SOIL ENZYME - SOIL AMENDMENT INTERACTIONS

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by

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This thesis is dedicated to my parents in grateful recognition of their support, encouragement and numerous sacrifices throughout my education.

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The soil, too, whether bare and dry or wooded and watered, hollow and hot, or high and cold.

Hippocrates (460 - 375 B.C.)
On Airs, Waters and Places.

ABSTRACT

A simple, rapid and reproducible method of assaying 1,3-β glucanase activity in soil has been developed, using laminarin as a substrate. It was founded upon a detailed analysis of the factors involved such as: quantity, type and age of soil; choice of: buffer, substrate concentration, microbial inhibitor, pH, temperature and incubation time, and methods of terminating the reaction. The broader implications of this methodological investigation are discussed in relation to soil enzyme assays in general.

Biochemical characterisation of the soil, 1,3-β-glucanase revealed a pH optimum of 5.4 (the same as the soil), a temperature optimum of 50° to 65°C, an activation energy of 49 kJ/mole and a Michaelis constant of 0.2 mg/ml. The predominant activity appeared to be exohydrolytic since glucose was the only detectable breakdown product. Activity was irreversibly reduced by 50% upon air-drying but thereafter remained constant for an indefinite period when the soil was stored at room temperature (21 ± 2°C). This remarkable stability was also observed in irradiated soil and long-term incubations of wet soil at a range of temperatures and suggests that 1,3-β-glucanase is a typical accumulated soil enzyme.

In addition, the possibility of using soil enzymes as monitors of agrochemical* effects in soil

* Throughout this thesis the term agrochemical is used to describe any material which is added to soil in an agricultural context and hence includes pesticides, fertilisers, lime and manures.

have been investigated in the laboratory. The choice of test enzymes, 1,3-β-glucanase and urease, was based partly on the considerable input of their substrates into soil; 1,3-β-glucans being cell wall components and cytoplasmic or vacuolar reserve materials in micro-organisms and plants, and urea being an agricultural nitrogen fertiliser as well as a mammalian excretory product.

Concentrations equivalent to 5 times the recommended field application rates of the pesticides, 2,4-D, diallate, glyphosate, benzoylprop ethyl and malathion, applied as formulations, had no effect on the activity of either soil enzyme under any of the incubation conditions tested. The latter included unamended air-dried, field-wetness and flooded soil and air-dried soil amended with NPK fertiliser, urea, pig slurry, ground limestone, (all at concentrations equivalent to field application rates) cellulose and glucose. All soil was maintained at 65% WHC (except the flooded soil) and incubated at room temperature for 14 to 90 days.

As far as the pesticides were concerned, these enzyme systems could only be disrupted by unrealistically high dosage rates (100 to 1000 ppm) of some of the active ingredients. Thus, 1,3-β-glucanase activity was enhanced by 2,4-D, inhibited by diallate, benzoylprop ethyl and malathion, but unaffected by glyphosate, whereas urease activity was inhibited by 2,4-D but unaffected by diallate, glyphosate and benzoylprop ethyl.

Of the non-pesticide amendments NPK fertiliser and urea had no effect on either enzyme. 1,3-β-Glucanase activity was enhanced by pig slurry, cellulose and glucose but inhibited by ground limestone, whereas urease activity was enhanced by glucose, inhibited by pig slurry but unaffected by ground limestone and cellulose. The activity of neither enzyme was altered by flooding the soil.

The isolation and enumeration of microorganisms capable of producing 1,3-β-glucanase and urease is described and the results are discussed in terms of interactions between such microorganisms and the agrochemicals, with particular reference to enzyme origin.

It is suggested that specific soil enzyme activity estimations have certain advantages over some of their more commonly used **alternatives** (such as counts and composition of microbial populations) in monitoring the effects of agrochemicals on the soil microflora.

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PART ONE - INTRODUCTION

A. SOIL ENZYMES

1. Introduction

Reviews by Skujins (1967, 1976), Kuprevich and Shcherbakova (1971), Kiss, Dragon-Bularda and Radulescu (1975) and Ladd (1978) reveal that approximately fifty enzyme systems have been assayed in soil. With the exception of the isomerases and ligases they represent all enzyme classification groups although the majority are hydrolytic and catabolic (Table 1).

It is important to emphasise the term enzyme systems because for many catalysts the observed activity does not represent the action of one particular enzyme. This point is admirably illustrated by dehydrogenase which is assayed by measuring the formation of a reduced dye resulting from the oxidation of generally unknown endogenous substrates (Benefield, Howard and Howard, 1977). Numerous enzymes must be represented in this non-specific assay. Although this is an extreme case, reservations also apply to other soil enzymes such as the proteases and nucleases whose activities have been measured without identification of the bonds hydrolysed or all the products formed.

Total soil enzyme activity can be broadly regarded as being the sum of one intracellular and two extracellular components (Burns, 1977). The first extracellular fraction is either free in the soil solution or

TABLE 1 Enzymes in Soil (amended from Skujins, 1976)

Oxidoreductases	Hydrolases	Lyases
Catalase	Acetylesterase	Aspartate decarboxylase
Catechol oxidase	α- and β-Amylase	Glutamate decarboxylase
Dehydrogenase	Arylsulphatase	Tryptophan decarboxylase
Diphenol oxidases	Asparaginase	Tyrosine decarboxylase
Glucose oxidase	Cellulase	
Peroxidase	Dextranase	
Polyphenol oxidase	α- and β-Galactosidase	Transferases
	α- and β-Glucosidase	Aspartate transaminase
Urate oxidase	Glutaminase	Glutamate transaminase
	Inulase	Leucine transaminase
	Invertase	Pyruvate transaminase
	Levanase	Valine transaminase
	Lichenase	Dextran sucrase
	Lipase	Levan sucrase
	Malathion esterase	Rhodanese
	Nucleotidases	
	Phosphatase	
	Phytase	
	Polygalacturonase	
	Polyphosphatase	
	Proteases	
	Urease	
	Xylanase	

attached to living cells, whilst the second is stabilised by association with the soil colloids, the clays and humic materials. Both extracellular components are made up of enzymes excreted by living cells and those released upon cellular death and subsequent lysis. Intracellular catalysis need not be restricted to living cells; Skujins and McLaren (1969) have suggested that enzymes continue to function within a cell even after its death. In such a situation the substrate and products pass into and out of the cell by passive diffusion. The contribution made by each of these fractions to total soil enzyme activity varies considerably from soil to soil and enzyme to enzyme (Fig. 1).

Kiss et al. (1975) used the expression "accumulated enzymes" to describe the activity independent of immediate microbial proliferation, whilst Skujins (1978) has coined the adjective "abiontic". From a comparison of substrate transformation in the presence and absence of chemicals preventing microbial growth it is generally agreed that accumulated enzymes have an important role to play in carbon, nitrogen, sulphur, phosphorus and other biogeochemical cycles; those essential transformations upon which life on this planet depends.

2. Origin

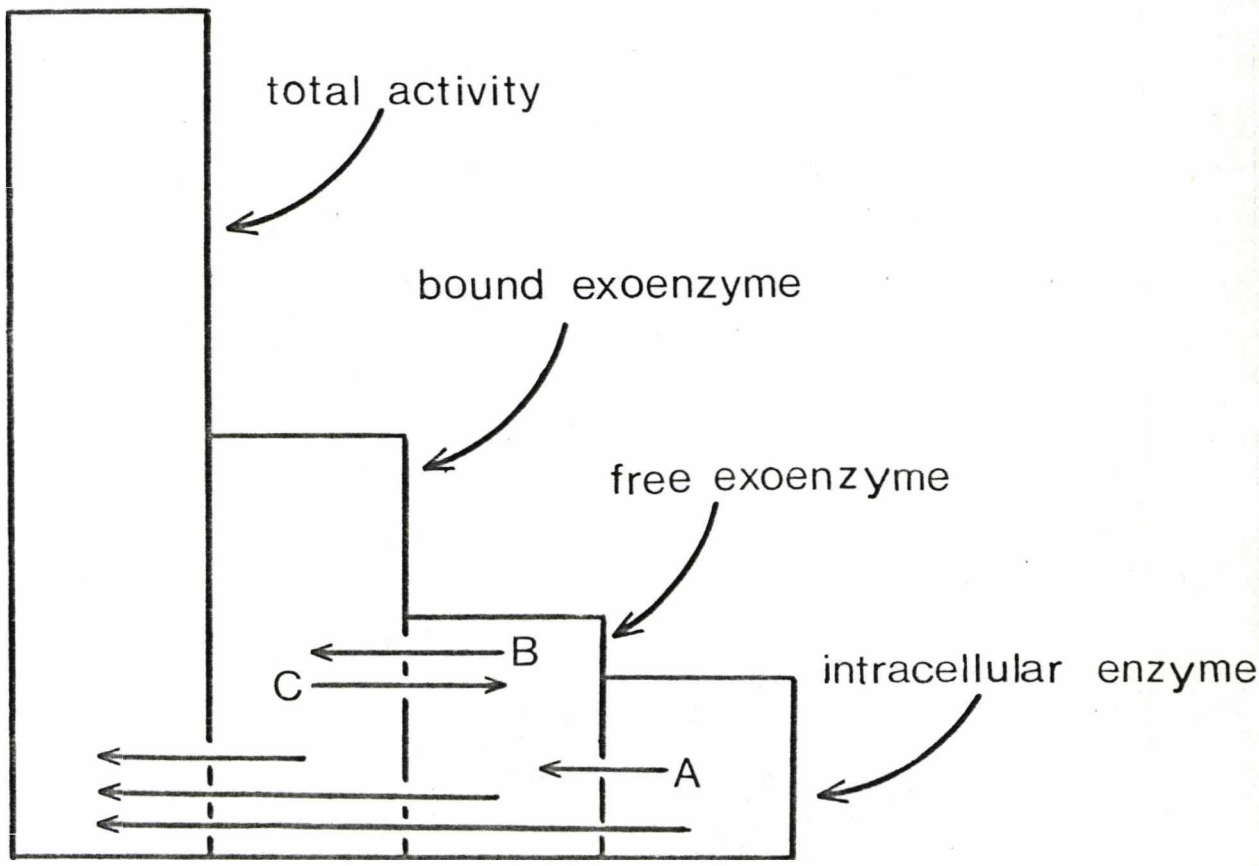
Although it would seem reasonable to propose that enzymes in soil originate from microbial, plant and animal sources, experimentally it has not proved

FIGURE 1 Components of total soil enzyme activity (from Burns, 1977)

A = Enzyme released from lysed cells and leakage from extant cells.

B = Extracellular enzyme becoming bound to soil colloids.

C = Leakage of bound enzyme from soil colloids.



possible to quantify the contribution of these sources to total activity. Attempts to determine the source of soil enzymes have shown relationships with the total microflora, specific groups of bacteria or fungi, organic carbon, pH, plant roots, soil fauna and even lichens and algae found on the soil surface.

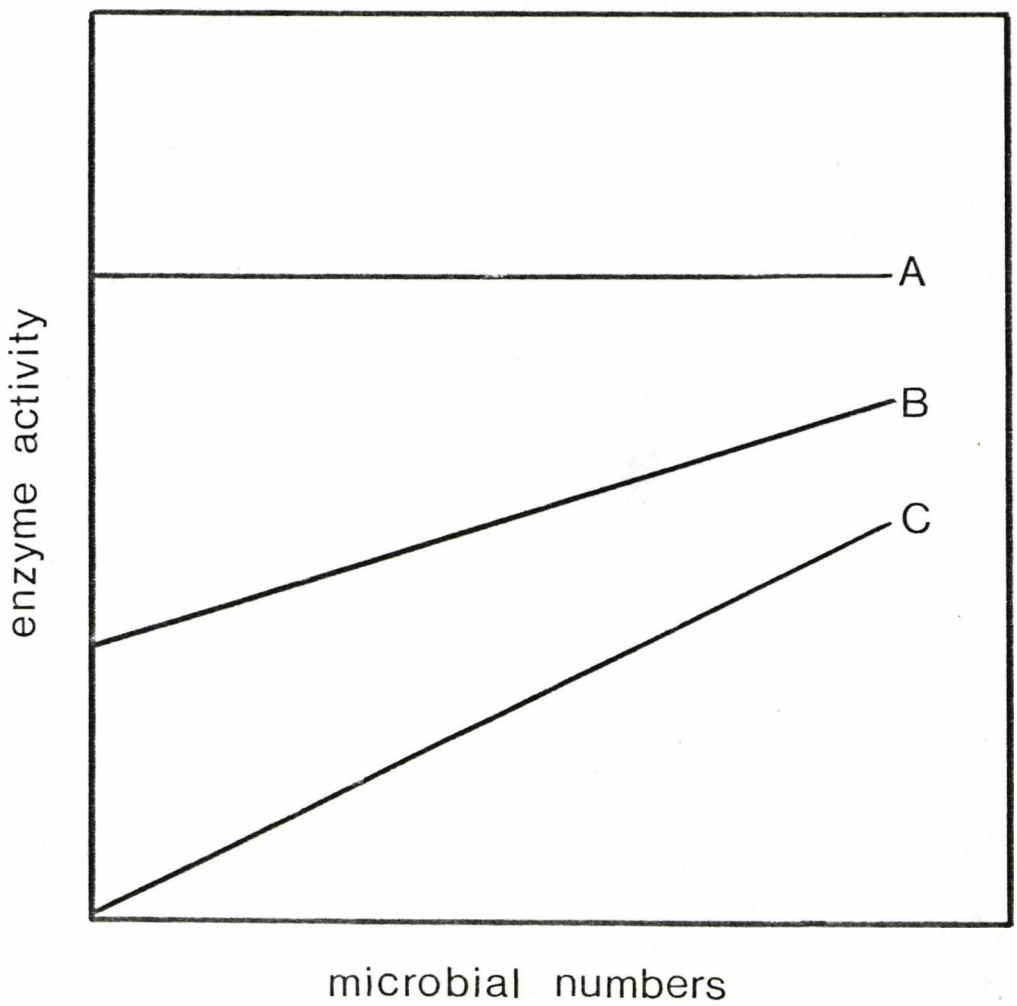
It is a widespread assumption that soil enzymes are primarily derived from microorganisms, although large fluctuations in microbial numbers are not necessarily reflected in enzyme activities (Stevenson, 1959; Skujins, 1973). Figure 2 describes a number of theoretical relationships between microbial numbers and enzyme activity. Response A shows that enzyme activity is completely independent of microbial proliferation. However, if there is a direct relationship between the activity of a particular enzyme and microbial proliferation then one would expect response B or C. A straight line through the origin (C), such that when the microorganisms are absent so is the activity, indicates that intracellular microbial activity, or short lived extracellular activity is the major contributor. Response B suggests that although the relationship of microorganisms to activity is still in evidence, a proportion of activity is independent of immediate microbial proliferation (McLaren, 1972). This proportion is thought to represent the two extracellular fractions. From studies on irradiated soil Pettit, Smith, Freedman, and Burns, (1976) proposed that approximately 60% of the total urease activity

FIGURE 2 Relationship between microbial numbers and enzyme activities (from Burns, 1977)

A = Enzyme activity independent of microbial proliferation

B = Enzyme activity related to microbial proliferation plus an independent background level.

C = Enzyme activity totally dependent upon microbial proliferation.



in their soil was in the extracellular bound form.

Evidence for the persistence of plant enzymes in soil is indirect and rests mainly on the effects of season, cultivation and the nature of plant cover on soil enzyme activities (Pancholy and Rice, 1973 a,b) and investigations with fresh plant homogenates (Ross, 1975 a,b), plant exudates or plant debris separated from soil (Ladd, 1972). Whether these changes are a direct effect of plant growth or the associated response of a rhizospheric microflora is uncertain.

Much of our knowledge of soil enzymes is based upon indirect evidence so it is especially pleasing to read Ladd's (1978) novel ideas concerning their origins. He describes an in situ technique which appears to have the potential for deducing the origin of soil enzymes from their location in intact cells and in partly decomposed but recognisable cell fragments. The sites of enzyme activity can be demonstrated at the ultrastructural level (to within 1 nm) using a combination of histochemistry and electron microscopy.

Such investigations will not only yield information concerning the source of soil enzymes but also about the extent to which their stability is due to protection within intact dead cells compared to extracellular protection by the soil colloids. Early results suggest that plant phosphatases persist in soils within decomposing plant materials. Cells deep within the tissue are less damaged and contain more sites of active phosphatases than cells nearer the tissue

surface.

3. Location and Persistence

The most striking difference between enzymes accumulated in soil and other free enzymes is that the former are stable under conditions which would rapidly denature the latter (Pettit et al., 1976). One would predict that free enzymes excreted by live cells or released from disrupted dead cells would be rapidly degraded or denatured in the hostile soil environment. In the absence of further synthesis the concentrations of these free enzymes would be expected to decline rapidly due to hydrolysis by extracellular microbial proteases or denaturation by the physico-chemical stresses encountered in the soil. Although a great deal of activity is lost in this manner, a certain proportion appears to survive for long periods.

It is now generally recognised that mechanisms exist in the soil whereby free enzyme can become stabilised and hence persist for long periods after the original source has been destroyed. For example, Skujins and McLaren (1968) showed that urease and phosphatase activities were present in several 9,000 year old permafrost soils which had no active microflora. Interestingly, these enzymes were not detected in 32,000 year old buried soil samples despite their contamination in transit. Less dramatically, Pettit et al. (1976) observed that jack bean urease lost 58% of its activity upon freezing, whereas soil urease activity

decreased by only 5%.

The stability of soil enzymes is thought to be due to the formation of clay-enzyme and humus-enzyme complexes. Clay minerals, which in essence are composed of alternating silicon oxide and aluminium oxide or hydroxide sheets, possess an overall negative charge and may expand (montmorillonite) or not expand (kaolinite) upon hydration. Enzymes can be adsorbed both onto and within the clay lattice and whilst this usually decreases enzyme activity (Skujins, Pukite and McLaren, 1974) it occasionally slows down their rate of inactivation by proteases and denaturing agents (Sorenson, 1969). Interlamellar entrapment is known to protect proteins from direct microbial attack (Pinck and Allison, 1951; Estermann, Peterson and McLaren, 1959). This adsorption may enhance activity by causing conformational changes of the enzyme (Burns, 1977).

