INOSITOL PHOSPHATE PHOSPHATASES OF MICROBIOLOGICAL ORIGIN.* THE ISOLATION OF SOIL BACTERIA HAVING INOSITOL PHOSPHATE PHOSPHATASE ACTIVITY

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Summary

The isolation of three soil bacteria exhibiting inositol phosphate phosphatase activity is described. One isolate, a member of the genus *Pseudomonas*, had pronounced phytase activity but the others had not. The latter were slightly active against pentaphosphates, however, and they easily dephosphorylated tetraphosphates and lower esters.

I. Introduction

Since the first report of the existence in soil of an inositol phosphate, apparently similar to the *myo*-inositol hexaphosphate, "phytin", present in plants (Dyer, Wrenshall, and Smith 1940), phosphate esters of this type have been shown to be important soil constituents (Anderson 1967; McKercher and Anderson 1968; Williams and Anderson 1968). It was observed by Pearson, Norman, and Ho (1942) that phytate phosphorus added to soil was slowly mineralized and, later, Jackman and Black (1952) detected low levels of phytase activity in a number of soils. Recently, Greaves and Webley (1969) attempted to demonstrate the existence of phytase activity in soil, using a modification of the method of Jackman and Black (1952). No activity was detected, even when greatly increased amounts of substrate were used. The earliest report of phytase activity in a microorganism likely to occur in soil is that of Dox and Golden (1911) who demonstrated it in the fungus *Aspergillus niger*. This was later confirmed by Casida (1959) who showed that several members of the genus *Aspergillus*, including some soil isolates, possessed phytase activity. There are numerous other reports of the presence of low levels of phytase activity in microorganisms (Sloane-Stanley 1961; Cosgrove 1967; Skujins 1967) but as has already been pointed out (Cosgrove 1967), all such reports should be assessed with caution, owing to the possibility that such activity is due mainly or entirely to the action of phosphatases on the lower phosphate esters often present in commercially available calcium phytate.

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* An enzyme will be considered to have "inositol phosphate phosphatase" activity if it is able to dephosphorylate completely any inositol phosphate possessing one or more orthophosphate groups esterified to the inositol nucleus. "Phytase" is *myo*-inositol hexaphosphate phosphohydrolase (E.C. 3.1.3.8). Other terms to be used are, for example, "inositol hexaphosphate monophosphatase", a system able to hydrolyse a hexaphosphate to a pentaphosphate; and "inositol pentaphosphate phosphatase", a system able to completely dephosphorylate a pentaphosphate to the free inositol.

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Recently, Shieh and Ware (1968) examined more than 2000 soil isolates, fungi and bacteria, but concluded that most had little or no extracellular phytase activity. The most active isolate was identified as *Aspergillus ficuum*. We have confirmed that this fungus produces an extracellular phytase and will report on the properties of the enzyme in a later paper. In contrast to the findings of Shieh and Ware (1968), Greaves and Webley (1969) showed that incubating sand moistened with a solution containing ammonium nitrate, sodium phytate, and a little dilute soil suspension enabled a large mixed bacterial population to develop. After 7 days, all inositol phosphates had been hydrolysed and many of the pure cultures subsequently isolated were found to have phytase activity. The bacteria included a wide range of types commonly found in soils.

Until now, only one phytase of microbial origin has been investigated at all thoroughly (Greaves, Anderson, and Webley 1967). The enzyme was obtained from a laboratory culture of *Aerobacter aerogenes*.

The study of the biodegradation of soil phytate constituents would obviously be facilitated by the availability of a highly active bacterial or fungal phytase, preferably one derived from a soil isolate. This paper describes the isolation from soil of three bacteria able to utilize *myo*-inositol phosphates as a carbon source. One of these, a *Pseudomonas* sp., was highly active against all *myo*-inositol phosphates, including the hexaphosphate; the others had no phytase activity but were able to dephosphorylate *myo*-inositol pentaphosphates and lower esters.

