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Lead Inhibition of Enzyme Synthesis in Soil

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Addition of 2 mg of Pb^{2+}/g of soil coincident with or after amendment with starch or maltose resulted in 75 and 50% decreases in net synthesis of amylase and α-glucosidase, respectively. Invertase synthesis in sucrose-amended soil was transiently reduced after Pb^{2+} addition. Amylase activity was several times less sensitive to Pb^{2+} inhibition than was enzyme synthesis. In most cases, the rate of enzyme synthesis returned to control ($-Pb^{2+}$) values 24 to 48 h after the addition of Pb. The decrease in amylase synthesis was paralleled by a decrease in the number of Pb-sensitive, amylase-producing bacteria, whereas recovery of synthesis was associated with an increase in the number of amylase-producing bacteria. The degree of inhibition of enzyme synthesis was related to the quantity of Pb added and to the specific form of lead. $PbSO_4$ decreased amylase synthesis at concentrations of 10.2 mg of Pb^{2+}/g of soil or more, whereas PbO did not inhibit amylase synthesis at 13 mg of Pb^{2+}/g of soil. Lead acetate, $PbCl_2$, and PbS reduced amylase synthesis at total Pb^{2+} concentrations of 0.45 mg of Pb^{2+}/g of soil or higher. The results indicated that lead is a potent but somewhat selective inhibitor of enzyme synthesis in soil, and that highly insoluble lead compounds, such as PbS, may be potent modifiers of soil biological activity.

Despite the fact that Pb is highly toxic and is present in significant quantities in many soils (3, 8, 9), there are few data on the effects of Pb on soil microorganisms. Tyler et al. (21) reported that 500 to 1,000 μg of lead acetate/g of meadow soil significantly decreased nitrate levels but had no effect upon ammonium levels. In their experiments, no supplemental nitrogen was added; consequently, the decrease in NO_3^- may have been the result of inhibition of soil organic matter decomposition combined with decreased activity of soil-nitrifying bacteria. Because Pb^{2+} is bound rapidly by soil components (2) and since it has been shown to be less toxic to bacteria than other heavy metals when added to culture media (1, 20), the initial hypothesis was that it would have little effect on soil microbes.

The approach used in this study was to examine the effect of Pb on enzyme synthesis when the appropriate substrate was added to soil. The rationale for this approach is that protein synthesis is a complex process that requires metabolically active cells, abundant energy, and precursors (e.g., nitrogen and phosphorous). If Pb destroyed cells or inhibited any catabolic or anabolic reaction required for protein synthesis, the observed result would be a decreased rate of enzyme synthesis.

A good correlation between soil enzyme activity and microbial numbers has been reported (16, 17). Amylase was selected as an indicator enzyme because a high percentage of bacteria in cultivated soils produce this enzyme (4).

The experiments described below were conducted to provide preliminary answers to three questions: (i) does Pb have any effect on soil microbes, (ii) can enzyme synthesis be used as an indicator of inhibition of soil biological processes, and (iii) do microorganisms influence the availability of Pb to biological systems?

MATERIALS AND METHODS

Soil samples. Drummer silt-loam soil, obtained from the Agronomy South Farm, University of Illinois, was used in all experiments. Soil was stored moist at room temperature (20 to 25°C) until used. Soil composition was 9.5% sand, 30.3% clay, and 60.2% silt (pH 6.0), and organic matter content was 5.9%. Other properties were cation-exchange capacity, 30.3 meq/g, and Pb-sorption capacity, 24.4 mg of Pb^{2+}/g of soil (12). The water content at one-third bar water tension was 0.339 g of water/g of soil. Soil samples were sifted through a 3-mm screen to remove large aggregates and undecomposed crop residues before use.

Sampling procedures. All experiments were performed on triplicate subsamples for each treatment. Duplicates were removed from each subsample at each time indicated, and all experiments were repeated at least twice. For example, each point in Fig. 1 represents the mean of 12 assays (three replicates × two samples × two experiments) at 0, 24, 25, 48, 72, and 96 h.