Humus, a complex colloidal gel, consists of high molecular weight polyphenols, aromatic amino acids, vitamins, polysaccharides and polyuronides. Its charge fluctuates according to pH and its constituent molecules, but like the clays is predominantly anionic. Humic materials are reported to have similar effects to clays on proteins and enzymes (Rowell, Ladd and Paul, 1973; Verma, Martin and Haider, 1975). Small amounts of enzymes have been extracted from soil and after partial purification have been shown to be associated with the humic material (Burns, El-Sayed and McLaren,

1972; Burns, Pukite and McLaren, 1972; Nannipieri, Cervelli, Pedrazzini, 1975; McLaren, Pukite and Barshad, 1975; Cacco and Maggioni, 1976; Ceccanti, Nannipieri, Cervelli and Sequi, 1978). Pettit et al. (1976) have shown that a urease-organic matter complex extracted from soil was more resistant to temperature inactivation and proteolysis than was pure jack bean urease.

It seems likely that enzymes become stabilised by humic materials in one of three ways: entrapment within the cross-linked gel during its synthesis, adsorption by ion exchange or hydrogen and covalent bonding, or by entering into a chemical relationship with it by actually becoming part of the humic polymer as occurs with some proteins (Verma, Martin and Haider, 1975) and pesticides (2,4-D). The bacteriostatic properties of the phenolic components of humus may offer additional protection against microbial attack.

It is difficult to determine whether it is the clay or organic matter which plays the more important role in stabilising extracellular enzymes because in most agricultural soils these two colloids are intimately associated to form the organo-mineral complex, but recombination and separation studies strengthen the case for organic matter. The long term protective influence of soil can be mimicked by artificial clay-enzyme-organic matter complexes (Estermann et al., 1959; Burns et al., 1972 a,b) while clay on its own has only an ephemeral effect (Skujins et al., 1974; Morgan and Cooke, 1976).

Organic matter enzyme analogues have been reviewed by Ladd and Butler (1975).

The fact that jack bean urease is rapidly inactivated on contact with soil (Moe, 1967; Roberge, 1970; Zantua and Bremner, 1977) suggests that either a native soil's capacity for protection is saturated and that any excess urease is broken down or that other mechanisms besides excretion are required for stabilisation, such as humification.

Paulson and Kurtz (1970) and Dalal (1975b) have claimed that comparisons of Michaelis constants and activation energies of both accumulated and microbial enzyme fractions in soil are evidence that soil enzymes occur at least partly as adsorbed extracellular complexes. In both cases the values were much greater for the accumulated than the microbial fraction. They argued that one might expect adsorption to alter both the enzyme's affinity for substrate and the energy requirement for formation of the enzyme-substrate complex.

Although it seems necessary for some of the mechanisms already mentioned to function for the stabilisation of truly extracellular enzymes, there is no reason why the stability of intracellular enzymes could not be due merely to their location within intact dead cells. Alternatively some stability might be conferred upon membrane bound enzymes which survive cell disruption in this state. These dead cells or stabilised enzymes associated with fragments of cell

debris could then adsorb to, or be enclosed within, the organo-mineral complex.

4. Estimating Activity

It is a scientific dogma that the results and hence conclusions are only as good as the methods from which they are derived. In other words, if the methodology is suspect one can have little confidence in the experimental findings.

The soil enzymologist not only has to cope with the basic problems of any enzyme assay: choice of buffer, pH optimum, substrate concentration, length of incubation period, stopping the reaction and determination of product etc., but also those specific to a medium like soil: prevention of microbial turnover of substrate or products, removal of soil at the end of the reaction, adsorptive influences of the soil colloids and extensive replication. Nor do the difficulties end here, because many of the problems associated with pure enzyme assays assume an increased degree of complexity when encountered with soil (see Methods and Materials).

Unfortunately many of these problems have proved insurmountable to some workers due in part to lack of thought, but primarily to ignorance of basic biochemical principles. For example, it is amazing to read of an assay for arylsulphatase in soil using 1mM p-nitrophenyl sulphate as substrate when the authors have reported Km values for the enzyme ranging from 1.37 to 5.69mM

(Tabatabai and Bremner, 1971). Thus we are presented with an enzyme assay in which the substrate concentration is manifestly rate limiting. Mistakes of this kind are by no means rare and probably reflect the large numbers of soil and agricultural scientists expanding into microbiology and biochemistry without prior training. Hopefully such errors will be eliminated with the influx of a new generation of biochemically-aware microbiologists and biochemists.

The problems involved in developing a colorimetric assay for one soil enzyme -1,3-β-glucanase- will be discussed in parts two and three of this thesis, but the fundamental issues which apply to any soil enzyme assay are outlined below.

Soil enzymes are assayed either by monitoring substrate disappearance or product appearance, but wherever possible the latter should be chosen. It is important that the substrate concentration should be in excess throughout the assay period to avoid substrate limitation of the enzyme and under such circumstances the ratio of converted to unconverted substrate is small. Hence, measuring substrate disappearance involves looking for small changes in substrate concentration and is less sensitive than monitoring product appearance. If the method used to detect the product also detects the substrate then the assay should be allowed to proceed until the value of the assay is large compared to the value of the substrate blank. Finally, immobilisation and adsorption of both substrate

and product should also be taken into account.

In soil it is not easy to distinguish between the various contributors to total enzyme activity. When measuring the activity of an accumulated enzyme it is necessary to prevent substrate turnover due to microbial proliferation. This is achieved by employing a short assay period (one hour) or, where a long term incubation period is necessary, a microbial inhibitor. However neither of these methods will prevent passive diffusion of substrate into latent cells which is why Kiss et al. (1975) include this activity in their definition of accumulated enzymes. In some assays the inhibitor has a second function, namely to prevent the microbial assimilation of product. This is particularly relevant when measuring the activity of enzymes producing the almost universal carbon substrate glucose in soil, since there would be no linear build up of this product in the absence of an inhibitor.

Four types of anti-microbial agents have been used: toluene, irradiation, azide salts and antibiotics. In keeping with my earlier criticisms of soil enzyme methodology, toluene, the one with the most disadvantages, is the one most commonly used (Ross, 1968; Dalal, 1975b). The use of toluene is not recommended for the following reasons:

1) It is decomposed by soil bacteria (Claus and Walker, 1964) and hence may cause an increase in enzyme activity.
2) It can give artificially high values for activity (Conrad, 1942b) by increasing the

permeability of cell membranes to substrates and products and stimulating microbial autolysis, thus setting free more enzyme (Thente, 1970).

3) Solvents in general are powerful inhibitors of soil urease activity (Lethbridge, Pettit, Smith and Burns, 1976).

The extensive literature concerning the use of toluene in soil enzyme assays implies that this reagent may cause more problems than it solves (Kiss and Boaru, 1965; Skujins, 1967, 1976; Kiss et al., 1975).

The most effective form of irradiation for sterilising soil is high energy ionising radiation - such as gamma rays. The major drawback to this method is that the dose required to completely eliminate proliferating microorganisms (ca 5 Mrads) usually inactivates a proportion of the enzyme activity (Pettit et al., 1976). Coupled with this, sterilising doses of gamma irradiation are known to induce physico-chemical changes within the soil such that the enzyme is no longer being assayed within its natural environment. Griffiths and Burns (1968) showed that soil aggregate stability was decreased by such doses and postulated that it was due to the depolymerisation of soil polysaccharide (a major contributor to soil humus). The effects of radiation on the microbial and biochemical properties of soil have been reviewed by McLaren (1969) and Cawse (1975).

Although rarely used in soil enzyme assays sodium and potassium azide (Pettit et al., 1976; Gibson and Burns, 1977) are a great improvement on the two previous

microbial inhibitors. It is well known that azides inhibit numerous microbiological transformations (Clifton, 1946) by interfering with oxidative enzyme systems such as cytochrome oxidase. They have been used to increase the persistence of the carbanilate herbicide chloropropham which is normally rapidly biodegraded in soil (Kaufman, 1977) and to retard the rate of urea hydrolysis (Bremner and Bundy, 1976). However these inhibitors are not faultless either and must be checked for inherent effects on the activity of the enzyme to be assayed because azides are classical enzyme inhibitors, particularly of peroxidases and oxidases. They also undergo chemical degradation in soil, especially under acidic conditions (Ketchersid and Merkle, 1976).

Antibiotics have only been employed sporadically in this context. Benefield (1971) used penicillin G to measure cellulase activity but most of the investigations have involved antibiotics which inhibit protein synthesis, thus directly preventing enzyme synthesis and microbial proliferation. Kiss et al. (1975) have discussed the use of such antibiotics (streptomycin and chloromycetin) in the study of carbohydrases (invertase, maltase and cellobiase) in soil.

There has been considerable debate about the choice and even the use of buffers in soil enzyme assays (Tabatabai and Bremner, 1972; Zantua and Bremner, 1975a; Burns, 1978). Those opponents of buffered assays argue that they are artificial and that

activity should be measured at soil pH to obtain an index of activity under natural conditions. However, since the whole assay is artificial compared to the field (substrate in excess, constant temperature, microbial inhibitor and soil reduced to structureless slurry) then this argument collapses. It is standard enzymological practise to perform enzyme assays in buffer and soil enzymes should be no exception. As long as it is understood that the assay measures the maximum potential of that enzyme under the stated conditions and not its in vivo activity then there is no problem. The practical advantage of the buffer method will be discussed with specific reference to soil 1,3-β-glucanase in the Methods and Materials section.

Many of the divergent findings in soil enzymology are obviously due in part to the vastly different methodologies. This variable methodology only serves to complicate the study of an already complex environment and there is clearly a need for standardisation to facilitate valid comparisons between the growing number of research communications.

5. Applications

Despite the fact that current research into soil enzymology is of a fundamental nature the subject has many potential applications in agriculture, medicine, industry, extraterrestrial life detection and even criminology.

In agriculture they can be used as monitors of

pollution by agrochemicals (Lethbridge and Burns, 1976; Burns and Lethbridge, 1976; Lethbridge, Bull and Burns, 1976) and heavy metal ions (Tyler, 1974). Indeed, because of their reproducibility they are a huge improvement on the notoriously inaccurate microbial counts in such studies. They may even serve as indices of soil fertility since they play an important role in nutrient cycling and in some cases reflect microbial activity (Howard, 1975).

Burns (1972) has suggested that the addition of immobilised enzymes to soil may stimulate the mineralisation of organic matter to produce plant materials and the decay of recalcitrant pesticides. Interestingly, there is a strong indication that accumulated soil enzymes are involved in the degradation of the organophosphate insecticides malathion (Getzin and Rosefield, 1971; Satyanarayana and Getzin, 1973; Gibson and Burns, 1977) and methyl parathion (Kishk, El-Essawi, Abdel-Ghafar and Abou-Donia, 1976).

Soil enzyme activities (and indeed biological activity in general) should be taken more into account when designing and recommending the use of synthetic fertilisers. They may be a useful aid to turnover or a nuisance in that they break it down too quickly, as is the case with urea (see Introduction, C) and have to be inhibited by the addition of yet another chemical which may in turn have effects on other systems within the soil.

Immobilised enzymes are already used extensively in medicine (production of semi-synthetic penicillins and steroid modification) and industry (conversion of starch to sugar and hydrolysis of lactose in milk or whey) yet many of these systems are costly to prepare and inefficient due to enzyme leakage (Zaborsky, 1973). An understanding of the factors involved in soil enzyme stability could herald the use of clays and humic materials as inexpensive immobilising agents.

Assaying for urease activity by monitoring radioactive carbon dioxide evolution from soil treated with labelled urea has been used by NASA in attempts to detect life on the Moon. The choice of urease is based on two criteria. Firstly, its substrate may have arisen abiologically and thus any primitive living system may possess enzymes capable of degrading it. Secondly, urease activity is known to survive in soil long after the cessation of life forms from which it originated. Such a monitoring device would therefore measure both existing and extinct biological activity. Finally, one rather bizarre application for soil enzymology has been proposed by Thornton and McLaren (1975) who suggested a role for soil enzymes in criminology, based on the fact that the Km for a particular enzyme varies from soil to soil. This might enable the forensic scientist to compare soil samples taken from a suspect with those from the scene of a crime.

B. UREASE

1. Introduction

Urease (urea amidohydrolase, EC. 3.5.1.5) catalyses the hydrolysis of urea to ammonia and carbon dioxide via ammonium carbamate [I] and thus

$NH_2CONH_2 + H_2O \rightarrow NH_2COONH_4 \rightarrow CO_2 + 2NH_3$ ..... [I]

participates in both carbon and nitrogen cycles. It is also involved in the terminal step of purine, pyrimidine and arginine degradation. Urease has been detected in many higher plants and microorganisms, particularly bacteria, several animals and even the gastric mucosa of man. Strictly speaking the term 'ureases' should be used because urease activity is exhibited by several protein species from many different sources (Reithel, 1971). Jack bean (Canavalia ensiformis) urease was the first enzyme to be crystallised (Sumner, 1926) and most of our knowledge of urea hydrolysing enzymes is derived from it. It is not specific for urea but will act upon hydroxyurea, dihydroxyurea, thiourea, semicarbazide and p-nitrophenyl carbamate (Bennett and Wren, 1977). It is probably a nickel metalloenzyme (Dixon, Gazzola, Blakeley and Zerner, 1975) with a molecular weight of ca.483,000 (Lehninger, 1970).

The first hint that soil possessed urease activity was obtained by Rotini in 1935, but it was left to Conrad (1940; 1942 a,b; 1943) to conclusively demonstrate both its presence and involvement in the

mineralisation of urea nitrogen. Since ureases from living sources are not alike it is not surprising that soil urease, whose origins probably vary considerably from soil to soil, differs significantly from such as jack bean urease (Bremner and Mulvaney, 1978). In general, optimum pH, Michaelis constant and activation energy values are higher than the corresponding values for jack bean urease. Unfortunately the majority of these comparisons have been based on work which is not directly comparable since it was carried out in a number of different laboratories under different conditions. However the few internal comparisons which have been performed at the University of Kent (Pettit et al., 1976) confirm these statements. It is worth noting however that kinetic data derived from heterogeneous soil environments and from homogeneous solutions are not directly comparable because of surface effects (Cervelli, Nannipieri, Ceccanti and Sequi, 1973; Irving and Cosgrove, 1976).

Urease is an intracellular enzyme (Sumner and Somers, 1947) and hence it is important to be aware that accumulated soil urease activity also includes that due to substrate turnover in non-proliferating cells.

2. Assay Methods

If one ignores the choice or even the use of a buffer and a microbial inhibitor, both of which have been discussed previously (see Introduction, A.4),

then the assays of urease in soil fall into three categories. These involve estimation of ammonium or carbon dioxide produced or urea decomposed. There are numerous ways of estimating ammonium production, (the most widely used method). These have involved direct colorimetric determination (McGarity and Myers, 1967), direct titrimetric determination (McLaren, Reshetko and Huber, 1957), and steam distillation followed by titration (Tabatabai and Bremner, 1972). Compared to the first two, the third is incredibly tedious and as a result less variables and replicates can be investigated in any one experiment. This is always a critical point in soil enzymology where large numbers of assays have to be performed. Bremner and Mulvaney (1978) have criticised methods involving the estimation of ammonium released on the grounds that some of this product might be removed by soil fixation or lost through volatilisation during the assay procedure.

The disappearance of urea determined colorimetrically has been used to assay soil urease activity by Conrad (1940) and Douglas and Bremner (1971). The criticisms of measuring enzyme activity by substrate disappearance have already been discussed (see Introduction, A.4.)

Carbon dioxide evolution has been the least used of all the methods. Skujins and McLaren (1969) and Pel'tser (1972) measured $^{14}CO_2$ released from ^{14}C labelled urea, whereas Norstadt, Frey and Sigg (1973) determined both 'hot' and 'cold' carbon dioxide production.

3. Factors Affecting Activity

(a) Soil Properties

Attempts to relate soil properties to urease activity have indicated that sandy and calcareous soils tend to have lower activity than heavy textured and non-calcareous soils, and that soils under dense vegetation tend to have high activity, whereas saline and gleyed soils tend towards low activity (McGarity and Myers, 1967; Skujins, 1967; Myers and McGarity, 1968; Skujins and McLaren, 1969). As expected there is far from total agreement in the literature when it comes to relating specific soil properties to urease activity but certain trends stand out. Activity in soil profiles decreases markedly with depth (Myers and McGarity, 1968; Musa and Mukhtar, 1969; Gould, Cook and Webster, 1973; Tabatabai, 1977) and increases as organic carbon content increases (McGarity and Myers, 1967; Myers and McGarity, 1968; Gould *et al.*, 1973; Dalal, 1975a; Tabatabai, 1977; Zantua, Dumenil and Bremner, 1977). There is no significant correlation between activity and soil pH (McGarity and Myers, 1967; Myers and McGarity, 1968; Pancholy and Rice, 1973a; Zantua *et al.*, 1977). Activity increases as cation exchange capacity increases (Dalal, 1975a; Zantua *et al.*, 1977).

Many other factors such as total nitrogen, clay, sand and silt content and surface area have been investigated but the findings are too diverse to allow

any generalisations.

(b) Environmental Factors

As is the case with soil properties there is no consensus of opinion concerning the effects of environmental factors on soil urease activity and this is only to be expected when vastly different soil types are being used.

Numerous studies have shown that urease activity in soil increases with temperature between 10°C and 40°C (Conrad, 1942b; Tanabe and Ishizawa, 1969; Gould et al., 1973; Dalal, 1975 a,b). Zantua and Bremner (1978) found that this increase continued up to 70°C but that activity fell off rapidly between 70°C and 80°C.