II. MATERIALS AND METHODS

(a) Sodium Phytate Solution for Media

Calcium phytate (Koch–Light Laboratories Ltd.) was converted to a solution of the neutral sodium salt (Cosgrove 1966). The solution was adjusted to 1% $C_6H_6O_{24}P_6Na_{12}$ on the basis of its phosphorus content before being used for the preparation of media. This solution contained appreciable amounts of esters other than the main constituent, *myo*-inositol hexaphosphate.

(b) Media

(1) Citrate–phytate medium: K_2HPO_4, 7·0 g; KH_2PO_4, 2·0 g; $MgSO_4.7H_2O$, 0·1 g; $(NH_4)_2SO_4$, 1·0 g; Difco yeast extract, 0·1 g; trisodium citrate, 0·5 g; 1000 ml 1% sodium phytate solution; adjusted to pH 7·0.

(2) Phytate medium: as for (1) but with trisodium citrate and yeast extract omitted.

(3) Phytate agar: as for (2) but with 1·5% agar added.

Media were sterilized by autoclaving for 15 min at 15 lb/in².

(c) Soil Samples

These are listed in Table 1. Pretreatments prior to inoculation of medium (1) are also given.

(d) Isolation of Phytase-producing Organisms from Soil

The citrate–phytate enrichment medium (1) (100 ml) in 500-ml Erlenmeyer flasks was inoculated with samples of soil (1 g) and shaken at 25°C for periods of up to 2 weeks. When microbial growth became apparent, aliquots of the suspensions (1·0 ml) were transferred to fresh portions of the medium and shaken for a further 6 days. Growth from these flasks was plated onto the phytate agar and incubated at 25°C. A selection of bacteria, based on colony morphology, was made from the agar plates. These were transferred to 100-ml Erlenmeyer flasks containing 10-ml portions of the liquid phytate medium and incubated at 25°C. Two isolates from soil E (SB2 and SB3) and one from soil C (SB4) made good growth in the phytate medium and were sub-

sequently tested for their ability to dephosphorylate *myo*-inositol phosphates. Growth was followed by measuring changes in the optical density of the culture at 625 nm. Cultures were maintained on slopes of phytate agar.

(e) Identification of Bacterium

The bacterium was examined using standard procedures (Society of American Bacteriologists 1957) and identified using the keys of Skerman (1957) and Hendrie and Shewan (1966).

(f) Ion-exchange Chromatography of Media and Culture Supernatants for Inositol Phosphates

When necessary, the medium was first centrifuged to remove bacterial cells. To an aliquot (5 ml) of the supernatant was added perchloric acid (0·1 ml, 70%) and the mixture was again centrifuged. An aliquot (2 ml) of the clear supernatant was diluted to 100 ml and a portion (2 ml) of this solution used for the chromatographic examination (Cosgrove 1963).

TABLE 1

SOME CHARACTERISTICS OF THE SOILS USED

Soil	Site	Type	pH	Phosphate (p.p.m. phosphorus)		Pretreatment
				Organic	Phytate	
A	Mt. Gingera, A.C.T.	Alpine humus	5·0	929	356	Air dried
B	Mt. Gingera, A.C.T.	Alpine humus	5·0	929	356	pH raised to 7·0 with lime and incubated at 25°C for 3 months
C	Crookwell, N.S.W.	Basaltic	6·5	251	27	Air dried
D	Robertson, N.S.W.	Krasnozem	5·9	955	3·6	Incubated at field capacity with 1% added sodium phytate at 25°C for 3 months
E	Garden soil, A.C.T.	—	6·5	—	—	None

III. RESULTS AND DISCUSSION

Growth of SB2 in phytate medium reached a maximum optical density (0·90 per 1 cm cell at 625 nm) after 17 hr but cultures of SB3 and SB4 grew sparsely and much more slowly, reaching a maximum density (0·20 and 0·39) after 72 hr. The elution diagram for the inositol phosphates in a sample of phytate medium before inoculation is shown in Figure 1 and the calculated values for the tetraphosphate plus lower esters, pentaphosphate, and hexaphosphate content of 100 ml of both inoculated and non-inoculated media are in Table 2. Ion-exchange chromatography of the culture supernatant from SB2 (17 hr) showed that all the inositol phosphates had been utilized. A similar examination of samples of media from cultures of SB3 and SB4 (72 hr) revealed that no tetraphosphates or lower esters were present; the pentaphosphate content was reduced in each case but the hexaphosphate content remained virtually unaltered (Table 2).