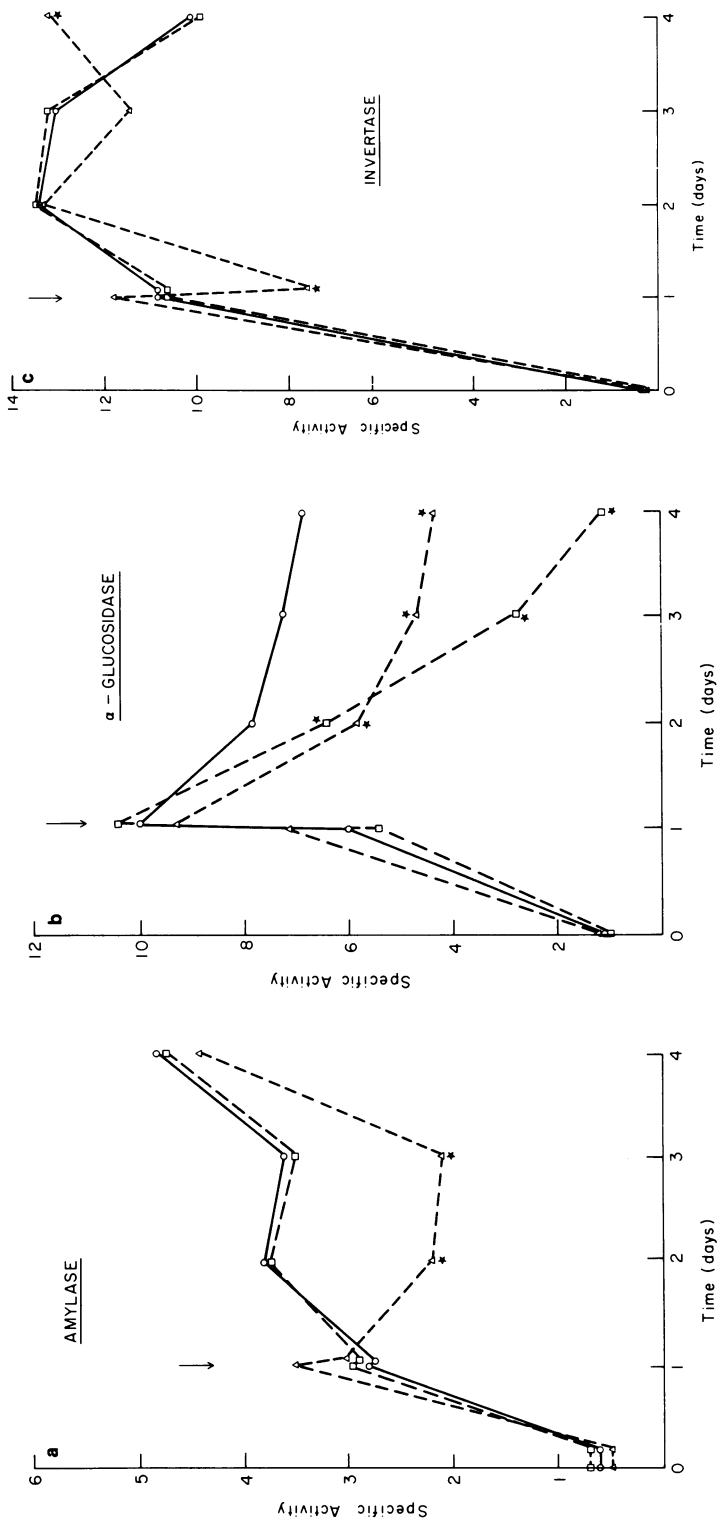

FIG. 1. *(a–c) Time course of enzyme synthesis in soil. Soil, NH_4Cl, and substrate were mixed at zero time and incubated for 24 h. Lead or calcium acetate at a final concentration of 1.5 mg of Pb^{2+} or Ca^{2+}/g of soil was added to the appropriate mixtures immediately after removing the 24-h samples. Values shown are means of six assays per sample time. Symbols: ○, no salt added; △, lead acetate added: □, calcium acetate added. Arrow indicates time at which lead or calcium was added to the appropriate samples. Values indicated by a star (★) at the same sample time are significantly different than control values ($P = 0.05$).*