Urease activity has even been detected at temperatures as low as -20°C but not at -30°C (Bremner and Zantua, 1975). It was postulated that the activity detected had occurred in unfrozen water at surfaces of soil particles. This was supported by the fact that jack bean urease also functioned at -20°C but only in the presence of clay or autoclaved soil.

There are surprisingly few reports in the literature concerning the effect of assay pH on soil urease activity. The optimum pH for activity has been reported as 7.3 (Vasilenko, 1962) 7.2 (Chunderova, 1970) and 6.5 (Pettit et al., 1976). However Tabatabai and Bremner (1972) and May and Douglas (1976)

quoted values of 8.8 and 9.0 respectively.

Air drying has been reported to increase (McGarity and Myers, 1967; Gould et al., 1973), decrease (Speir and Ross, 1975) and have no effect (Zantua and Bremner, 1977) on soil urease activity.

4. Stability

The preservation and pretreatment of soils for experimental purposes is a controversial topic of long standing in soil microbiology and nowhere is there more contention than in soil enzymology. This problem has received attention from Zantua and Bremner (1975b). They found that the following drying or storage treatments of field moist soil had no effect on soil urease activity: freeze drying at -60°C for 60 h, air drying at 22°C for 48 h, oven drying at 55°C for 24 h, storage at 20°, 5°, -10° or -20°C for times ranging from 1 day to 3 months. They also found that activity remained constant when air dried soils were stored at 21° to 23°C for up to one year. More recently (Zantua and Bremner, 1977) they reported no loss of activity when field moist soils were air dried and stored at 21° to 23°C for 2 years and that the following treatments of the field moist soil had no effect on urease activity: drying for 24 h at temperatures ranging from 30° to 60°C, storage for 6 months at temperatures ranging from -20° to 40°C and incubation under aerobic or waterlogged conditions at 30° or 40°C for 6 months. However incubation of

rewetted air dried soils led to a rapid reduction in activity, after which time a plateau was reached. This suggests that air drying of field moist soil leads to the release of urease from microorganisms and protected sites within the organo-mineral complex and that this free urease is rapidly degraded by micro-organisms and accumulated proteolytic enzymes or in-activated by physico-chemical forces when the soil is rewetted. Similar results were obtained under water-logged conditions and repeated wetting and drying cycles did not lead to a further decrease in activity.

Others have observed different responses to Zantua and Bremner (1975 a,b). Air dried storage at room temperature of 6 out of 7 soils studied by Skujins and McLaren (1969) led to a decrease in urease activity. Lloyd and Sheaffe (1973) noticed marked fluctuations of activity in aerobically incubated moist soil.

Soil urease exhibits remarkable temperature stability. It has been detected in soil heated for 24 h at 75°C but not at 105°C (Zantua and Bremner, 1977). Some activity survived 85°C for 48 h (Conrad, 1940). In longer term experiments with azide treated (to prevent replenishment of the accumulated fractions) wet soil Pettit et al. (1976) found that urease survived for 24 h at 70°C and that about 10% of the activity remained after 14 days at 45°C. Jack bean urease was completely inactivated by day 3 at this temperature. Slower declines in enzyme activity were

also noted at 37°, 25° and 4°C and, as expected the rate of inactivation decreased as incubation temperature decreased.

The addition to soil of the proteolytic enzymes trypsin and pronase, which rapidly hydrolyse jack bean urease, has no effect on soil urease activity (Burns et al., 1972 a,b; Pettit et al., 1976; Zantua and Bremner, 1977).

Pettit et al. (1976) have investigated the stability of soil urease to high energy gamma irradiation. Even after a dose of 20Mrads, 40% of the original activity remained in air dried soil. The enzyme was more susceptible to inactivation by gamma irradiation in wet soil than it was in air dry soil (Burns, Gregory, Lethbridge and Pettit, 1978) (see Results and Discussion, A.1.g).

C. UREA

The position of urea as an agricultural nitrogen fertiliser, a mammalian excretory product and a breakdown product of the nucleic acid bases makes it a key compound in the nitrogen cycle of the soil.

The ability to hydrolyse urea is possessed by many bacteria including species of Bacillus, Micrococcus, Pseudomonas, Achromobacter, Corynebacterium and Clostridium, together with some actinomycetes and filamentous fungi. The ecological importance of such microorganisms was realised as early as the turn of the century by Beijerinck (1901) in his enrichment

culture studies with urea bacteria to which he gave the name Urobacillus pasteurii.

Mammals excrete waste or excess nitrogen primarily in the form of urea and it has been estimated that the amount of urea voided daily by sheep is 45 g, that by cattle is 140 g; compared to 30 g by humans (Gasser, 1964). It is difficult to assess the usefulness of this urea as a fertiliser since only a small proportion of the total area grazed by cattle receives urine during one year so that the nutrients contained in the excreta are unevenly distributed. Even with the more ordered applications of famryard slurries the residues have usually been stored and therefore much of the urea has been decomposed.

Over-rapid hydrolysis of urea can give rise to two environmental problems. The confinement of animals to feedlots can cause an air pollution problem because large amounts of ammonia are produced as a result of the heavy urea deposition. Moreover this ammonia may leach down the soil profile, enter the drainage water and promote eutrophication of lakes or streams nearby (Hutchinson and Viets, 1969). Presumably this only occurs when the adsorptive capacity of the soil is saturated.

One of the most striking features of fertiliser practise during the past twenty years has been the rapid increase in the use of urea (Cooke, 1969; Tomlinson, 1970; Harre, Garmen and White, 1971; Engelstad and Hauck, 1974). Cooke (1969) even went so

far as to say, "Certainly no other existing solid is likely to become more important than urea now is, nor is another material likely to be cheaper and more easily made". It is the major agricultural nitrogen carrier in Africa, Asia and South America and during 1970 accounted for 15% of all fertiliser nitrogen used in the United States (Engelstad and Hauck, 1974).

The advantages of urea as a fertiliser are: high nitrogen content (45% as compared to 35% for ammonium nitrate) which reduces transport, storage and spreading costs, highly soluble and suitable for applying in solution either directly to the soil or as a foliar spray and manufacture does not involve the fire, explosion or pollution hazards associated with the nitrates.

The disadvantages are that it is hygroscopic and may cake on storage and is perhaps too rapidly hydrolysed on contact with soil. This latter point has widespread repercussions. The pH of soils receiving urea rises initially as ammonia is produced and the conversion may proceed so rapidly that free ammonia is lost to the atmosphere. Volatilisation is appreciable only when the soil's buffering capacity is low. Alkaline zones are temporarily present even in unlimed acid soils. The ammonium is then converted to nitrous and nitric acids in the nitrification process so that the pH falls. The long term effect of continuous urea applications is in fact a decline in pH and after a number of years this has to be corrected

by liming.

Seeds, germinating seedlings and young plants can be injured or even killed by the initial increase in pH and ammonium concentration. In such cases damage can also result from nitrite toxicity. Nitrite is an intermediate product in the biological transformation of ammonium to nitrate and does not ordinarily accumulate in the soil. However under alkaline conditions its rate of conversion to nitrate is slowed down and toxic levels can build up.

If the urea is incorporated into the soil by tillage during or soon after application most of the ammonia will remain in the soil as the ammonium ion and will be available for crop use. If the urea is allowed to remain on the soil surface however, during warm drying conditions significant amounts of nitrogen can be lost as ammonia to the air.

There are three approaches to reducing the problems associated with urea fertiliser. They have involved attempts to: 1) increase the capacity of the soil for adsorption of ammonium, 2) reduce the rate at which urea becomes available for hydrolysis and 3) reduce the rate of urea hydrolysis in soil.

The first approach is restricted to the use of urea in the form of acidic derivatives such as urea nitrate or phosphate. Such compounds hydrolyse readily in water to give acidic solutions. The resulting fall in soil pH increases the adsoprtion capacity of the soil for ammonium produced during the

subsequent urea hydrolysis. Bremner and Douglas (1971b) showed that urea phosphate not only reduced the gaseous loss of ammonia from soil but that it also retarded the enzymatic hydrolysis of urea.

Obviously, the rate of urea hydrolysis is determined by substrate availability which may be retarded in two main ways, either by chemical combination to prepare urea derivatives of low solubility or by coating particles of urea with some inert water resistant coating. Fertilisers of this type are termed slow or controlled release nitrogen fertilisers; they have been reviewed by Prasad, Rajale and Lakhdive (1971). Combinations of urea with aldehydes such as formaldehyde or crotonaldehyde has proved successful and more recently urea-stearic acid complexes (Paulson and Kurtz, 1969a) and ureaform, a mixture of methyleneurea polymers of different lengths (Hadas, Kafkafi and Peled, 1975), have also been evaluated as slow release nitrogen carriers. Various inert coatings of urea, such as sulphur (Dalal, 1975c) and plastic (Mahendrappa and Salonius, 1974), have also proved successful in retarding hydrolysis.

The third approach to retarding urea hydrolysis in soil involves applying the fertiliser in combination with another compound. This has received much attention (Moe, 1967; Bremner and Douglas, 1971a, 1973; Bundy and Bremner, 1973) and many compounds have been patented as soil urease inhibitors. Most of them have previously been shown to be potent

inhibitors of plant and microbial ureases (e.g. heavy metal salts, dithiocarbamates, dihydricphenols and quinones). Bremner and Douglas (1971a) investigated the ability of more than 100 compounds to inhibit soil urease. They found that the most effective organic compounds were phenols and quinones and the most effective inorganic compounds were silver and mercury. Several potent inhibitors of plant and microbial ureases reduced activity by no more than 25% (e.g. N-ethylmaleimide, acetohydroxamic acid and cupric sulphate). Bundy and Bremner (1973) found that the effectiveness of 34 substituted p-benzoquinones as soil urease inhibitors depended upon their substituent groups. Methyl-,chloro-,bromo and fluoro-substituted p-benzoquinones had a marked inhibitory effect, whereas phenyl-,t-butyl- and hydroxy substituted p-benzoquinones had little if any effect. Since the parent compound p-benzoquinone (which is inexpensive to prepare) was only slightly less effective than the best substituted inhibitors 2,3 dimethyl-, 2,5 dimethyl- and 2,6 dimethyl- p-benzoquinone they concluded that p-benzoquinone was the most promising for large scale agricultural use.

More recently the effects of hydroquinone have been reported to be identical to those of p-benzoquinone (Bremner and Mulvaney, 1978). Since the hydroquinone is cheaper than p-benzoquinone and considerably less expensive than other compounds proposed as soil urease inhibitors it would appear that

hydroquinone is the most promising soil urease inhibitor investigated to date.

If such inhibitory compounds are to find widespread use in agriculture it is not sufficient that they are cheap to manufacture; they should also be soluble in water, so they move with the urea and should not have deleterious side effects on other biological processes, nor accumulate in the soil. Volk (1961) suggested that the reason why copper, a potent inhibitor of microbial ureases, had no effect on urea hydrolysis in soil was that it was readily immobilised by the soil and hence the urea diffused from the copper effect zone.

D. 1,3-β-GLUCANASES

1. Introduction

1,3-β-glucanases (1,3-β-glucan 3-glucanohydrolase, EC. 3.2.1.6 - hereafter called glucanase) depolymerise 1,3-β-glucans by exo- and endohydrolytic action. Cleavage of these polysaccharides can proceed by either activity or a combination of them both. Such enzymes have commonly been called laminarinases since laminarin (≡ laminaran) is frequently used as the substrate. The endohydrolase produces a series of oligosaccharides called laminaridextrins, whereas the exohydrolase yields glucose as its product. Laminarinases have been reviewed by Bull and Chesters (1966).

2. Occurrence

Glucanases are ubiquitous enzymes (Table 2) and have been identified in bacteria, fungi, algae, higher plants and invertebrates (Chesters and Bull, 1963a; Bull and Chesters, 1966).

The majority of glucanase investigations have been carried out with microorganisms and the enzymes are known to be produced extracellularly in the bacteria and fungi (Bull, 1972). They are constitutive in fungi (Chesters and Bull, 1963a) but are inducible in some bacteria (Tanaka and Phaff, 1965). In a thermophilic streptomycete synthesis was found to be semi-constitutive (Lilley and Bull, 1974; Lilley, Rowley and Bull, 1974). The fact that in this same organism synthesis occurred extensively during logarithmic growth, with no sudden release in maximum population phase (when autolysis occurs), indicated active secretion (Lilley *et al*, 1974). However in the fungus *Saprolegnia monoica* the enzymes were released on autolysis (Fevre, 1977) suggesting a different role for the glucanases in these two organisms.

3. Function

Four major functions have been attributed to glucanases: extracellular lysis of microbial cells and degradation of plant and microbial debris, cell wall plasticity, intracellular mobilisation of food reserves and digestive metabolism of invertebrates.

One of the most common interactions between

TABLE 2 The distribution of 1,3-β-glucanases (see also Chesters and Bull, 1963a; Bull and Chesters, 1966)

Source	Reference
1. Bacteria	
Arthrobacter sp. & *Bacillus circulans*	Doi, Doi & Nakamura (1976)
Cytophaga johnsonii	Bacon, Gordon, Jones, Taylor & Webley (1970)
Flavobacterium dormitator var. *glucanolyticae*	Nagasaki, Nishioka, Mori & Yamamoto (1976)
Streptomyces sp.	Lilley, Rowley & Bull (1974); Lilley & Bull (1974)
2. Fungi	
Mucor hiemalis	Miyazaki, Yadomae, Yamoda & Oikawa (1977)
Saprolegnia monoica	Fevre (1977)
Coniothyrium minitans	Jones, Gordon & Bacon (1974)
Myrothecium verrucaria & *Trichoderma viride*	Chesters & Bull (1963a)
Poria cocos	Nagasaki, Saito, Yamamoto (1977)
Physarum polycephalum	Farr, Schuler & Horisberger (1973)
Schizosaccharomyces pombe	Barras (1972)
Saccharomyces cerevisiae	Farkas, Biely & Bauer (1973)
Arthroascus, *Cryptococcus*, *Endomyces*, *Phaffia* & *Rhodotorula*	Meyer & Phaff (1977)
3. Algae	
Euglena gracilis	Barras & Stone (1969)
Cladophora rupestris	Duncan, Manners & Ross (1956)
Rhodymenia palmata, *Laminaria digitata* & *Ulva lactuca*	Duncan, Manners & Ross (1956)

4/

TABLE 2 (Contd)

Source	Reference
4. Higher Plants	
Avena sp.	Heyn (1969)
Hordeum sp.	Anderson, Cunningham & Manners (1964)
5. Invertebrates	
Spisula solidissima (the surf clam)	Lindley, Shallenberger & Herbert (1976)
Sea urchin eggs	Epel, Weaver, Muchmore & Schimke (1969)

populations of microorganisms in any natural environment is enzyme-induced lysis; one organism eliminating another by cell wall digestion. Since microbial cell walls are composed of a variety of polymers (e.g. cellulose, chitin, mucopeptide and 1,3-β-glucans) lysis is carried out by a barrage of enzymes of which the glucanases may form only a part. The importance of glucanases has been demonstrated in the lyses of: Aspergillus oryzae, Fusarium solani and Aspergillus nidulans hyphal walls by a streptomycete (Skujins, Potgieter and Alexander, 1965; Bull, 1970); Saccharomyces cerevisiae by Fusarium solani and an unidentified fungus (Jones and Webley, 1967) and the soil yeasts Cryptococcus albidus and Cryptococcus terreus by a non-fruiting myxobacterium Cytophaga johnsonii and a streptomycete (Jones, Bacon, Farmer and Webley, 1969). In every case the lytic organisms were isolated from soil.

Besides their lytic action these enzymes are involved in the degradation of plant and microbial debris containing 1,3-β-glucans.

The growth of bacterial, fungal and primary plant cell walls involves the action of hydrolytic enzymes creating regions within the existing polymeric system for the insertion of newly synthesised cell wall material. Fevre (1977) found that glucanase activity involved in the growth and branching of the fungus Saprolegnia monoica was mainly localised at the edge of the colony where these processes occur. It has

been suggested that glucanases might have a role to play in cell wall growth of Schizosaccharomyces pombe (Barras, 1972) and coleoptiles of Avena sativa (Heyn, 1969).

Glucanases are also concerned with intracellular mobilisation of food reserves such as pachyman (a 1,3-β-glucan) in the fungus Poria cocos (Reese and Mandels, 1959) and the alga Euglena gracilis (Barras and Stone, 1969) and unidentified 1,3-β-glucans in Penicillium italicum (Santos, Sanchez, Villanueva and Nombela, 1978).

4. In Soil

There has been only one direct attempt to measure glucanase activity in soil (Hussain, 1976). This is surprising since there is likely to be a significant input of both substrate and enzyme into soil (see Introduction, sections D and E). A discussion of the majority of Hussain's results in the context of this project is not valid since they were obtained from continuously perfused soil columns amended with fungal mycelium. However, it is interesting to note that the decline in activity down a soil profile was more pronounced with glucanase than with urease.

Jones and Webley (1968) incorporated fungal cell walls containing or lacking 1,3-β-glucans into kaolinite aggregates and incubated them on soil. Glucanase activity was only detected in the aggregates amended with cell walls rich in 1,3-β-glucans. Kiss

et al. (1975) have suggested that the enzyme activity in soil is confined to zones colonised by lytic micro-organisms.

Although in the majority of cases this enzyme only functions extracellularly, it is synthesised intracellularly, and in fungi is externalised in vesicles (Fevre, 1977). At first glance it might seem that intracellular breakdown of substrate in non-proliferating cells, always a complication in soil urease estimations, is unlikely to contribute to the assay of accumulated soil glucanase because the substrate is too large to penetrate the cell wall and cytoplasmic membrane. However it might become important (always assuming that the intracellular glucanase is active) in the late stages of the assay when short chain oligosaccharides which might be able to enter the cell are produced, or if the cell wall and plasma membrane were to rupture. Thus, although the contribution of intracellular substrate turnover in non-proliferating cells to total accumulated soil enzyme activity will be smaller in assays of glucanase than of urease, conceptually it can not be ruled out altogether.

