisture: (%)	15		4.8		7.1		14.5		

The "wet" soils were stored at 2°C in closed polythene bags. The dried soils were air dried and kept at room temperature.

The C.V. in the determination of K_m was about 20–30%.

* These samples were freeze-dried and kept at room temperature.

Table 5. Results of phosphatase determinations in freeze dried pig slurry at pH 5 and pH 8

K_m (μmoles l^{-1})	C.V. of K_m (%)	r	V_{max} (μmoles $g^{-1} \cdot h^{-1}$)	n	pH	P_{diss} (μmoles P l^{-1})
650	7	0.995	800	8	5	6500
1000	10	0.990	1000	12	8	3300

K_m and V_{max} are the kinetic parameters given in equation 1.

C.V. is the coefficient of variation of K_m.

r is the correlation coefficient between $a \cdot S_o$ and $\ln(1 - a)$ in equation 1.

n is the number of analyses.

P_{diss} is the concentration of inorganic P in the buffer solution during incubation with the dried pig slurry.

Some characteristics of the freeze dried pig slurry: total P: 2%, organic P: 0, 35%; organic matter: 72%.

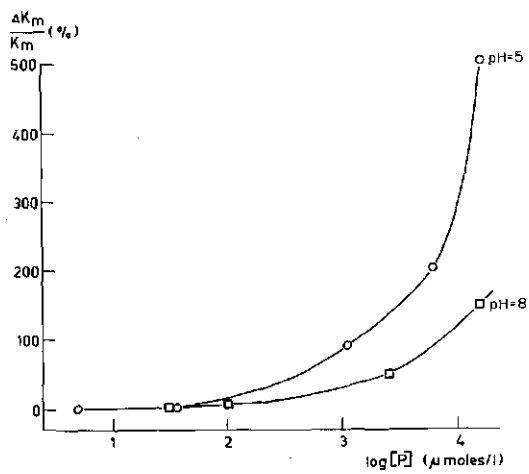

Fig. 4. Plot of the relative change in the soil phosphatase Michaelis constant K_m (equation 1) as a function of the total inorganic phosphorus concentration in incubated soil and buffer mixtures.

$H_2PO_4^-$ concentrations were calculated from inorganic P and pH measurements for soil samples solutions, incubated without substrate and were found to be significantly higher when using dried soils but never exceeded 100 μmoles l^{-1}. This might explain the slightly higher K_m in the case of dried soils.

Phosphatase activity was also determined in pig slurry. Centrifugation of pig slurry at 2000 *g* resulted in a significant loss of phosphatase activity from solution and centrifugation at 40,000 *g* resulted in a com-

Table 6. Relation between phosphatase concentration (V_{max} μmoles/g·h), total P (P_t), organic P (P_{org}) and organic matter (O.M.) of the sandy soils described in Table 1. (P_t, P_{org} and O.M. in % of D.M.)

Equation		C.V. (%)	r
V_{max}	$= 15 \cdot (O.M.)$	15	0.91
V_{max}	$= 2800 \cdot (P_{org})$	20	0.86
P_{org}	$= 0.47 \cdot (P_t)$	5	0.97

Table 7. Comparison of soil phosphatase constants given in the literature

K_m range (moles l^{-1})	Substrate (moles l^{-1})	T (°C)	Buffer Type	pH	Reference
10^3–5×10^3	10^3–2×10^4	37	universal	6.5	Tabatabai and Bremner (1971)
3×10^2–5×10^3	5×10^3–2×10^4	37	acetate (0.5 M)	4.7	Cervelli *et al.* (1973)
100–300	10^2–3×10^3	25	maleate (10 mM)	6.9	Brams and McLaren (1974)
200–300*	10^2–10^3	37	maleate (40 mM)	6.5	Irving and Cosgrove (1976)
50–200	50–500	30	acetate (50 mM)	5.0	Our data
5×10^2–10^3	50–500	30	tris (50 mM)	8.0	Our data

* Estimated from their data.

plete loss of phosphatase activity from solution. This indicates that the phosphatase enzyme is almost completely adsorbed to the solids in the pig slurry. Freeze-drying of pig slurry did not affect phosphatase activity (Table 5). In fresh slurry and the same slurry after freeze drying no significant difference in phosphatase activity (on a dry weight basis) was found.

The values of P_{diss} (Table 5) can be used to calculate the amount of inhibition by phosphate ($\Delta K_m/K_m$) from Fig. 4. Values of K_m, corrected for inhibition by phosphate, are then found to be: 200 μmoles l^{-1} for acid phosphatase and 650 μmoles l^{-1} for alkaline phosphatase. These values are in reasonable agreement with the values found for soil phosphatase. Also the values of V_{max} for acid and alkaline phosphatase are not significantly from each other, pointing again to the presence of one type of enzyme.

Using the data for the dried sandy soils, correlations were calculated between V_{max}, organic matter, organic P and total P (Table 6). The data for V_{max} in pig slurry appear to fit these equations fairly well if the corresponding data for organic matter and organic P (Table 5) are used. The ratio between total and organic P in slurry is, however, entirely different from the ratio in soils.

DISCUSSION

Some published values for K_m and the conditions under which they were obtained are compared with our values in Table 7. It can be seen that not much agreement exists. This may be due to many factors, e.g. different buffer anions and concentrations, pH, temperature, and too high substrate concentrations. Adsorption must also be accounted for properly. Cervelli *et al.* (1973) for instance corrected for both the adsorption of *p*-nitrophenol and *p*-nitrophenylphosphate. The correctness and accuracy of their procedure are in doubt as they assumed the phosphatase reaction takes place only in solution and not at the soil surface.

We conclude that when using *p*-nitrophenylphosphate as substrate in soils or animal wastes useful data of phosphatase activity are obtained when low substrate concentrations are used and adsorption of *p*-nitrophenol as well as inhibition by $H_2PO_4^-$ are corrected for. Working with low substrate concentrations causes the extinction of *p*-nitrophenol in the extract to be swamped by the extinction of other (mainly organic) compounds dissolved from the sample. To improve precision under these circumstances it is essential to separate *p*-nitrophenol by chromatography from the other compounds. This we have done by HPLC using a column containing cellulose powder.

Correction for adsorption of enzyme and substrate is difficult to evaluate and can best be omitted and taken as an inherent property of phosphatase in the matrix concerned (e.g. soil or slurry).

To improve comparison of results, circumstances under which phosphatase activities are determined, should be standardized. For this purpose the buffer and substrate concentrations mentioned in this paper can be used. Also, as is evident from our data, conditions of storage of samples are important and should be standardized.

In the soils and pig slurry used a definite correlation exists between the phosphatase activity (V_{max}), and organic P and organic matter contents.

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