Enzyme assays. Procedures described below represent modifications of methods devised by von Hofmann and Hoffmann (7) and by Tabatabai and Bremner (18). All enzyme assays were conducted with 2 g of soil to which 0.3 ml of toluene was added 15 min before the addition of buffer and substrate. Amylase assays used 0.1 M sodium acetate buffer (5 ml, pH 5.0) containing 50 mg of starch. After a 24-h incubation at 25°C, 10 ml of distilled water was added, and the soil suspension was centrifuged at 12,350 × *g* for 10 min. Reducing-sugar analyses on the supernatant were done according to Nelson (15). Invertase was assayed in a manner identical to amylase, except that 0.1 M sodium acetate buffer, pH 5.5, containing 18 mM sucrose was used, and the incubation period was 3 h. Alpha-glucosidase was determined at pH 7.0 in 0.1 M citrate buffer containing 3.3 mM *o*-nitrophenyl-α-D-glucopyranoside (ONP-glucoside, Sigma Chemical Co.). After a 24-h incubation at 25°C, 5 ml of *n*-butanol was added, and the mixture was shaken vigorously. The butanol-water emulsion was centrifuged at 12,350 × *g* for 5 min, and the butanol phase was diluted with *n*-butanol containing 10 μl of 5 N NaOH/ml of butanol. Essentially quantitative recovery of ONP (>95%) was achieved and little of the highly pigmented soil organic matter was partitioned into the *n*-butanol layer.

Enzyme activities are reported as specific activity (units per gram of oven-dried soil), where 1 U of activity corresponds to the release of 1 μmol of product/24 h. Enzyme activity values were corrected for apparent product formation in the absence of substrate.

Enzyme synthesis. The appropriate substrate (1 g), NH_4Cl (0.16 g), and soil (100 g) were combined in a 250-ml flask, and sufficient sterile distilled water was added to bring the soil to about 40% water-holding capacity. In some experiments, Pb was added before, or coincident with, substrate; in other cases, Pb was added after 24 h of incubation of soil plus substrate. Calcium salts were usually used in these experiments to assess the effect on enzyme synthesis of the metal-associated anions or of the increased ionic content of the soil mixtures. Starch, maltose, and sucrose were used to promote synthesis of amylase, α-glucosidase, and invertase, respectively. All incubations were performed at 25°C, and samples were removed at times indicated in the text. Salts were added to soil as dry, finely ground powder, with thorough mixing to distribute the salt.

The effect of Pb on amylase synthesis and amylase activity was distinquished in the following manner. Mixtures (in triplicate) of starch (4 g), NH_4Cl (0.8 g), and soil (400 g) were prepared as described in the previous section. After a 24-h incubation, the mixtures were split into four 100-g portions. One portion received no Pb, whereas Pb at a final concentration of 2, 4, or 8 mg of Pb^{2+}/g of soil was added to the other portions. After a further 24-h incubation, amylase activity in all samples was determined. Two-gram portions of the soil samples that had previously received no Pb were amended with Pb to a final concentration of 0, 2, 4, or 8 mg of Pb^{2+}/g of soil, and amylase activity was determined. Those samples to which Pb was added, followed by a further 24 h of incubation, would indicate the combined effect of Pb on amylase synthesis and activity, whereas the samples that received Pb only in the amylase assay mixture would indicate the effect of Pb on amylase activity only.

Culture media. Viable bacteria were enumerated by dilution and plating on a medium containing (in grams per liter): starch, 10; tryptone, 0.2; K_2HPO_4, 0.28; KH_2PO_4, 0.28; $MgSO_4 \cdot 7H_2O$, 0.18; and cycloheximide (Sigma Chemical Co.), 0.1. Cycloheximide was added to suppress growth of soil fungi (14). Fungi were enumerated on modified rose bengal agar (11) containing (in grams per liter): starch, 10; peptone, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; rose bengal, 0.03; and streptomycin, 0.2. A 10-fold dilution series with six plates per dilution was used for all samples. Plates were counted after 6 to 7 days of incubation at 28°C.

Pb resistance. The level of resistance to Pb was determined by supplementing the bacterial medium (above) with lead acetate. Lead acetate and medium components were autoclaved separately and mixed after sterilization. Isolates were randomly selected from streak plates of Pb-free medium and transferred with sterile toothpicks onto plates containing lead acetate.