Research into soil glucanase activity has a potentially important application in the control of soil-borne plant pathogens. A well-known approach to such problems is to induce changes in the microbial population by soil amendment such that the pathogen is eliminated or destroyed. Mitchell (1963) attempted to

stimulate a mycolytic microflora in soil by adding the two fungal cell wall type constituents chitin (a 1,4-β-glucan) and laminarin. This treatment resulted in a decline in disease severity caused by pathogenic Fusaria which are known to contain 1,4-β- and 1,3-β-linked glucans in their cell walls. However Pythium debaryanum and Agrobacterium tumefaciens which do not possess these types of polysaccharides in their cell walls were not suppressed. Natural sources of these polysaccharides such as ground lobster shells and Laminaria fronds were equally as effective. Although actinomycetes were dominant in chitin treated soils suggesting that antibiosis may have been involved in the fungal suppression (Mitchell and Alexander, 1962), they were themselves reduced in laminarin treated soil (Mitchell, 1963). Mitchell (1963) concluded that he had specifically induced a chitinase and laminarinase producing microbial population capable of digesting those fungal cell walls which contain these relevant polymers. This had previously been shown to be the case for chitin (Mitchell and Alexander, 1962). Thus it appears that 1,4 and 1,3-β-glucanases are active in soil mycolysis and may have a significant role to play in the control of soil-borne plant pathogens. This approach may be interpreted by some as a potential alternative to the over-used chemical control of plant pathogens. However it is important to recall that glucanases, or indeed any other enzyme in isolation will not bring about wall digestion.

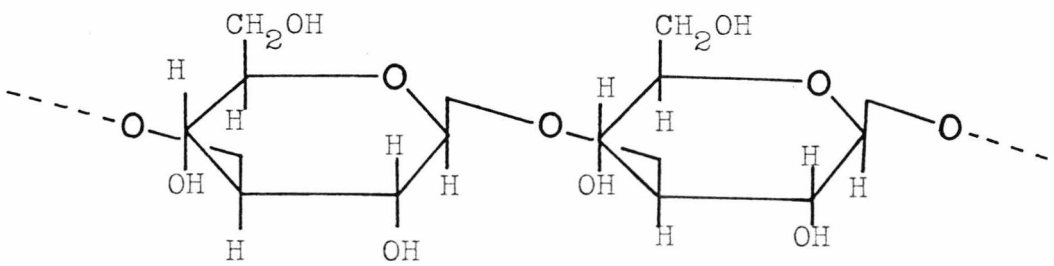
In contrast to the glucanases, other polysaccharidases including: the α- and β-amylases, cellulase, lichenase, inulase, xylanase, dextranase, polygalacturonase and levanase have received much attention from soil enzymologists. Another enzyme concerned with fungal cell wall turnover, chitinase, is conspicuous by its absence from this list.

E. 1,3-β-GLUCANS

Glucans are polymers in which the sole component is glucose. They are the most widely occurring of all the polysaccharides and include cellulose, starch, and glycogen and vary considerably in the degree of branching and type(s) of glucosidic linkage.

1,3-β-glucans occur widely in microorganisms and higher plants where they function as cell wall components and cytoplasmic or vacuolar reserve materials. In some β-glucans the 1,3-β-glucosidic linkage (Fig. 3) is exclusive

FIGURE 3 The 1,3-β-glucosidic linkage



e.g. paramylon, the reserve food in Euglena. Most frequently however, it occurs as a component of heterogeneous β-glucans where it can either be the major linkage e.g. the cell wall glucan of Saccharomyces cerevisiae - 85%(Manners, Masson and Patterson, 1973) or the minor linkage e.g. barley β-glucan - 30% (Luchsinger, Chen and Richards, 1965). The distribution of natural 1,3-β-glucans is shown in Table 3. The degree of molecular branching and/or the presence of the other β-glucosidic linkages tends to increase the solubility of 1,3-β-glucans by preventing regular molecular alignment.

TABLE 3 The distribution of 1,3-β-glucans (amended from Bull and Chesters, 1966)

Filamentous fungi	Yeasts	Algae	Gymnosperms
Aspergillus	Aureobasidium	Astasia	Cycas
Claviceps	Candida	Eisenia	Pinus
Helotium	Cryptococcus	Euglena	
Microsporum	Saccharomyces	Laminaria	Angiosperms
Penicillium	Schizosaccharomyces	Ochromonas	Avena
Plectonia		Peronema	Hordeum
Poria	Lichens		Petunia
Schizophyllum	Cetraria		Phaseolus
Sclerotinia			Vitis

In the fungi the 1,3-β-glucan component is probably the most widely distributed of all the wall polysaccharides and has been referred to as the R-glucan by some authors (see for example Rosenberger, 1976). When present it comprises 15 to 30% of the total wall polysaccharide. While absent from the hyphae of the zygomycetes it appears to be a wall component in the other species of filamentous fungi examined and in many yeasts. The fungal R-glucan is not a straight chain glucose polymer like Euglena paramylon or plant callose but contains some 1,6-β branches (Zonneveld, 1971). In this context it is interesting that an extracellular 1,3-β-glucan mucilage with 1,6-β branches containing a single glucose residue is produced by Claviceps fusiformis (Buck, Chen, Dickerson and Chain, 1968) and Schizophyllum commune (Wessels, Kreger, Marchant, Rosenburg and Devries, 1972). Rosenberger (1976) has speculated that the branched R-glucan might be important in conferring strength and rigidity upon the fungal cell wall by forming cross-links with other fungal wall polymers vis a vis the cross-linking of polysaccharides by peptide bridges in bacteria.

In angiosperms the 1,3-β-glucan callose appears to be of wide occurrence and is especially evident in vascular tissues and reproductive structures, albeit in small amounts.

Most of the work on the 1,3-β-glucanases has been carried out using laminarin as substrate. This water

soluble 1,3-β-glucan is the main carbohydrate food reserve of the marine brown algae (Phaeophyceae). Extensive structural investigations have been carried out on the laminarin extracted from the fronds of two Laminaria species (Bull and Chesters, 1966). It is essentially a linear 1,3-β linked glucan backbone with occasional 1,6-β branch points. Approximately 30 to 50% of the molecules are terminated at the reducing end by a non-reducing D-mannitol residue (M chains) in 1,6-β linkage. The remainder (G chains) are terminated by reducing D-glucose residues. The quantity of D-mannitol varies from species to species but it has never been shown to be more than 3%.

Values for the molecular weight have varied from 2600 to 4000 depending on the method used and these values correspond to 14 and 22 glucose residue per molecule respectively. The majority of the determinations lie at the top end of the scale. Di- and oligosaccharides produced on partial chemical or enzymic hydrolysis of laminarin are named after the parent polysaccharide. Thus laminaribiose, laminaritriose and laminaritetraose are composed of two, three and four glucose units respectively.

Two different forms of laminarin, the so-called soluble and insoluble forms, have been isolated from Laminaria digitata and Laminaria hyperborea (cloustoni) respectively. The insoluble form will only dissolve to any extent in warm water (50°C), however once in solution it can be cooled to room temperature and will

only precipitate out of solution gradually over a long period of time. The soluble form has a lower reducing power and higher degree of branching. Percival (1970) has outlined the experiments which have led to the characterisation of laminarin.

F. AMENDMENTS

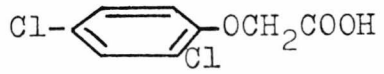
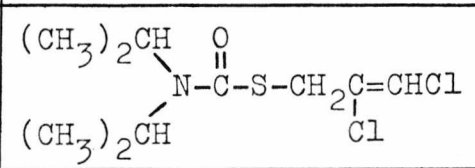
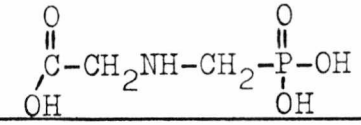
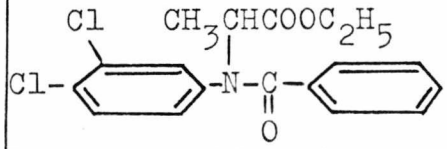
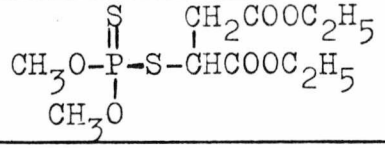
The aim of this project was not only to devise a simple, rapid and reproducible method of assaying 1,3-β-glucanase activity in soil and characterise this enzyme biochemically, but to investigate the effect of various soil amendments (on their own and in combination) on the activity of this and another soil enzyme, urease.

1. Pesticides

a) 2,4-Dichlorophenoxyacetic acid (2,4-D)

2,4-D (see Table 4) is the parent member of the phenoxyalkanoic acid group of herbicides which find extensive use in the selective control of broad-leaf weeds, particularly in cereal crops and lawns. These compounds are plant growth regulators and exert their effect by causing abnormal growth responses, hence the trivial names auxin or hormone herbicides. They are applied to soil, taken up by the roots and translocated to their sites of action. They are commonly used as their salts (sodium, potassium, trimethylamine), alkylesters (methyl-, isopropyl-, butyl-) or low water volatile esters (butoxyethanol, tetrahydrofurfuryl-)

TABLE 4 Pesticides used in this project

Trivial name	Systematic name	Structure
2,4-D	2,4-dichlorophenoxyacetic acid	
Diallate	S-(2,3-dichloroallyl) diisopropylthiol carbamate	
Glyphosate	N-(phosphonomethyl) glycine	
Benzoylprop ethyl	Ethyl N-benzoyl-N-(dichlorophenyl)-2-aminopropionate	
Malathion	O,O-dimethyl-S-(1,2-dicarboxyethyl) phosphorodithioate	

and only rarely as the parent acid. According to the United Nations Food and Agriculture Organisation production year book of 1974, 27 x 10^6 kilograms of 2,4-D were consumed in 1973, making it the most extensively used of all pesticides.

The conclusion that 2,4-D breakdown in soil is primarily a microbial process is based on substantial evidence (Audus, 1964; Kaufman and Kearney, 1976). Early workers reported that conditions favourable to microbial growth such as high moisture, temperature and organic matter were also favourable to 2,4-D disappearance. 2,4-D breakdown does not occur in soil sterilised by autoclaving or with sodium azide. Before degradation takes place there is a lag period during which microbial enrichment occurs. Lag periods vary from a few days to 4 weeks and the time for total disappearance may be as little as 7 days or as much as 14 weeks. Such variability is common in soil microbiology. Microbial numbers and species capable of degrading 2,4-D will vary from soil to soil and the incubation conditions used will influence the activity of these microorganisms. Additional doses of herbicide decompose with a shortened lag period and this state of enrichment is sometimes maintained for a year in stored moist soil.

Members of several microbial genera isolated from soil including: Pseudomonas, Corynebacterium, Achromobacter, Arthrobacter, Flavobacterium, Nocardia and Aspergillus are capable of utilising 2,4-D as a sole carbon and energy source when grown

in pure culture (Higgins and Burns, 1975). Foster and McKercher (1973) reported a positive correlation between microbial numbers and the rate of 2,4-D breakdown. Pemberton and Fisher (1977) have shown that in a Pseudomonas species the ability to degrade 2,4-D is plasmid coded.

Persistence is not only a function of microbial activity but, along with mobility, is dependent on the nature of adsorbing materials present and pH. However acidic pesticides, like 2,4-D, are generally not adsorbed by clay minerals, although they can be adsorbed to a limited extent by organic matter.

The vapour pressures of acidic pesticides are very small and in general 2,4-D loss from soil through volatilisation is negligible.

b) Diallate

Diallate (see Table 4), a thiolcarbamate herbicide, is a mitotic poison used for the pre-emergence control of wild oats (Avena fatua) in various crops. Because of its volatility diallate must be incorporated into the soil immediately after application. Carbamate herbicides are of particular importance in agriculture because of their effectiveness at low levels of application, their low mammalian toxicity and their general short term persistence in soil.

What little work has been done suggests that soil microorganisms play a considerable role in the disappearance of diallate from soil. Negligible loss

from autoclaved soils was reported by both Banting (1967) and Smith (1970). Breakdown was preceded by a 6 to 7 day lag period (Banting 1967) and the half-life varied from 4 to 8 weeks depending on the moisture content of the soil (Smith, 1970). Anderson and Domsch (1976) recorded a 50% loss from microbiologically active soils after 4 weeks and a 50% loss from autoclaved soil after 20 weeks, when 2.5 ppm were applied. They attributed the loss in sterile soil to non-biochemical degradation and incomplete extraction of residues.

A number of fungi which will degrade diallate in pure culture have been isolated from soil: Phoma eupyrena, Penicillium jonthinellum, Trichoderma harzianum (Anderson and Domsch, 1976) and Fusarium oxysporium (Kaufman and Blake, 1973).

It appears that at low temperatures microbial decay is the major contributor to diallate disappearance from soil, whilst above 22°C volatilisation becomes increasingly important.

c) Glyphosate

Glyphosate (see Table 4) is a post-emergence, broad spectrum, systemic herbicide which controls a wide range of annual and perennial grasses, broad-leaf weeds and vines. A few days prior to planting it is sprayed onto the weeds and is translocated from the leaf and stem tissue throughout the plant. Uptake by roots from soil is negligible (Sprankle, Meggitt & Penner 1975a). It kills by disrupting the

basic metabolic processes, particularly aromatic amino acid biosynthesis (Tucker and Phillips, 1975).

When the chemical comes in contact with the soil it is inactivated by one or a combination of the following factors: microbial degradation, chemical degradation or adsorption (Sprankle, Meggitt and Penner, 1975a). The initial step in this inactivation appears to be due to rapid binding to the soil and not microbial degradation, since autoclaving the soil did not prevent glyphosate inactivation (Sprankle, Meggitt and Penner, 1975a). There are several reports that the initial rapid inactivation by adsorption is followed by slow microbial degradation. Both free and bound glyphosate are subject to decay (Sprankle, Meggitt and Penner, 1975b; Nomura and Hilton, 1977; Torstensson and Aamisepp, 1977; Rueppel, Brightwell, Schafer and Marvel, 1977).

Persistence varies considerably from soil to soil. Nomura and Hilton (1977) observed that after 60 days five soils treated with 5 to 50 ppm lost 66%, 58%, 35%, 1.2% and 8% as carbon dioxide respectively, whereas Reuppel, Brightwell, Schaefer and Marvel (1977) reported losses as carbon dioxide of greater than 90% after 12 weeks in two samples compared to 25% in another - all soils being initially treated with 4 and 8 ppm gly phosate. In peat 333 ppm disappeared completely (without a lag period) in 12 days at 27°C and 20 days at 12°C (Quilty and Geoghegan, 1976).

The absence of a lag in the evolution of $^{14}CO_2$ from ^{14}C glyphosate-treated soil, the slow constant rate of microbial degradation of the herbicide and the lack of success in isolating microorganisms which will break down glyphosate in pure culture are all indicative of cometabolism (Sprankle et al., 1975b; Torstensson and Aamisepp, 1977). Quilty and Geoghegan (1976) observed that glyphosate disappearance in peat was inhibited by the antifungal agents nystatin and griseofulvin and concluded that one or several fungal species were responsible for its degradation.

A minor role for chemical degradation has been indicated by Torstensson and Aamisepp (1977) and Sprankle et al. (1975b) who detected slight disappearance in both autoclaved and sodium azide-treated soils.

Glyphosate appears to bind to soil through the phosphonic acid moiety as inorganic phosphate competes with it for adsorption sites (Sprankle et al., 1975a, Hance, 1976).

d) Benzoylprop Ethyl

The herbicide benzoylprop ethyl (see Table 4), better known by its tradename 'Suffix', is used for the post-emergence control of wild oats (Avena spp.) in spring and winter wheat. It acts by suppressing the growth of the wild oats thus allowing the crop to compete more effectively. The herbicide is applied

to the foliage and entry is primarily via the leaves from where it is translocated to the sites of action; the shoot meristematic and cell elongation zones. The selectivity between wheat and wild oats is based on the rate at which the compound is hydrolysed to the active molecule N-benzoyl-N- (3,4 dichlorophenyl) -2-aminopropionic acid and then inactivated in the plants. At the recommended application rate wheat is unaffected (Bell and Moberley, 1972).

Beynon, Roberts and Wright (1974) treated various soils with 20 ppm benzoylprop ethyl and found that the half-life varied from one week in sandy loam and clay loam soils to 12 weeks in peat soil. Persistence increased as the organic matter content of the soil increased but they could detect no correlation between degradation rate and either the clay content or the pH of the soils. They concluded that benzoylprop ethyl is strongly adsorbed by soil organic matter.

e) Malathion

Malathion (see Table 4) is a safe, general purpose, organophosphorus insecticide suited for household, home garden, vegetable and fruit insect control and for the control of mosquitoes, flies and lice of public health importance. Organophosphorus insecticides have gradually replaced all but the most important organochlorine compounds (e.g. DDT, aldrin and dieldrin) because broadly speaking they are less persistent and consequently present fewer

toxic residue problems. They exert their insecticidal effect by inhibiting the enzyme acetylcholine esterase.

It is apparent from the number of contradictory reports that malathion disappears from soil *via* a number of different routes, including chemical hydrolysis, microbiological decay and extracellular enzyme breakdown. After comparing malathion loss from sterile (5 Mrads irradiation) and non-sterile soils, Konrad Chesters and Armstrong (1969) suggested that degradation is primarily a non-biological hydrolytic event related to adsorption and alkalinity. Indeed malathion is readily hydrolysed in highly alkaline solutions whilst in neutral and acid environments hydrolysis is extremely slow. In estuarine water the rate of disappearance increased with salinity and there was no difference between non-sterile and autoclaved samples (Walker, 1976).