It is clear from these results that, of the three bacteria examined, only SB2 possesses phytase activity. SB3 and SB4 evidently belong to a group of bacteria

having inositol pentaphosphate phosphatase activity; the previously postulated existence of such organisms (Cosgrove 1967) is therefore confirmed.
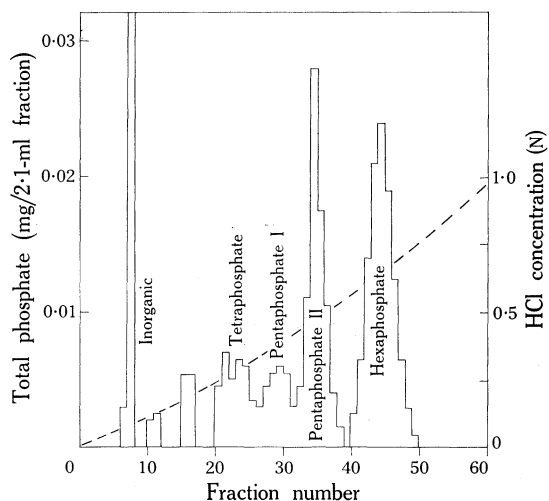

Fig. 1.—Elution pattern from a Dowex-1 (chloride form) column of medium (2). The broken line indicates the HCl gradient.

Greaves and Webley (1969) examined many of their isolates by inoculating them from slopes into a medium containing 0.1% ammonium nitrate plus 0.1% myo-inositiol hexaphosphate (pH 6.8). After incubation at 25°C for 7 days all cultures liberated between 50 and 70% of the substrate phosphorus as inorganic phosphorus.

TABLE 2

INOSITOL PHOSPHATES IN PHYTATE MEDIUM

Values expressed as milligrams phosphate per 100 ml medium

Isolate Used for Inoculation	Incubation Time (hr)	Tetraphosphates and Lower Esters	Penta-phosphate I	Penta-phosphate II	Hexa-phosphate
—*	0	108·0	68·7	192·5	275·0
SB2	17	0	0	0	0
SB3	72	0	47·5	166·2	273·2
SB4	72	0	30·0	131·2	274·1

* Before inoculation of medium.

No attempt was made to follow the rate of disappearance of hexaphosphate in the medium, but it appears likely, from other results recorded by Greaves and Webley (1969), that 70% release of inorganic phosphorus did not represent complete

dephosphorylation of all ester phosphates. We have tested SB2 on the same medium and have examined the culture filtrate by ion-exchange chromatography. After incubation for 40 hr, diphosphate, triphosphate, and a trace of tetraphosphate were detectable, but after 64 hr no ester phosphorus of any sort remained. It would appear that cultures of SB2 can dephosphorylate *myo*-inositol hexaphosphate more rapidly than any of the bacterial isolates examined by Greaves and Webley (1969). The active bacterium, SB2, has the following characteristics:

Rods, $0 \cdot 6$–$0 \cdot 7$ by $1 \cdot 4$–$2 \cdot 2$ μm occurring singly and in pairs; motile, possessing two flagella at one pole; Gram-negative. Nutrient agar colonies after 48 hr at 30°C: circular, 2 mm diameter, entire, umbonate, smooth glistening, translucent, buff coloured without diffusible pigments. Peptone water: marked turbidity with thin pellicle and ring. Aerobic, facultative, grows well at 30°C, no growth at 42°C. Produces acid from glucose but not from lactose. Oxidative in Hugh and Liefson's medium, containing glucose but not lactose. Oxidase positive. Growth on inositol as sole source of carbon. Liquid cultures produce faint blue-green pigment which fluoresces in ultraviolet light.

It is therefore a *Pseudomonas* sp.

IV. Acknowledgment

V. References

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