Lead analysis. Soil samples were extracted 24 h in 0.1 M sodium acetate, pH 5.0. Soil was removed by centrifugation at 10,000 × *g* for 10 min. This procedure extracts biologically available Pb, primarily Pb that is water soluble or exchangeable (10). The supernatant fraction was filtered through 0.45-μm membrane filters (Millipore Corp.) to remove suspended material, diluted with an equal volume of concentrated HNO_3, and digested for 3 h at 90°C. Pb was determined by atomic absorption spectrometry (A. M. Hartley, personal communication).

Chemicals. Reagent-grade chemicals were used throughout this investigation. Lead salts of low water solubility were soaked in several changes of distilled water for 2 to 3 days and dried at 80°C before use.

RESULTS

A time course of enzyme synthesis in soil and the effect on this of lead acetate or calcium acetate is shown in Fig. 1. The results indicated that lead was an inhibitor of amylase synthesis and had a significant effect on invertase synthesis. However, amylase specific activity was not significantly lower in Pb-amended samples than in control samples 48 h after Pb addition. Invertase activity in Pb-amended soil returned to control levels 24 h after Pb addition. Calcium acetate did not affect amylase or invertase synthesis but did cause severe inhibition of α-glucosidase synthesis.

The significant reduction of α-glucosidase synthesis by both lead acetate and calcium acetate suggested that either acetate or divalent

cations in general inhibit synthesis of this enzyme. Because α-glucosidase synthesis was affected by ions other than Pb, further studies of this enzyme were not done. Invertase synthesis was not examined in more detail because synthesis was not as markedly inhibited by Pb as was amylase synthesis. Hence, of the enzymes examined, amylase appeared to be the most suitable for these studies by virtue of the absence of an effect of presumably nontoxic ions (e.g., Ca^{2+} and acetate) and the greater Pb sensitivity of amylase synthesis.

That the primary effect of Pb was on enzyme synthesis, and not enzyme activity, is shown in Fig. 2. At 2 mg of Pb^{2+}/g of soil, there was no significant inhibition of amylase activity, but the quantity of amylase formed was reduced to 26% of control levels. These data indicate that relatively low Pb levels were sufficient to inhibit enzyme synthesis without affecting enzyme activity. For the soil used, 2 mg of Pb^{2+}/g of soil represents only 8% of the Pb-sorption capacity of the soil.

The decline and subsequent recovery of amylase synthesis after lead addition (Fig. 1) suggested two possible mechanisms of Pb action: (i) inhibition of protein synthesis before Pb immobilization in the soil, with recovery occurring when the available concentration had been reduced to subtoxic levels, or (ii) destruction of the major Pb-sensitive, amylase-producing organisms followed by growth of Pb-resistant producers that initially made only a minor contribution to total amylase production. To determine the correct mechanism, viable bacteria and fungi were counted at the same time that enzyme assays were performed. The data in Fig. 3 show a rapid decline in amylase-producing bacteria after Pb addition, with a rise in the viable count coincident with increased amylase levels. The abundance of Pb-sensitive bacteria decreased rapidly after Pb addition, and the abundance did not return to preaddition values during the experiment (Table 1). The decline in abundance of Pb-sensitive bacteria did not occur if samples were not amended with lead. Fungal numbers did not change significantly during the interval of 0 to 48 h (Fig. 3). Because the dilution procedure used for fungal enumeration is primarily a measure of fungal spore numbers (22), an effect of Pb on fungal synthesis of amylase cannot be excluded. However, the nearly identical curves for amylase synthesis (Fig. 1a) and numbers of amylase-producing bacteria (Fig. 3) strongly suggest that soil bacteria, not soil fungi, were affected by Pb amendment.