There is considerable evidence for microbial degradation of malathion. Konrad *et al*. (1969) reported that in aqueous soil-free systems inoculated with soil extract 5 ppm malathion disappeared rapidly after a 7 day lag period, whereas in intact soil 50 to 90% had gone after only 24 h. They concluded that in soil complete chemical degradation of the insecticide had occurred before the microbial population had a chance to adapt to it. Malathion disappeared twice as rapidly from non-sterile estuarine sediments

than from autoclaved ones (Walker, 1976). Such reports have been supported by the isolation of micro-organisms from soil which are capable of malathion degradation in pure culture. These include Trichoderma viride and a Pseudomonas sp. (Matsumura and Boush, 1966) and an Arthrobacter sp. (Walker and Stojanovic, 1974). Seven out of 15 microorganisms isolated from a salt marsh had the ability to utilise malathion as a sole source of carbon and energy and the remainder were capable of degrading it when supplied peptone (Bourquin, 1977). Merkel and Perry (1977) proposed that microbial degradation may involve cometabolism since heptadecene and n-heptadecane increased the rate of $^{14}CO_2$ production from soil treated with ^{14}C malathion. Glucose, glycerol, glycerophosphate, casein hydrolysate, acetate, succinate, citrate and pyruvate had no effect.

Getzin and Rosefield (1968) observed that malathion disappeared faster in non-sterile soils and in soils sterilised by 4 Mrads irradiation than in heat sterilised soil and concluded that a temperature sensitive substance was involved in the decomposition. Subsequently they were able to extract a heat labile, water soluble, organic substance which after partial purification was shown to possess typical enzymic properties (Getzin and Rosefield, 1971; Satyanarayana and Getzin, 1973). They called this catalyst malathion esterase and it possessed the characteristics of a typical extracellular accumulated soil enzyme.

For example, it retained 70% of its activity when heated at 80°C for 15 min and was resistant to proteolysis by pronase. No loss of activity occurred during extended storage of enzyme solutions at 4°C or frozen at -10°C. Gibson and Burns (1977) suggested that a stable exoenzyme associated with the colloidal organic matter was the most important single factor involved in the rapid breakdown of malathion in the soil studied.

2. Fertilisers

a) Nitrogen, Phosphorus, Potassium (NPK) Compound Fertilisers

A compound fertiliser contains the three essential plant nutrients nitrogen, phosphorus and potassium in varying proportions. Thus a 1:1:1 fertiliser contains as chemical equivalents, one unit total nitrogen, one unit P_2O_5 and one unit K_2O.

In Britain more nitrogen is now used in the form of compound fertilisers than as 'straight' fertilisers containing only nitrogen. The main form of nitrogen in most solid compounds is ammonium nitrate often with mono- or diammonium phosphate. Urea is an important constituent of many liquid N-compounds when it is mixed with ammonium nitrate and ammonium phosphates.

The majority of phosphate in compound fertilisers is water soluble. In all cases it is equivalent in efficiency to the phosphate in superphosphate and is readily available for plant uptake. The very small

amount of insoluble phosphate is of little agronomic value. Single superphosphate and triple superphosphate are prepared by the action of concentrated sulphuric and phosphoric acid respectively on rock phosphate. In single superphosphate the phosphate is present as the mono- (30%) di- (19%) and tri- (3%) calcium phosphates. Monocalcium phosphate is water soluble, dicalcium phosphate is not water soluble, but citrate soluble and hence is also available to plants, whereas the tricalcium phosphate dissolves in soil solution extremely slowly and is only available to plants over a number of years.

Most potassium fertiliser is sold in the soluble form as a constituent of a compound fertiliser, the principal one being potassium chloride or muriate of potash.

To prevent deterioration of physical condition during storage most commercial fertilisers are now pelleted and coated with clays or waxes. These pellets disintegrate slowly on contact with the soil solution.

b) Urea

See Introduction, C.

c) Farm Slurry

Animal slurries are suspensions of faeces in urine mixed with varying quantities of extraneous water. The faeces contain most of the excreted phosphorus and

insoluble nitrogen and potassium. Slurry is generally stored in a holding tank or lagoon. Providing the collection and storage system is efficient, losses of phosphorus and potassium are negligible, but under exposed conditions large amounts of nitrogen may be lost as ammonia. Disposal of slurry after collection is normally by direct application to the land which permits the nutrients to be recovered in crops.
The collection and disposal of animal excreta as semi-liquid slurries instead of as the more traditional farmyard manure results from rises in labour costs and the need to increase animal output by greater stocking density. Thus there are three types of farm slurry: cow, pig, and poultry.

When properly used as manure slurries can be a valuable source of plant nutrients (Table 5).

TABLE 5 Composition of undiluted farm slurries (from MAFF, 1975)

Source of slurry	Percentage by weight		
	N	P_2O_5	K_2O
Cow	0.5	0.2	0.5
Pig	0.6	0.2	0.2
Poultry	1.7	1.4	0.7

However, they should not be used indiscriminately otherwise problems may arise (McAllister, 1977).

Frequent dressings of slurry often cause a build up of nutrients in soil particularly phosphorus, potassium and occasionally organic matter. Slurry from pigs whose food contained copper compounds has increased the amount of copper in the soil. These and other accumulations can lead to crop disorders and pollution of water supplies by leaching and run off.

Another damaging side effect of heavy slurry dressings is reduced aeration of the soil. The finer slurry solids (under 250 μm) mostly contain microbial polysaccharides, produced as a result of microbial growth during storage, which are easily decomposed aerobically. As a result oxygen consumption is extensive and at the same time gaseous diffusion is restricted by blocked soil pores.

Slurries also contain methane, ethylene and volatile fatty acids produced by anaerobic microbial activity in the rumen. Volatile fatty acids can inhibit plant growth (Lynch, 1977, 1978) and their persistence under anaerobic conditions is prolonged. It is probable that even moderate slurry dressings will lead to anaerobic zones in the soil, the effect being greater if the soil is initially wet or poorly drained.

3. Lime

In agriculture the imprecise term 'lime' covers those materials containing calcium or calcium and

magnesium which are commonly applied to the land to reduce soil acidity. Included in this definition, therefore, are calcium oxide (burnt or quick lime), calcium hydroxide (slaked lime) and calcium carbonate. Calcium carbonate is typically in the form of chalk or limestone, although it may be an industrial waste product from processes such as sugar beet purification (sugar lime) or water softening. Most limestones are calcitic, which means that the carbonate is primarily in the form of the calcium salt. Those containing magnesium carbonate are called dolomitic. A secondary function of lime is to supply the essential plant nutrients calcium and magnesium.

The object of liming is to bring the pH of a soil to the optimum value for the crop. In general terms this means that with arable crops a pH of 6.5 is aimed at, whereas in grassland the required pH is 6.0. Soil is usually limed to a depth of 15 cm. The amount of lime that must be added to a soil to achieve the desired pH is called the 'lime requirement' and is determined in the laboratory. However it is generally true that if this dressing is given based on the <u>in vitro</u> estimation, the pH does not change as expected (Russell, 1973). It is commonly necessary to apply two to three times this amount. This is called the liming factor and depends on the evenness with which the lime is incorporated into the soil, the rate at which it dissolves and reacts with soil particles and the rate of leaching through the soil. Thus lime

requirement is only an approximate guide.

The rate at which pH is increased depends on the composition and particle size of the liming material. Natural limestones and chalk must be crushed before they will react with the soil but it is not necessary to grind them to a fine powder. Forty per cent of the material in ground limestone must pass through a 0.15 mm mesh sieve and such a material will exert about 90% of its effect within the first two weeks.

The time interval between spreading the lime and sowing the crop should be as long as possible, especially when heavy dressings are used or when acid sensitive crops are grown. Only under the extreme circumstances of crop failure due to soil acidity is lime applied after planting.

The quality of all forms of lime is assessed on their neutralising value (NV); that is their ability to neutralise acid under standard laboratory conditions. This is expressed as the percentage calcium oxide in the material, or its equivalent if both calcium and magnesium are present. Thus when a ground limestone is said to have an NV of 50 this means that it will neutralise the same amount of soil acidity as a liming material containing 50% calcium oxide by weight. Subject to their being in an effective state of fineness all forms of lime are similar in their effects on soils, provided quantities applying the same NV value are applied. Thus 20 cwt burnt lime (NV 80) are

approximately equivalent to 25 cwt hydrated lime (NV 70) or 30 cwt ground limestone or chalk (NV 50). In the field situation however the usual application rates of burnt lime and ground limestone or chalk are one and two tons per acre respectively.

4. Cellulose and Glucose

Cellulose is an insoluble, linear polysaccharide composed of at least 5000 1,4-β linked D-glucose residues. It forms the bulk of the cell wall material in higher plants and as such is the most widespread and abundant naturally occurring organic polymer. Consequently a large input of this polysaccharide into soil is to be expected; however the analyses show that it represents only a small fraction of humus, often less than 1% ($^{w}/_{w}$) (Gupta, 1967) because it is broken down by extracellular enzymes produced by soil microorganisms (Alexander, 1977) and accumulated soil enzymes (Benefield, 1971; Ross, 1974; Kiss, et al., 1975; Skujins, 1976).

It has been suggested that the microbial degradation of this glucose polymer involves at least three groups of extracellular enzymes. The first group of cellulases (C1) disrupt the cellulose microfibrils causing a loss of tensile strength. Cx cellulases (composed of endo- and exo-β-glucanases) perform the subsequent depolymerisation to low molecular weight oligosaccharides and the disaccharide cellobiose. The third group of enzymes, the β-glucosidases, catalyse

the hydrolysis of those di- and oligosaccharides to glucose. Where cellobiose is the substrate the enzyme responsible is called cellobiase. With the production of glucose and almost certainly the low molecular weight oligosaccharides the reaction becomes intracellular (Enari and Markkanen, 1977).

Whilst most higher animals are unable to degrade cellulose (except those having cellulolytic micro-symbionts) cellulases are possessed by a variety of bacteria, actinomycetes and fungi (Table 6).

TABLE 6 Some soil microorganisms capable of degrading cellulose.

Bacteria	Actinomycetes	Fungi
Achromobacter	Micromonospora	Alternaria
Bacillus	Nocardia	Aspergillus
Cellulomonas	Streptomyces	Cladosporium
Cellvibrio		Coprinus
Clostridium		Fusarium
Cytophaga		Penicillium
Pseudomonas		Trichoderma
		Verticillium

Cellulose itself contains no nitrogen and so the speed at which degradation takes place is very dependent upon the nitrogen content of the soil

(Imshenetsky, 1968). In the early stages of decomposition nitrogen is removed from the soil by the cellulose utilisers but it is returned later on when they themselves die and are decomposed (Tribe, 1961). The addition of inorganic nitrogen in the form of ammonium or nitrate salts enhances the rate of cellulose breakdown (Alexander, 1977).

It is not unreasonable to predict that since glucose is the hydrolytic product, then cellulose additions to soil will have considerable effects on the non-cellulolytic as well as the cellulolytic microflora.

As expected glucose disappears very rapidly when added to soil. Macura and Kubatova (1973) applied 6000 ppm glucose to soil in the presence and absence of an unspecified amount of ammonium phosphate. In the presence of the nitrogen source all the glucose had disappeared by day one and even in its absence glucose could not be detected by the second day. When added in combination with fructose, galactose, lactose, cellobiose and xylose; glucose did not repress the synthesis of enzymes concerned with degrading these sugars but it did inhibit the activity of the lactose and galactose enzymes. Thus in glucose-lactose mixtures and glucose-galactose mixtures the lactose and galactose were not utilised until all the glucose had been used up, whereas in glucose-fructose, glucose-cellobiose and glucose-xylose mixtures the fructose, cellobiose and xylose were

utilised concomitantly with glucose.

Investigations into the metabolism of ^{14}C glucose to $^{14}CO_2$ have revealed conversion rates of 60 to 80% in 14 days (Cheshire, Mundie and Shepherd, 1969) and 63% in 6 weeks (Martin, Haider, Farmer and Fustec-Mathion, 1974) using 0.5% and 0.2% ^{14}C glucose respectively.

There is evidence that glucose can be converted to gluconic acid by accumulated glucose oxidase activity in soil (Ross, 1968, 1974).

G. Effects of Amendments on Soil Enzyme Activities

1. Pesticides

a) Introduction

The word pesticide is an all embracing term to describe the chemicals which are now widely used in agriculture to reduce losses in food production caused by weeds, insects, nematodes, rodents and fungal or bacterial diseases. The extent to which they are used is illustrated by the consumption of herbicides, admittedly the largest group, in the U.K. in 1973. If all the active ingredient (8300 tonnes) had been evenly mixed into the top 0.5 cm of the treated land (4.15 million hectares) this would have produced a herbicide concentration of 30 ppm throughout that soil (Greaves, Davies, Marsh and Wingfield, 1976). At the same time considerably larger quantities of formulation additives, such as wetting agents and

solvents, are applied to the soil. Even foliar applied chemicals may eventually find their way into the soil. Despite advances in biological pest control the importance of these chemicals is unlikely to diminish in the foreseeable future.

There is general agreement amongst soil ecologists that when such large quantities of anti-biological materials are being introduced into the soil there is considerable potential for disruption of this complex environment. A great deal of detailed experimentation is required to determine whether this potential is ever realised in practise.

b) Effects on Soil Microorganisms

Microorganisms make an invaluable contribution to soil fertility, being involved in such crucial processes as nutrient cycling, humification and soil aggregation. It is therefore important to understand not just the metabolism of pesticides but also other relationships between pesticidesand the microbiology and biochemistry of the soil.

There is an enormous volume of literature on this subject, indeed at least sixteen review articles have been published in the last eighteen years (Table 7).

These reports have revealed that some pesticides when applied at excessive rates can have dramatic effects, both stimulatory and inhibitory, on the numbers, species composition and activities of soil microorganisms. However, no long term disruption

TABLE 7 Reviews concerning effects of pesticides on soil microorganisms

Pesticides	Herbicides	Insecticides
Bollen (1961)	Fletcher (1960, 1966 a,b)	Tu & Miles (1976)
Martin (1963)	Audus (1964, 1970)	
Alexander (1969)		Fungicides
Helling, Kearney & Alexander (1971)	Cullimore (1971)	Wainwright (1977)
Parr (1974)	Grossbard (1976)	Microbiocides
	Greaves, Davies, Marsh & Wingfield (1976)	Kreutzer (1963)

has been reported when field application rates are investigated. Exceptions to this are the fumigants and some fungicides which are applied to soil in higher doses than the herbicides or insecticides, to specifically inhibit all or some microorganisms (Alexander, 1969; Helling et al., 1971; Wainwright, 1977).

These findings are reassuring, particularly for the pesticide manufacturers, but must not lead to the complacent assumption that harmful disruptions will never occur. Since attempts to predict pesticide effects have been unsuccessful (Domsch and Paul, 1974) there is no substitute for a practical test to

monitor these foreign compounds for soil effects.

Ideally it would be desirable to base the assessment of pesticide effects on soil microorganisms on one indicator alone, i.e. something analogous to the demonstration of water contamination by sewage using indicator organisms. This has not proved possible because the various microbial activities do not respond to a pesticide in the same manner. Pesticide effects in soil have been investigated using a vast array of parameters ranging from counts and composition of microbial populations, to carbon turnover (organic matter, plant material, lignoid material, cellulose, glucose etc), to nitrogen turnover (mineralisation, ammonification, nitrification, denitrification, nitrogen fixation), to soil enzyme activity estimations (dehydrogenase, phosphatase, urease etc). Axenic and mixed cultures of microorganisms grown in artificial media have also been used in such studies despite the disadvantage that the results can not be extrapolated to the soil environment. The choice of indicator activities is often based on the aims of the research. If it is orientated towards discovering whether a particular chemical is likely to be a hazard in the field situation then large scale monitoring using relatively simple and rapid techniques is needed (Grossbard, 1973). However, if the research is of a more fundamental nature then rapidity need not be an over-riding factor.

Much of this work has been performed with little regard for the amount of pesticide that is likely to be found in the field and, whilst it is necessary to incorporate a safety margin into the concentrations used in such experiments (to account for their uneven distribution in the soil - see for example Robinson (1976)), the greatly exaggerated rates used by some (in excess of 1000 ppm) are hardly justified, unless like Stojanovic, Kennedy and Shuman (1972) the results are discussed, somewhat hypothetically, in terms of accidental spillages or the disposal of pesticides and pesticide containers.

The picture is further complicated by discrepancies between the results obtained for the same pesticide on the same microbial characteristic but in different soils. Unfortunately this variation will never be eliminated because no two soils are alike, however it would be a step forward if all the laboratories working in this area were to adopt a standard series of tests, thus creating a desirable uniformity which would facilitate valid comparison of results. The feasibility of such standardisation is now under test in West Germany. If this proves successful it is likely to lead to long overdue EEC legislation concerning the testing of novel compounds prior to their registration.

c) Effects on Soil Enzymes

From the reviews by Kiss *et al.* (1975), Greaves *et al.* (1976) and Grossbard (1976) it is apparent that

soil enzyme activity estimations *per se* have only played a minor role in monitoring pesticide effects in soil and this is particularly true in the western world. The soil enzymes to have received the most attention are: invertase, catalase, dehydrogenase, phosphatase, protease and urease, in short those which have been extensively characterised from an enzymological point of view. In keeping with the rest of the pesticide soil microenvironment interaction research the herbicides have received the most attention.

Table 8 summarises the reports involving soil urease. The concentrations quoted are those given by the authors because conversion to the same units might be misleading. The conversion factor for a dose per unit area to ppm will vary with the type of chemical and conditions in which it is used. Solubility and the degree to which a pesticide is adsorbed will determine the depth of penetration which will in turn affect the weight of soil per hectare. However, if it is assumed that most of the pesticide is located in the top 5 cm of the soil then kg/ha can be approximated to ppm by multiplying by 1.4 (see Methods and Materials). Concentrations are expressed in terms of active ingredient except where indicated by an asterisk. In these instances the original papers were not available to the author and the results have been extracted from the reviews of Grossbard (1976), Greaves *et al.* (1976) or Grossbard and Davies (1976).

TABLE 8 Effects of pesticides on soil urease activity

Pesticide	Concentration/ Dose	F/L	Effect	Reference
1. Herbicides				
Aphalon	2-1000 ppm	L	0	Krezel & Musial (1969)
Atrazine	100-1000 ppm	L	0	Krezel & Musial (1969)
Atrazine	4 kg/ha[1]	F	-	Voets, Meerschman & Verstraete (1974)
Atrazine	3 kg/ha	F	0	Kruglow, Gersz, Piercewa, Bay-Bienko & Michajlow (1975)
Atrazine	200 ppm	L	-	Pel'tser (1972)
Aresin	2-1000 ppm	L	0	Krezel & Musial (1969)
Chlorophos	200 ppm	L	-	Pel'tser (1972)
Chloropropham	1000 ppm	L	-	Krezel & Musial (1969)
Chloropropham	1 ppm*	L	-	Grossbard & Davies (1976)[2]
Dalapon	10 kg/ha	F	+	Namdeo & Dube (1973a, b, c)
Dalapon	200 ppm*	L	-	Zinchenko & Osinskaya (1969)[4]
2,4-D	20 ppm	L	-	Zinchenko & Osinskaya (1969)
2,4-D	2 kg/ha*	F	+	Zinchenko, Osinskaya & Prokudina (1969)[5]
Dinoseb	50 ppm*	L	-	Zinchenko & Osinskaya (1969)[4]
Dinoseb	5 kg/ha*	F	+	Zinchenko et al. (1969)
Diuron	4-20 ppm	L	-	Cervelli, Nannipieri, Giovannini & Cerna (1976, 1977)
Diuron	unspecified*	L	+	Zinchenko & Osinskaya (1969)[3]

Eptam	2-1000 ppm	L	0	Krezel & Musial (1969)
Fenuron	4-20 ppm	L	-	Cervelli et al. (1976, 1977)
Linuron	4-20 ppm	L	-	Cervelli et al. (1976, 1977)
Linuron	100 ppm*	L	-	Grossbard & Davies (1976)[2]
Monuron	4-20 ppm	L	-	Cervelli et al. (1976, 1977)
Methurin	3 kg/ha*	F	-	Zinchenko et al. (1969)[4]
Methurin	unspecified*	L	-	Zinchenko & Osinskaya (1969)[3]
Neburon	4-20 ppm	L	-	Cervelli et al. (1976, 1977)
Paraquat	3.75 kg/ha	F	+	Namdeo & Dube (1973a, b, c)
Paraquat	100-400 ppm	L	0	Giardina, Tomati & Pietrosanti (1970)
Prometryne	2-1000 ppm	L	0	Krezel & Musial (1969)
Pyrazone	4-8 kg/ha*	F	-	Zinchenko et al. (1969)[4]
Siduron	4-20 ppm	L	-	Cervelli et al. (1976, 1977)
Simazine	100-1000 ppm	L	0	Krezel & Musial (1969)
Simazine	50 ppm*	L	+	Zinchenko & Osinskaya (1969)[4]
Simazine	2 kg/ha	F	+	Manorik & Malichenko (1969)[5]
Simazine	10 kg/ha	F	-	Manorik & Malichenko (1969)
Tenoran	2-1000 ppm	L	0	Krezel & Musial (1969)
TCMB	50-300 ppm	L	-	Voets & Vandamme (1970)
Tillam	2-1000 ppm	L	0	Krezel & Musial (1969)
2,3,6-TBA	unspecified*	L	-	Zinchenko & Osinskaya (1969)[3]
2,4,5-T	1000 ppm	L	0	Burns & Lethbridge (1976)

TABLE 8 (Contd)

Pesticide	Concentration/ Dose	F/L	Effect	Reference
Trifluralin	3-30 kg/ha	L	-	Tyunyayeva, Minenko & Pen'kov (1974)
Tordon	1000 ppm	L	0	Burns & Lethbridge (1976)
Avadex	1.4 kg/ha			
Pyramin	5 kg/ha	F	-	Verstraete & Voets (1974)
Betanol	1.2 kg/ha			
2. Insecticides				
Accothion	50-1000 ppm	L	-	Lethbridge & Burns (1976)
Malathion	50-1000 ppm	L	-	Lethbridge & Burns (1976)
Thimet	50-1000 ppm	L	-	Lethbridge & Burns (1976)
3. Fungicides				
Perenox	200-32,000 ppm	L	-	Bhavanandan & Fernando (1970)
Perenox	0.03-0.56 kg/ha	F	0	Bhavanandan & Fernando (1970)
4. Nematocides				
3 amino-1,2,4 triazole	Unspecified	L	-	Gauthier, Ashtakala & Lenoir (1976)

1 = 14 annual applications; 2 = Personal communication from Krezel & Musial;

3 = Cited by Greaves et al. (1976); 4 = Cited by Grossbard (1976);

5 = Cited by Grossbard & Davies (1976); F = Field experiment;

L = Laboratory experiment; + = Stimulation; - = Inhibition;

0 = No effect.

Of the 48 reports cited 28 involved inhibition, 13 involved no effect and only 7 involved stimulation of activity. So urease appears to be particularly sensitive to pesticide inhibition (Grossbard, 1976) and it is not only the doses at the top end of the concentration spectrum which cause this reduction in activity. Cervelli, Nannipieri, Giovannini and Perna (1976, 1977) observed that urease inhibition by the substituted urea herbicides fenuron, monuron, diuron, siduron and neburon (in itself an interesting result from the point of view of herbicide fertiliser interactions in the field) increased with inhibitor concentration over the range 4 to 20 ppm. The inhibition observed (10 to 40%) was of the mixed type in which both Km and maximum velocity are modified and the kinetic data take on the characteristics of both competitive and non-competitive inhibition. Further evidence for urease inhibition by substituted urea herbicides comes from a personal communication to Grossbard and Davies (1976) by Krezel and Musial, in which they reported inhibition with 100 ppm linuron.

In contrast to this sensitivity to the substituted urea herbicides, urease has been reported to be unaffected by 1000 ppm treatments of: simazine, atrazine, prometryne, aphalon, aresin, tenoran, tillam and eptam (Krezel and Musial, 1969) and 2,4,5-trichlorophenoxyacetic acid and tordon (Burns and Lethbridge, 1976).

With the exception of linuron and methurin, wherever there is more than one report for a pesticide there is never total agreement as to the response, although this is only to be expected considering the variation in experimental techniques used. The four reports concerning atrazine illustrate these contradictions admirably. Pel'tser (1972) reported inhibition at 200 ppm, whereas Krezel and Musial (1969) could detect no effect in the range 100 to 1000 ppm. In field experiments 3 kg/ha had no effect (Kruglow, Gersz, Piercewa, Bay-Bienko and Michajlow, 1975), whereas 14 annual applications of 4 kg/ha reduced activity by 36 to 64% (Voets, Meerschman and Verstraete, 1974).

The only report of herbicide combinations that is cited is the one by Verstraete and Voets (1974) in which a mixture of avadex (diallate), pyramin and betanol at 1.4, 5 and 1.2 kg/ha respectively reduced activity by an unspecified amount in a field investigation.

Table 9 summarises the effects of the herbicide 2,4-D on some soil enzymes other than urease. Only in the case of dehydrogenase (Lenhard, 1959) did it reduce activity when applied at 100 to 700 ppm.

Soil enzyme-agrochemical interactions have been discussed from a theoretical point of view by Cervelli, Nannipieri and Sequi (1978).

TABLE 9 The effect of 2,4-D on soil enzymes other than urease

Dose/ Concentration	Enzyme	F/L	Effect	Reference
2-4 kg/ha*	Amylase	L	0	Bliyev (1973)
2-4 kg/ha*	Catalase	L	+	Bliyev (1973)
2 kg/ha	Catalase	F	+	Zinchenko et al. (1969)
150-1100 kg/ha	Dehydrogenase	L	+	Klein, Loh & Goulding (1971)
50 ppm	Dehydrogenase	L	+	Lenhard (1959)
100-700 ppm	Dehydrogenase	L	-	Lenhard (1959)
2 kg/ha	Invertase	F	+	Zinchenko et al. (1969)
2-4 kg/ha*	Invertase	L	0	Bliyev (1973)

* = In the presence of 20-60 kg dalapon/ha;

F = Field experiment;

L = Laboratory experiment;

\+ = Stimulation;

\- = Inhibition;

0 = No effect.

2. Fertilisers, Lime and Organic Amendments

a) Effects on Soil Enzymes

The effects of fertilisers, lime and organic amendments in general on soil polysaccharidases and urease have been reviewed by Kiss (1978) and Bremner and Mulvaney (1978) respectively. Table 10 summarises the reports involving urease. As with the pesticides, dose per unit area has not been converted to ppm but so that comparisons can be made kg/ha can be converted to ppm by multiplying by 0.45 assuming uniform incorporation to a depth of 15 cm (see Methods and Materials).

The bulk of this work has been carried out in the laboratory with little attempt to reflect the likely concentrations of these materials found in the field. In contrast to urease-pesticide interactions there are several discernable trends and this can be explained in part by the fact that fewer variables have been investigated in greater detail. In general, organic amendments such as sugars, polysaccharides, manures, composts and plant debris increase urease activity, whilst the addition of its substrate urea has no effect (Bremner and Mulvaney 1978). In the case of liming materials the results are more variable, particularly for calcium carbonate where stimulatory (Verstraete and Voets, 1977), inhibitory (Volk, 1966; Pel'tser, 1972) and no effect (Zantua and Bremner, 1978) responses have been recorded. Both investigations using calcium hydroxide (Moe, 1967; Pel'tser, 1972) recorded a decrease in

TABLE 10 Effect of fertilisers, lime and organic amendments on soil urease activity

Amendment	Concentration/ Dose	L/F	Effect	Reference
1. N P K or S				
Ammonium carbonate	875 ppm N	L	+	Conrad (1943)
Ammonium carbonate	500 ppm N	L	0	Zantua & Bremner (1978)
Ammonium nitrate	500 ppm N	L	0	Zantua & Bremner (1978)
Ammonium sulphate	500 ppm N/S[1]	L	0	Zantua & Bremner (1978)
Ammonium sulphate	336 kg/ha N	F	?[2]	Bhavanandan & Fernando (1970)
Calcium ammonium nitrate	336 kg/ha N	F	+	Bhavanandan & Fernando (1970)
Calcium nitrate	50 ppm N	L	+	Conrad (1942a)
Calcium sulphate	500 ppm S	L	0	Zantua & Bremner (1978)
Diammonium phosphate	500 ppm N/P[1]	L	0	Zantua & Bremner (1978)
Dicalcium phosphate	500 ppm P	L	0	Zantua & Bremner (1978)
Ground rock phosphate	500 ppm P	L	0	Zantua & Bremner (1978)
Magnesium sulphate	500 ppm S	L	0	Zantua & Bremner (1978)
Monoammonium phosphate	500 ppm N/P[1]	L	0	Zantua & Bremner (1978)
Monocalcium phosphate	500 ppm P	L	0	Zantua & Bremner (1978)
Potassium chloride	500 ppm K	L	0	Zantua & Bremner (1978)
Potassium chloride	5000 ppm K	L	+	Vasilenko (1962)

Potassium nitrate	500 ppm N/K[1]	L	0	Zantua & Bremner (1978)
Potassium sulphate	500 ppm S/K[1]			Zantua & Bremner (1978)
Sodium nitrite	500 ppm N	L	0	Zantua & Bremner (1978)
Unspecified nitrate	100 ppm N	L	0	Musa & Mukhtar (1969)
Unspecified NPK and S	11.2 kg/ha N	F	0	Khan (1970)
	5.9 kg/ha P			
	16.7 kg/ha K			
	9.0 kg/ha S			
Unspecified N and S	11.2 kg/ha N	F	0	Khan (1970)
	9.0 kg/ha S			
2. Lime				
Calcium carbonate	1300-10,000 ppm	L	-	Pel'tser (1972)
Calcium carbonate	2509 kg/ha	F	-	Volk (1966)
Calcium carbonate	4000-10,000 ppm	L	0	Zantua & Bremner (1978)
Calcium carbonate[3]	25,455 kg/ha	F	+	Verstraete & Voets (1977)
Calcium hydroxide	2240 kg/ha	L	-	Moe (1967)
Calcium hydroxide	800-4000 ppm	L	-	Pel'tser (1972)
Calcium oxide	1000-4000 ppm	L	+	Conrad (1943)
Calcium oxide	2000-4000 ppm	L	+	Zantua & Bremner (1978)
Unspecified	unspecified	L	+	Wang, Tseng & Puh (1966)

TABLE 10 (Contd)

Amendment	Concentration/ Dose	L/F	Effect	Reference
3. Urea				
	50 ppm N	L	0	Conrad (1942a)
	5000 ppm N	F	0[4]	Vasilenko (1962)
	unspecified	L	0	Tanabe & Ishizawa (1969)
Plus 1000 ppm glucose	13 ppm N	L	+	Paulson & Kurtz (1969b)
	336 kg/ha N	F	+	Bhavanandan & Fernando (1970)
	500 ppm N	L	0	Lloyd & Sheaffe (1973)
Plus 2000 ppm glucose	500 ppm N	L	+	Lloyd & Sheaffe (1973)
	100-200 kg/ha N	F	+	Namdeo & Dube (1973a, b, c)
	10-500 ppm N	L	0	Zantua & Bremner (1976)
4. Glucose				
	50,000 ppm	F/L[5]	+	Vasilenko (1962)
	50,000 ppm	L	+	Wang, Tseng & Puh (1966)
	2000-10,000 ppm	L	+	Tanabe & Ishizawa (1969)
	6250 ppm	L	+	Musa & Mukhtar (1969)
	10,000-30,000 ppm	L	+	Laugesen (1972)
	200-5000 ppm	L	+	Zantua & Bremner (1976, 1978)

5. Manures and Composts

Rotted compost	33,750 ppm	L	+	Conrad (1942a)
Fresh compost	33,750 ppm	L	+	Conrad (1942a)
Coarse rotted manure	33,750 ppm	L	+	Conrad (1942a)
Farmyard manure	23,641 ppm	L	+	Balasubramanian, Siddaramappa & Rangaswami (1972)
Beef cattle manure	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Dairy cattle manure	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Sheep manure	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Sewage sludge	20,000 - 30,000 ppm	L	+	Zantua & Bremner (1976, 1978)
Green farmyard manure	20,364 kg/ha	F	+	Verstraete & Voets (1977)
Unspecified manure	45,000 kg/ha	F	0	Khan (1970)

6. Plant debris

Coarse alfalfa hay	33,750 ppm	L	+	Conrad (1942a)
Straw	33,300 ppm	L	+	Conrad (1942a)
Heat dried alfalfa	33,750 ppm	L	+	Conrad (1942a)
Corn mulch	4480 kg/ha	L	+	Moe (1967)
Rice straw	10,000 ppm	L	+	Delaune & Patrick (1970)
Maize stalk	65,359 ppm	L	+	Balasubramanian, Siddaramappa & Rangaswami (1972)
Pongamia cake	27,322 ppm	L	+	Balasubramanian, Siddaramappa & Rangaswami (1972)

TABLE 10 (Contd)

Amendment	Concentration/ Dose	L/F	Effect	Reference
Corn	5000-10,000 ppm	L	+	Zantua & Bremner (1976, 1978)
Orchard grass	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Alfalfa	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
7. Miscellaneous				
Casein	50 ppm N	L	0	Conrad (1942a)
Cellulose	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Gelatin	50 ppm N	L	0	Conrad (1942a)
Nucleic acid	50 ppm N	L	0	Conrad (1942a)
Peptone	5000 ppm N	L	+	Vasilenko (1962)
Peptone	2000 ppm	L	+	Tanabe & Ishizawa (1969)
Starch	100,000 ppm	L	+	Chin & Kroontje (1963)
Starch	unspecified	L	+	Tanabe & Ishizawa (1969)
Starch	5000 ppm	L	+	Zantua & Bremner (1976, 1978)
Sucrose	1584 ppm	L	+	Conrad (1942a)

1 = Could be either because information provided is ambiguous; 2 = Interpreted by Bhavanandan and Fernando (1970) as slight enhancement but no control values quoted; 3 = Sugar lime; 4 = Interpreted as slight stimulation, followed by slight inhibition, followed by slight stimulation by Vasilenko (1962); 5 = Air dried soil treated in laboratory but returned to the field for incubation. F = Field experiment; L = Laboratory experiment; + = Stimulation; - = Inhibition; 0 = No effect.

activity, whilst the two involving calcium oxide reported stimulation (Conrad, 1943; Zantua and Bremner, 1978). Mineral fertiliser treatments have either increased activity (Conrad, 1942a, 1943; Vasilenko, 1962; Bhavanandan and Fernando, 1970) or had no effect (Musa and Mukhtar, 1969; Khan, 1970; Zantua and Bremner, 1978).

PART TWO - METHODS AND MATERIALS

A. The Soil

A silt loam soil (Hamble series) from Spring Grove Farm, Wye, Nr. Ashford, Kent, was used for all the experiments (Table 11). Samples were collected from the surface 15 cm, hand crumbled, air-dried and sieved. The fraction which passed through a 2.36 mm mesh was stored in dark glass bottles at room temperature (21 $\pm$ 2°C) until required for experiments.

TABLE 11 Soil Analysis

pH	% C	% N	Organic matter	Sand	Silt	Clay	100% W.H.C.* (ml/g)
5.4	2.20	0.54	6.4%	16%	64%	20%	0.72

* = water holding capacity

pH was determined using a glass electrode in a soil: distilled water slurry (1:2.5), % C and N using a Hewlett Packard F and M 185 automatic analyser, % organic matter by combustion, texture by sedimentation using a hydrometer (Bouyoucos, 1927) and water holding capacity using a Hilgard cup (Pramer and Schmidt, 1964).