The conditions used for the preceding experiments differ substantially from natural habi-

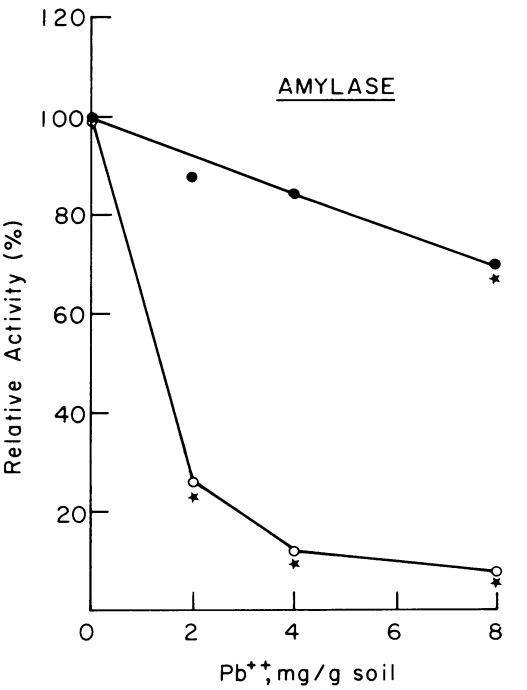

FIG. 2. *Relative amylase synthesis and activity as influenced by lead concentration. Relative activity or synthesis is expressed as enzyme units/g of soil* $(+Pb^{2+}) \div$ *enzyme units/g of soil* $(-Pb^{2+}) \times 100$. *Each point is the mean of six assays per Pb concentration. Symbols: ●, amylase synthesis; ○, amylase activity. Values for Pb-amended samples indicated by a star (★) are significantly less than values for Pb-free samples.*

tats in both the form of Pb added and the manner of addition of Pb. Most Pb is incrementally added to soil in natural environments as insoluble salts such as $PbSO_4$, PbO, or $Pb_3(PO_4)_2$, or as soluble $PbCl_2$ (5, 19). Consequently, Pb in native soils may be added in a form that is not available to biological systems and Pb may not be added during a period of intense biological activity. When added to soil, soluble Pb is rapidly bound to soil minerals and organic matter (2; F. J. Stevenson, personal communication). The effect of Pb after allowing time for Pb adsorption to soil was examined by amending moist, sifted soil with 8.2 mg of Pb (as $PbCl_2$)/g of soil and incubating the samples for 14 days at room temperature. Control samples were also incubated for 14 days, but no Pb was added. At this time, starch plus NH_4Cl was added, and portions were removed periodically and assayed for amylase activity. If Pb were biologically unavailable after the 14-day period, no effect on amylase synthesis would have been observed. The results obtained indicated that Pb was an

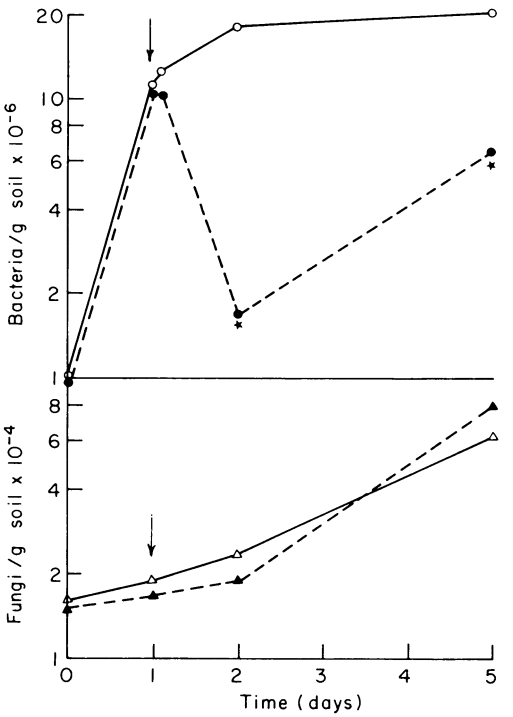

FIG. 3. *Effect of lead upon viable bacterial and fungal counts. Soil mixtures and incubation conditions were identical to those described to Fig. 1, except that calcium acetate treatment was omitted. Bacterial and fungal counts were determined at the indicated times. Symbols: ○, bacteria in $-Pb^{2+}$ samples; ●, bacteria in $+Pb^{2+}$ samples; △, fungi in $-Pb^{2+}$ samples; ▲, fungi in $+Pb^{2+}$ samples. Arrow indicates time at which lead was added to the appropriate samples. Values indicated by a star (★) at the same sample time are significantly different than control values (P = 0.05).*

effective inhibitor for at least 14 days after adding it to soil. The 14-day preincubation before starch addition also affected the ability of soil organisms to synthesize amylase in samples to which Pb was not added, but the reduction in the rate was more severe in Pb-amended soil (data not shown).