B. Enzymology

1. Glucanase

a) The assay

Quadruplicate samples (1 g) of air-dried soil were pre-incubated with 2 ml, 0.1% w/v aqueous sodium azide (a microbial inhibitor) in 20 ml screw-capped universal bottles. After 30 min the reaction was started by adding 4 ml, 1% w/v laminarin (Sigma, Koch-Light or British Borax Co.) in 0.1 M sodium acid maleate buffer pH 5.4, such that the absolute laminarin concentration was 6.7 mg/ml. Separate controls lacking laminarin (soil control) and soil (substrate blank) were also set up but only in duplicate (see Methods and Materials, B.1.b.7.). The reasons for these controls were to take into account the inherent extractable reducing sugar concentration of the soil, absorbance due to humate extraction and the reducing value of the laminarin end groups (see Introduction, E.).

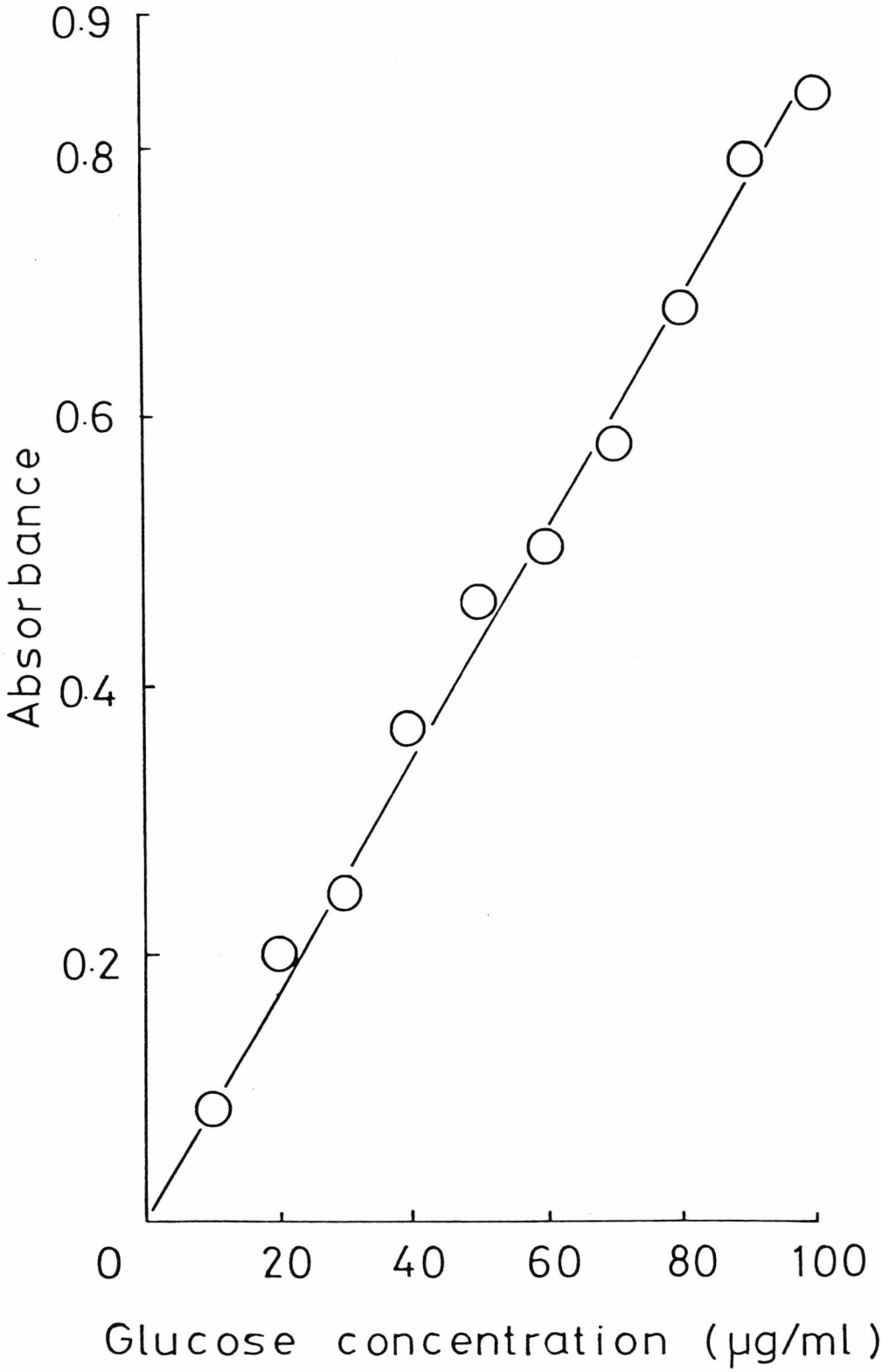
Treatments and controls were incubated at 25°C on an orbital shaker for 17 h after which the reaction was terminated by placing the bottles in a 100°C water bath for 20 min (Thornton and McLaren, 1975). When the reaction mixtures had cooled to room temperature they were decanted into plastic tubes and centrifuged at 20,000 x g for 10 min (16 x 15 ml rotor) in an MSE HS 18 refrigerated (4°C) centrifuge to remove the soil.

Each supernatant was assayed in duplicate for reducing sugars using the Nelson-Somogyi copper reduction method (Spiro, 1966). To 1 ml supernatant, suitably diluted with buffer to bring it within the reducing sugar concentration range of the standard curve (see below), was added 1 ml alkaline copper reagent (see below). After "Rota-Mixing" the glass test-tubes were covered with Oxoid caps and placed in a 100°C water bath for 10 min (necessary for the reducing sugars to reduce the cupric sulphate to cuprous oxide). When the contents of the tubes had cooled to room temperature 1 ml arsenomolybdate reagent (see below) was added followed by 3 ml distilled water, "Rota-Mixing" at each stage. The absorbance of the resulting blue colour was measured within 60 min at 520 nm using 1 cm, 3 ml cuvettes in a Pye Unicam SP 500 spectrophotometer. Reducing sugar concentration, as glucose equivalents, was calculated from a glucose standard curve (0 to 100 µg/ml) freshly prepared in buffer every day (Fig. 4). The correlation coefficient of the straight line was always greater than 0.990. The computed coefficient of variation for the assay ranged from 2 to 8%.

The alkaline copper reagent (Somogyi, 1952) was prepared as follows:

Solution one - 15 g potassium sodium tartrate and 30 g anhydrous sodium carbonate were dissolved in 300 ml water followed by 20 g sodium bicarbonate.

FIGURE 4 Glucose standard curve.



A solution of 180 g anhydrous sodium sulphate in 500 ml water was heated to expel air and after cooling was combined with the first solution and made up to 1 litre with water.

Solution two - 5 g cupric sulphate and 45 g anhydrous sodium sulphate were dissolved in water and diluted to 250 ml. Just prior to use, 4 volumes of solution one were combined with 1 volume of solution two.

The potassium sodium tartrate is a copper chelating agent, the anhydrous sodium sulphate prevents reoxidation of the cuprous oxide by the atmosphere and the sodium carbonate-bicarbonate buffer provides the alkaline conditions.

The arsenomolybdate reagent (Nelson, 1944) was prepared by adding 21 ml concentrated sulphuric acid, with stirring, followed by 3 g sodium arsenate in 25 ml water, to a solution of 25 g ammonium molybdate in 450 ml water. The solution was stored in a glass-stoppered bottle and just prior to use was diluted with 2 volumes 0.75 M sulphuric acid. The function of this reagent was to react with the cuprous oxide formed in the previous stage, producing a stable blue complex whose optical density could then be measured.

b) Development of the Assay

1) Selection of Buffer

In any colorimetric assay it is important that the optical density of the control is small compared to the treatment. Therefore, in a colorimetric soil enzyme assay such as glucanase, it is essential to select a buffer which does not extract large quantities of humic material.

Six buffers were evaluated in relation to their extraction of organic matter using the normal assay procedure, except that no substrate was used and that the supernatants were scored visually for organic matter extraction (Table 12).

TABLE 12 Suitability of buffers for use in colorimetric soil enzyme assays

Buffer	Organic matter extraction	pH range (at 25°C)
Citrate (0.1 M)	+++	3.0 - 6.2
Citrate-phosphate (0.05 M)	++++	2.6 - 7.0
Citrate-phosphate (0.05 M) + 0.5 M $CaCl_2$	+	2.6 - 7.0
Citrate-phosphate (0.05 M) + 0.5 M $MgSO_4$	++	2.6 - 7.0
Phosphate (0.1 M)	+++	5.7 - 8.0
Acetate (0.1 M)	-	3.6 - 5.6
Succinate (0.1 M)	-	3.8 - 6.0
Maleate (0.1 M)	+	5.2 - 6.8

In addition, the citrate-phosphate buffer, which on its own extracted very high levels of organic matter, was investigated in the presence of 0.5 M calcium chloride and 0.5 M magnesium sulphate as floculating agents; the purpose being to halt colloid dispersal and hence humate extraction. Citrate, citrate-phosphate plus magnesium sulphate and phosphate buffers were all discarded on the groundsof high organic matter extraction. Citrate-phosphate plus calcium chloride was subsequently discarded for causing precipitation at two stages during the assay (heating to halt the reaction and on addition of the alkaline copper reagent in the Nelson-Somogyi reducing sugar determination). From the remaining three buffers sodium acid maleate was selected for use in the assay, because at that time the effect of pH on activity had not been investigated so all the work was being performed at pH 5.8, the pH at which Lilley and Bull (1974) measured the glucanase activity of their thermophilic streptomycete. This pH falls in the middle of the sodium acid maleate buffering range, whereas it is outside that of sodium acetate buffer and only just within the range of sodium succinate buffer. Even when the pH optimum of the enzyme was found to be 5.4 it was decided to stick with sodium acid maleate buffer because this pH is within its buffering range, plus the fact that the small amount of organic matter extracted did not lead to unacceptably high controls. The optical density, after reaction with the Nelson-Somogyi

reagents, of the assay supernatant diluted 5 times usually fell into the range 0.4 to 0.5, whereas that of the undiluted soil control was always less than 0.1.

To check whether sodium acid maleate buffer had any effect *per se* on soil glucanase a comparison of enzyme activity in distilled water and buffer was made (Table 13). At the same time, activity in acetate, succinate and citrate-phosphate buffers was measured. The pH of the buffers was 5.4, whereas that of the distilled water was 6.5. Maleate, acetate and succinate buffers had no effect on activity but citrate-phosphate had a considerable stimulatory effect (57%).

TABLE 13 Comparison of glucanase activity in distilled water and four buffers. Activity is expressed in μ moles of reducing sugars as glucose equivalents/g soil/h

Buffer	Glucanase activity
Distilled water	0.42
Sodium acetate (0.1 M)	0.44
Sodium acid maleate (0.1 M)	0.39
Sodium succinate (0.1 M)	0.43
Citrate-phosphate (0.05 M)	0.66

The pH of the soil-buffer mixture was measured at the beginning and end of the assay to check for efficiency of buffering and whereas the buffers held their pH very well the pH of the soil-distilled water mixture increased from 5.3 to 5.9 over the 17 h incubation period. Thus, because water has no buffering capacity the pH of the soil-water mixture changed during the assay. The increase in pH may have been a function of the sodium azide, since Skipper and Westerman (1973) reported that 400 and 800 ppm sodium azide increased soil pH (5.1) in unbuffered systems to 5.7 and 6.3 respectively. Just how this pH shift was brought about is unclear since if it was caused directly by the sodium azide then an immediate rather than a gradual response would have been expected. Alkaline breakdown products are a possibility, although in acidic solution azides are hydrolysed to hydrazoic acid. In practise this pH shift had no noticeable effect on glucanase assays in water because its activity varies little over the range pH 5 to 6 (see Results and Discussion, A.1.a), however this may not be the case for all other soil enzymes.

The buffer versus water debate in soil enzymology has already been touched upon in the introduction (see section, A.4) and it is now appropriate to summarise the many advantages of using buffers in soil enzyme assays.

a) Enzymes should be assayed at their optimum pH - this is standard procedure in biochemistry. In the case of glucanase this coincided with the soil pH but in many instances, such as urease (Pettit et al., 1976) and phosphatase (Pettit, Gregory, Freedman and Burns, 1977) whose pH optima are 6.5 and 6.7 respectively in pH 5.4 soil, this will not be the case and a buffer must be used to bring the reaction to its optimum pH.

b) Enzymes must be assayed at constant pH simply because activity is pH dependent. Water itself has no buffering capacity and if the substrate, products or microbial inhibitor are acidic or basic to such an extent that the soils own buffering capacity can not cope then pH will change if a buffer is not used. Acidic or alkaline products may arise from the action of enzyme on its substrate (urease produces both ammonia and carbonic acid), humate extraction or breakdown of the microbial inhibitor.

c) Many soils do not have a pH of 5.4 $\pm$ 0.5 but by using a buffer to poise the pH the glucanase assay remains constant and a comparison of activity in different soils is possible.

d) Comparisons are often made between soil enzymes and purified enzymes from other sources but the latter can not be reliably assayed in distilled water. In

this laboratory the distilled water pH varies unpredictably between 6 and 8.

e) Soil pH may change in the field due to crop growth, manuring, liming or addition of fertilisers or in the laboratory due to experimental treatments such as autoclaving, irradiation or addition of pesticides (see Results and Discussion, B.3.b.2). If one is looking at possible effects of these treatments it is imperative to assay at the original pH otherwise more than one variable is under test. Even when air-dried soil was re-wetted and incubated at 25°C for 70 days pH fluctuated between 5 and 6 from week to week (see Results and Discussion, B.1.b.4).

The only circumstance under which a soil enzyme should be assayed in water is in comparative studies to make sure that the buffer to be used has no effect on activity.

2. Length of Incubation Time and the Need to Use a Microbial Inhibitor

The advantages and disadvantages of the various microbial inhibitors used in soil enzymology have already been discussed. Sodium azide was chosen because, although far from ideal, it has less disadvantages than the others (see Introduction, A.4).

The production of reducing sugars from laminarin was monitored at regular intervals up to 145 h (Fig. 5) in the absence (2 ml distilled water) and in

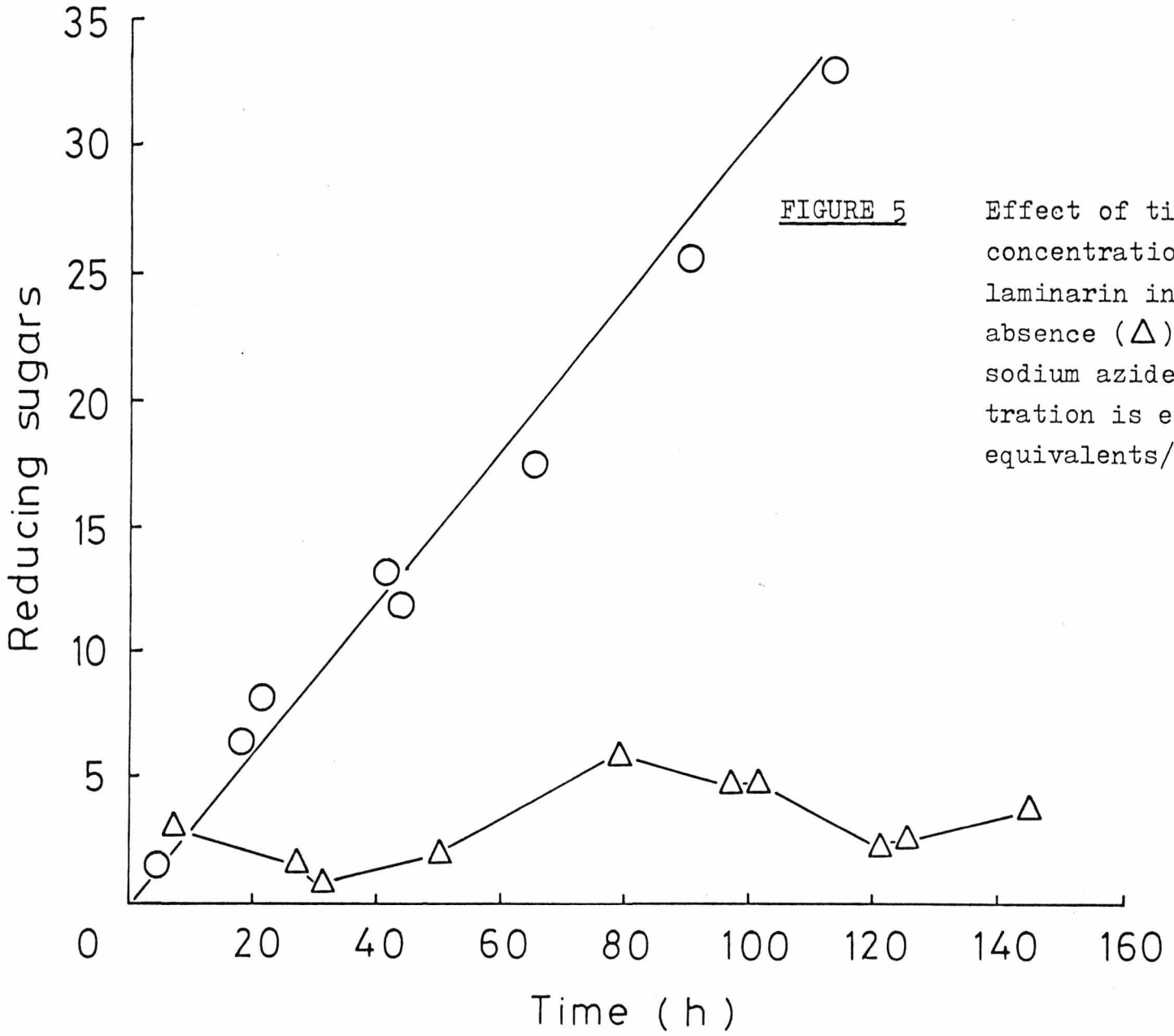


FIGURE 5

Effect of time on the reducing sugar concentration of soil incubated with laminarin in the presence (O) and absence (Δ) of the microbial inhibitor sodium azide. Reducing sugar concentration is expressed as μ moles glucose equivalents/g soil.

the presence of sodium azide (2 ml, 0.1% w/v). Apart from the incubation time the procedure was the same as for a normal glucanase assay. In the sodium azide-treated soil reducing sugar concentration increased linearly with time (correlation coefficient 0.99) and there was no sign of a tail off, suggesting that the substrate concentration being used (6.7 mg/ml) was not limiting, even after 113 h. In contrast, the reducing sugar concentration in the non-azide-treated soil fluctuated wildly with time. These fluctuations were probably accounted for by a combination of laminarin breakdown by extracellular glucanases associated and unassociated with microbial proliferation, microbial metabolism and immobilisation of laminarin breakdown products, the chief one being glucose (see Results and Discussion, A.1.h) and possibly catabolite repression of glucanase synthesis by glucose (Lilley and Bull, 1974; Santos et al., 1978). Subsequent work has shown that added glucose disappears very rapidly from soil in the absence of sodium azide but not at all in its presence (see Results and Discussion, B.1.b.5). Thus, in the case of glucanase the microbial inhibitor prevents not only substrate turnover by proliferating microorganisms but also the microbial metabolism of laminarin breakdown products. For these reasons it is impossible to measure glucanase activity in a non-sterile soil.