Since $PbCl_2$ and lead acetate are water soluble, 10 mg/ml of water and 460 mg/ml of water, respectively (6), experiments were carried out to determine the influence of salt solubility on Pb inhibition and to establish the amount of initially insoluble Pb needed to decrease amylase synthesis. These questions were answered by adding PbS (water solubility = 8.6×10^{-4} mg/ml), $PbSO_4$ (water solubility = 4.2×10^{-2} mg/ml), or PbO (water solubility = 1.7×10^{-2} mg/ml). Lead oxide did not inhibit amylase synthesis when added at 13 mg of Pb^{2+}/g of soil, whereas $PbSO_4$ at 10.2 mg of Pb^{2+}/g of soil reduced amylase synthesis to 35% of control levels after 48 h. Lead sulfide, in spite of its low water solubility, was as inhibitory as $PbCl_2$ at equivalent Pb concentrations (Table 2). Lead chloride was less inhibitory than lead acetate when added at <900 μg of Pb^{2+}/g of soil. The degree of inhibition was not related to soluble Pb^{2+} concentrations in the soil, as soluble Pb levels for a particular salt changed very little over the experimental period (Table 3). In addition, soluble Pb was highest when $PbSO_4$ was added, but this salt was a less potent inhibitor than lead acetate, $PbCl_2$, or PbS.

There was no difference in amylase synthesis between soils receiving no salt and soil amended with 1 mg of MnS/g of soil or 1.3 mg of $CaSO_4$/g of soil. However, 1.3 mg of $CaCl_2$/g

TABLE 1. *Level of Pb resistance of amylase-producing bacteria before and after addition of lead acetate to soil*[a]

Time (h)	No. of isolates resistant to:		
	<500 μg of Pb^{2+} per ml	>500, <1,000 μg of Pb^{2+} per ml	>1,000 μg of Pb^{2+} per ml
24	11	28	46
25	3	53	40
48	1	43	47
120	0	58	39

[a] Soil samples from which isolates were selected were treated as described in the legend to Fig. 1. Lead acetate (1.5 mg/g of soil) was added immediately after taking the 24-h sample.

TABLE 2. *Comparative inhibition of amylase synthesis by lead acetate, chloride, and sulfide*

Hours after Pb addition	Pb concn (μg of Pb^{2+}/g of soil)[a]	Amylase sp act		
		Lead acetate[b]	$PbCl_2$	PbS
24				
	0	1.80a[c]	1.80a	1.80a
	450	0.72b	1.97a	1.64a
	900	0.62b	1.24a	1.50a
	1800	0.44c	0.49bc	0.73b
48				
	0	3.95a	3.95a	3.95a
	450	3.21ab	5.53c	2.95b
	900	2.72b	3.11a	4.95c
	1800	1.84b	0.93d	1.66b

[a] Soil was amended with 1 g of starch and 0.16 g of NH_4Cl/100 g of soil and the indicated lead salt at zero time. Enzyme assays were performed on samples after 24- or 48-h incubations.

[b] Pb sources added.

[c] Numbers for the same sample time followed by the same letter are not significantly different (P = 0.05). Values given are the means of 6 or 12 samples.

TABLE 3. *Soluble lead levels in soil amended with various lead salts*

Lead salt added[a]	Soluble Pb (μg/g of soil)	
	0 h[b]	48 h
Lead acetate	7.9	7.9
Lead chloride	6.8	5.6
Lead sulfate	10.5	10.1
Lead sulfide	2.6	2.6
None	<0.8	<0.8

[a] Soils were amended with starch and the indicated lead salt (1.8 mg of Pb^{2+}/g of soil) and sampled immediately or after 48 h of incubation. Lead was extracted and assayed as described in Materials and Methods.

[b] Time after Pb addition.

of soil significantly increased the rate of amylase synthesis over samples receiving no salt. Since calcium acetate did not affect synthesis (Fig. 1a), the chloride ion was the most probable stimulant of amylase. The lack of effect of MnS or $CaSO_4$ indicates that the inhibition observed with PbS (Table 2) and $PbSO_4$ was due to Pb, not to the anions, S^{2-} or SO_4^{2-}.

DISCUSSION

Except for PbO, significant inhibition of amylase synthesis was observed when various Pb salts were added to soil. The major effects of Pb were on the number of amylase-producing bacteria (Fig. 3) and amylase synthesis (Fig. 1a), findings which indicated that the most likely mechanism of Pb action was destruction of amylase-producing cells, and not solely inhibition of protein synthesis. The different degrees of Pb sensitivity of amylase, α-glucosidase, and invertase synthesis strongly suggest that these enzymes are produced by different groups of soil organisms, each of which is more or less sensitive to destruction by Pb.

The experimental procedure used makes it unlikely that recovery of amylase synthesis was due to induction of a Pb resistance mechanism similar to that reported in cadmium-resistant *Escherichia coli* (13). Cells tested for Pb resistance were isolated on Pb-free medium before testing their level of resistance. The large number of generations required for a single cell to give rise to a macroscopically visible colony should have been sufficient to allow cells to return to an uninduced state. Therefore, the conclusion that the recovery of amylase synthesis is the result of changes in the nature, rather than activity, of the soil population seems justified. Soluble Pb^{2+} levels remained constant during a 48-h period after lead addition (Table 3).

The loss of Pb-sensitive isolates after Pb addition suggests that contamination of soil with Pb may bring about marked qualitative changes in the soil population. Pb-induced qualitative changes in the soil population could be more important in natural habitats than the transient inhibition of enzyme synthesis. For example, if the Pb-resistant members of the population were plant pathogens, or if the Pb-sensitive organisms were either normal competitors of plant pathogens or the major group required for some degradative function other than starch hydrolysis, major changes in disease incidence or substrate degradation would occur. This aspect of lead pollution will be examined in the future.

The lag period before inhibition by PbS is observed indicated that Pb in this compound is initially biologically inert, but is converted to a reactive form upon incubation with a nutrient source. It is possible to estimate the quantity of PbS that was converted to a biologically available form by reference to Fig. 2. Using amylase inhibition, the amount of Pb required to reduce amylase synthesis to 50% of control values was 1.5 mg of Pb^{2+}/g of soil. Based on the data in Table 2, the quantities of lead as $PbCl_2$ and PbS required to obtain 50% inhibition were 1.26 mg of Pb^{2+}/g of soil and 1.72 mg of Pb^{2+}/g of soil, respectively. It should be noted that this estimate of the amount of Pb converted to a biologically available form is expressed as "the amount giving an effect equivalent to 1.5 mg of Pb/g of soil as lead acetate" and does not necessarily indicate that the actual amount of available Pb was 1.5 mg/g of soil. The small quantities of "available Pb" (Table 3) indicate that the inhibition observed in our studies may have been caused by as little as 5% of the total Pb added. The extent and duration of Pb inhibition did not appear to be related to water solubility of the Pb compound added. There was little difference in the degree of reduction of amylase synthesis when either lead acetate or PbS was added, in spite of the fact that lead acetate is about 500,000 times more water soluble than PbS.

The experimental approach utilized in this study may be applicable to analysis of the effects of pollutants other than heavy metals. There are several advantages over conventional procedures such as plating. First, by assaying a single batch of soil, the large variation between samples that is so common is reduced. For example, the standard deviation for the enzyme assays was ±15% or less of the mean of 6 to 12 replicates; this narrow range is much less than found with the typical plating procedures, where the standard deviation is frequently ±50% of the mean. The enhanced preci-

sion enables one to detect quite small perturbations in soil biological activity, a result that is particularly desirable if long-term effects of small quantities of a pollutant are being examined. Second, the effect of a pollutant on soil organisms catalyzing specific reactions can be examined simply by adding the appropriate substrate and following enzyme levels over time. This technique would be particularly applicable if a trait were a nearly universal attribute of a microbial group, such as chitin hydrolysis by actinomycetes or decomposition of aromatics by *Pseudomonas*.

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