The fact that there is a continuous build up of reducing sugars in the azide treated soil is a strong

indication that the sodium azide is effective in preventing microbial proliferation in soil, but nevertheless verification was sought by plating 0.1 ml aliquots of the slurry onto nutrient agar. Surprisingly, heavy growth was observed on these plates but when the procedure was repeated, this time using nutrient agar containing approximately 1000 ppm (w/w) sodium azide, the plates remained free of growth for up to 3 weeks. Skipper and Westermann (1973) and Gibson and Burns (1977) have observed the same phenomenon and it may indicate that the sodium azide was adsorbed onto the surface of the soil colloids such that on untreated agar some spore-forming microorganisms (unaffected by the sodium azide) germinated and grew away from the zone containing the sodium azide. In contrast, microbial spores failed to germinate on treated agar because there was no azide-free zone to escape to. This hypothesis could be tested by microscopical analysis of the organisms that grew on the untreated agar but was not deemed necessary in the present context.

The 17 h incubation period for the routine assay was chosen because the variation in short term assays (up to 8 h) was unacceptably high due to the fact that the difference in reducing sugar concentration between the assay and soil control plus the substrate blank (laminarin possesses reducing end groups) was not large enough. After 17 h (a convenient overnight period) the reducing sugar concentration of the assay

supernatant (about 300 µg/ml) was large compared to that of the soil control (0 to 10 µg/ml) and substrate blank (70 to 80 µg/ml).

Soil which had been autoclaved on three occasions for 30 min at 20 lb psi and incubated for 24 h at 25°C between each one, to encourage heat resistant spores to germinate, possessed no activity.

3. Stopping the Reaction

Three methods of stopping the reaction were examined, namely centrifuging at 20,000 x g for 10 min, heating in a 100°C water bath for 20 min and the addition of 1 ml 10 mM silver sulphate. Silver is a well documented inhibitor of microbial glucanases (Chesters and Bull, 1963b; Nagasaki et al., 1976, 1977; Miyazaki et al., 1977). Centrifugation is essential anyway so as to remove the soil and provide a clear supernatant for colorimetric assay. Many soil enzymologists remove the soil by filtering but for a large number of assays this is far too time consuming.

The results of this comparison are summarised in Table 14. Glucanase activity is expressed in µ moles reducing sugars as glucose equivalents/g soil without a time factor to facilitate explanation.

When the assay slurry was centrifuged after 17 h incubation (1) analysis of the supernatant revealed a glucanase activity of 5.10 µ moles reducing sugars/g soil and there was no significant increase in this

TABLE 14 The effectiveness of centrifugation, silver sulphate and heat treatment in terminating the glucanase reaction. Activity is expressed in μ moles reducing sugar as glucose equivalents/g soil.

	Material being assayed	Length of incubation (h)	Treatment	Subsequent incubation (h)	Glucanase activity
1.	Soil	17	Centrifuge		5.10
2.	Supernatant from 1	24			5.23
3.	Soil	41	Centrifuge		12.67
4.	Soil	17	$AgSO_4$ + centrifuge		5.37
5.	Soil	17	$AgSO_4$	24 then centrifuge	13.00
6.	Soil	41	$AgSO_4$ + centrifuge		13.40
7.	Soil	0	$AgSO_4$	17 then centrifuge	5.03
8.	Soil	17	Heat + centrifuge		5.17
9.	Supernatant from 8	24			5.17
10.	Soil	17	Heat	24 then centrifuge	5.17
11.	Soil	41	Heat + centrifuge		12.90
12.	Soil	0	Heat	17 then centrifuge	0

value when this supernatant was reincubated at 25°C for 24 h (2). Thus centrifuging alone irreversibly halted activity in the supernatant (compare 1 and 2 with 3) by physically removing the enzyme which therefore must be retained in the pellet.

Silver sulphate had no effect on glucanase activity. Reaction mixtures to which silver sulphate was added after 17 h incubation and which were subsequently incubated for a further 24 h without centrifuging (5) had very similar activities to those incubated for 41 h straight off (compare 5 with 3 and 6). Glucanase activity was not significantly reduced even when silver sulphate was added at time zero followed by 17 h incubation (compare 7 with 1 and 4). The complete absence of any inhibitory effect by silver ions suggests that they do not penetrate to the site of the enzyme. This lack of penetration may be a result of their positive charge. Pettit (1978) has reported similar findings for phosphatase. Urease however is severely inhibited by silver (Burns et al., 1978) suggesting that urease may reside in a different location to glucanase and phosphatase within the soil matrix.

When the assay mixture was heated in a 100°C water bath for 20 min prior to centrifuging, analysis of the supernatant revealed a glucanase activity of 5.17 μ moles reducing sugars/g soil (8). This value remained constant when this supernatant and even the heat-treated soil suspension were reincubated for a

further 24 h (compare 8, 9 and 10). Heating therefore irreversibly terminated the enzyme reaction. When the assay mixture was heated at time zero and then incubated for 17 h no activity was detected (12).

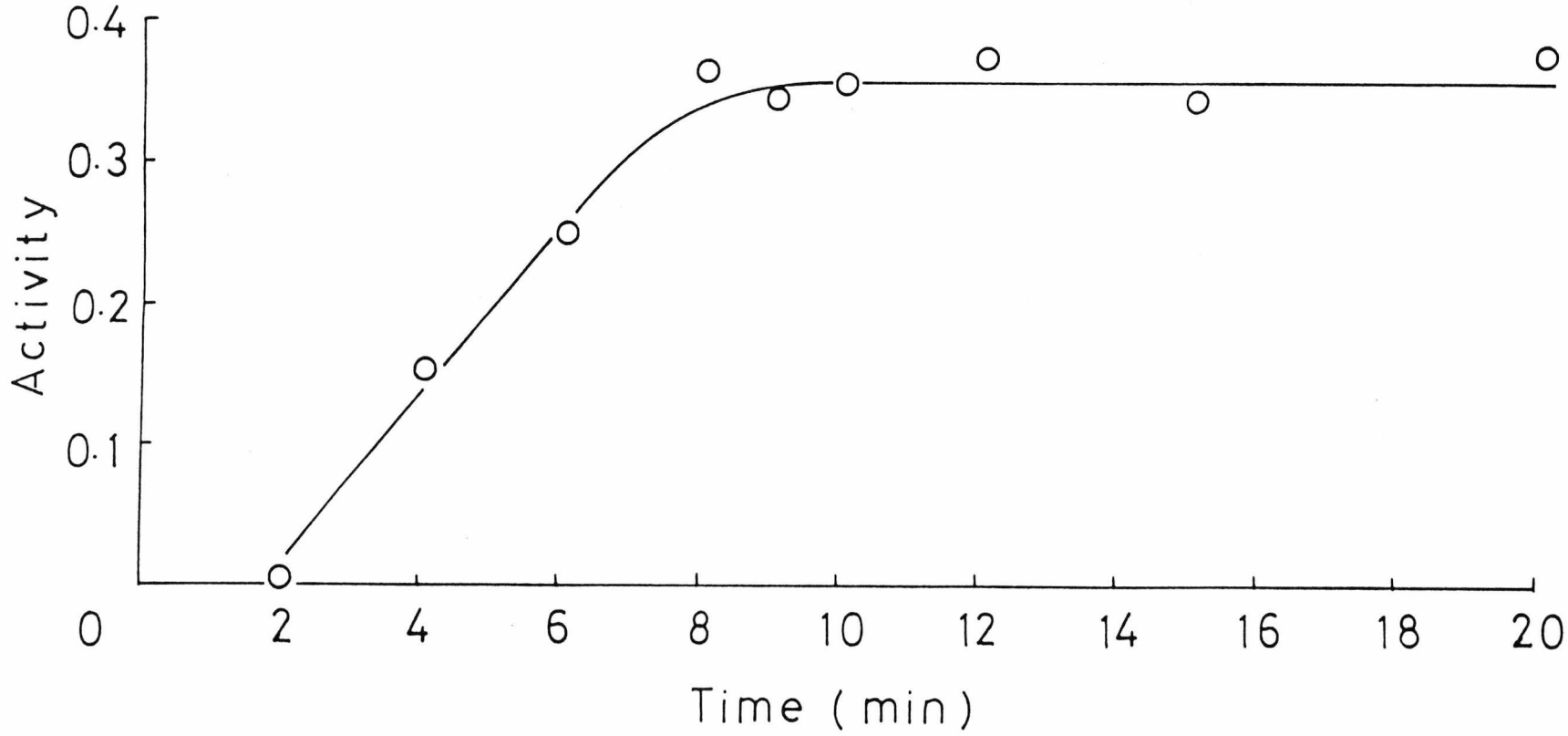
Thus, both the heating and centrifugation treatments irreversibly halted the glucanase reaction but the former was adopted as the standard method since far more assays could be stopped simultaneously by the heat treatment (50) compared to the centrifugation treatment (16).

4. Optimisation of the Nelson-Somogyi Copper Reduction Method for the Determination of Reducing Sugars

The various monosaccharides reduce the copper in the Nelson-Somogyi reaction at different rates. Spiro (1966) has recommended that a 10 min heating period in a 100°C water bath is sufficient for complete oxidation of glucose. To verify this, glucose standard curves were prepared using 10, 20, 30, 40, 50, 60 and 120 min heating periods. The inclination angles of these plots were almost identical thus confirming Spiro's (1966) recommendation.

To determine the length of heating period required to oxidise all the reducing sugars produced during laminarin breakdown a typical assay was carried out using a range of heating times up to 20 min at the reducing sugar estimation stage (Fig. 6). All the reducing sugars produced during the assay were oxidised in 9 min, so a standard 10 min heating period was

FIGURE 6 Optimisation of the heating period required for copper reduction in the Nelson-Somogyi method of reducing sugar determination. Glucanase activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.



adopted.

One interesting point to emerge from this experiment was that the reducing sugar value of the substrate blank increased throughout the 20 min heating period, albeit very slowly after 8 min, suggesting either that complete oxidation of the laminarin is a slow process or the laminarin was being slowly hydrolysed under the alkaline conditions (the pH of the alkaline copper reagent was 10.8).

5. Choice of Substrate

Substrate from four sources was examined: purified laminarin extracted from Laminaria hyperborea (Sigma, Koch-Light), crude laminarin (British Borax Co.) and barley β-glucan (Novo Industri A/S). Barley β-glucan turnover was impossible to assess because of precipitate formation on addition of the arsenomolybdate reagent during reducing sugar determination.

Laminarin was dissolved by warming the substrate buffer mixture to 50°C. The resulting solution was allowed to cool to room temperature, made up to volume and in the case of crude laminarin filtered before being added to the soil. Laminarin is known to come out of solution very slowly at 25°C, the assay temperature, and dry weight determinations showed that 11% precipitated over the 17 h assay period, but this loss was nowhere near causing substrate limitation.

Activity in the presence of crude laminarin was 92% of that recorded using both pure substrates. The development of the assay and enzyme characterisation were carried out with purified substrate, whereas crude laminarin was used in the amendment work. The change over was enforced due to the lack of commercially available purified laminarin after April 1976.

6. The Fate of Glucose Produced During Laminarin Degradation

If the reducing sugars, produced when laminarin is depolymerised by extracellular soil glucanases, are themselves enzymatically converted to compounds which no longer possess a reducing end group, then glucanase activity measurements will be an underestimate of the real values. Ross (1974) has suggested that glucose oxidase activity may interfere with soil enzyme assays in which the production of glucose is measured by converting it to the non-reducing gluconic acid. Disappearance of glucose via microbial utilisation is not a problem since the assays are performed in the presence of a microbial inhibitor.

To examine the fate of glucose under the conditions of the glucanase assay a typical assay was set up except that the laminarin was replaced by 1 mg glucose/g soil, a typical weight of reducing sugar produced from laminarin by 1 g soil during the 17 h incubation. This assumed that all the reducing

sugar produced during the assay was glucose and subsequent experiments have indicated that this is the case (see Results and Discussion, A.1.h). 96% of the added glucose was detected at the end of the 17 h incubation period suggesting that if there is any accumulated glucose oxidase in this soil its activity is negligible under the conditions of the glucanase assay and hence has little or no effect on glucanase determinations.

7. Extent of Laminarin Adsorption to Soil

The routine glucanase assay does not take substrate adsorption (if any) into account because the soil control and substrate blank were set up separately to conserve the expensive laminarin. It is unlikely that laminarin is adsorbed by the soil colloids because it is a non-charged molecule, however it was necessary to check this.

Laminarin adsorption was investigated by comparing the reducing sugar value of the usual soil control plus substrate blank with a control which combined the two. This combined control was set up by adding the laminarin after the sodium azide treated soil had been heated in a 100°C water bath for 20 min to denature the enzyme. After 17 h incubation the reducing sugar value of the combined control was 93% of the sum of the two separate controls indicating that laminarin adsorption had very little effect on soil glucanase determinations under the conditions of the assay.

2. Urease

a) The Assay

The method used was that devised by McLaren et al., (1957) and modified by Pettit et al., (1976) and Burns et al., (1978). In the outer ring of a Conway microdiffusion dish 1 g air-dried soil was pre-incubated with 1 ml, 0.2% w/v aqueous sodium azide as a biostatic agent and 2 ml 0.5 M tris-maleate buffer pH 7.0. Boric acid indicator (3 ml) composed of 2% w/v boric acid, 2% v/v ethanolic indicator (0.084% w/v bromocresol green and 0.16% w/v methyl red) and 0.005% v/v decon, was pipetted into the central well. After 30 min the reaction was started by adding 1 ml 6 M urea (AR grade, Fisons) followed by gentle stirring with a glass rod. The dishes were covered with ground-glass lids and incubated at room temperature (21 $\pm$ 2°C) for 60 min, after which the reaction was terminated by adding 0.5 ml 10 mM silver sulphate and 1 ml 45% (3.26 M) potassium carbonate, with gentle stirring in between. Enzyme activity is only halted completely in the presence of both these chemicals (Pettit, 1978). The primary function of the potassium carbonate was to supply potassium ions to exchange with the ammonium ions from the anionic soil colloids by preferential adsorption and thus liberate any ammonia produced by enzyme action. The lids were replaced immediately following potassium carbonate addition and 18 h allowed for ammonia diffusion. The ammonia evolved from the soil was absorbed by the boric acid

indicator and measured by back-titrating with 200 mM followed by 20 mM hydrochloric acid. Controls lacking urea (soil control) were also set up to measure the inherent ammonia levels of the soil. The aqueous urea was found to be stable to non-biological hydrolysis over the assay period and so substrate blanks (no soil) were not routinely performed. The treatments and soil controls were both performed in triplicate. The computed coefficient of variation for the assay ranged from 2 to 6%.

C. Microbiology

1. Growth Media

a) General Purpose

1) Nutrient Agar (NA)

After soaking 12 g nutrient agar granules (Oxoid) in 400 ml deionised water for 15 min, the resulting mixture was autoclaved at 15 lb psi for 15 min in a pressure cooker.

2) Glucose Soil Extract Agar (GSEA)
(modified from Hussain, 1976)

Glucose	1.0g
Dipotassium hydrogen phosphate	0.5 g
Sodium dihydrogen phosphate	0.25 g
Ammonium nitrate	0.25 g
Soil extract	100 ml
Oxoid agar No. 3	12 g
Deionised water	900 ml
pH	7.0

The pH was adjusted from 7.4 to 7.0 with a few drops of 1 M orthophosphoric acid and all the components were autoclaved together at 15 lb psi for 10 min, because a longer period may have caramelised the glucose. The soil extract was prepared by autoclaving 250 g air-dried soil with 600 ml deionised water at 15 lb psi for 30 min (James, 1958). The liquid was clarified by centrifuging at 21,000 x g for 20 min in a refrigerated (4°C) MSE HS 18 centrifuge (6 x 250 ml rotor).

3) Bunt and Rovira's Agar (BARA)

This medium has the advantage that slight modification (see below) will convert the basic bacteriological medium (Bunt and Rovira, 1955) into one specific for fungi (M.P. Greaves, 1976 - personal communication). The basic recipe was as follows:

Lab M balanced peptone	1.0 g
Oxoid powdered yeast extract	1.0 g
Dipotassium hydrogen phosphate	0.4 g
Diammonium hydrogen phosphate	0.5 g
Magnesium chloride. 6 H_2O	0.05 g
Magnesium sulphate. 7 H_2O	0.2 g
Calcium chloride	0.2 g
Ferric chloride 1 drop of a 1% w/v solution	
Soil extract	250 ml
Oxoid agar No. 3	12 g
Deionised double distilled water	750 ml

To prevent phosphate precipitation prior to autoclaving deionised double distilled water was used and a concentrated solution of the two phosphates (about 15 ml) was slowly run down the side of a beaker from a 10 ml pipette into a rapidly stirring dilute solution of the remaining salts. Any slight precipitation that did form disappeared when the pH was adjusted from 7.2 to 7.0 (bacterial agar) or 5.5 (mycological agar) with 1 M hydrochloric acid (HCl was used in preference to orthophosphoric acid because the latter caused extra precipitation problems). The bacterial agar was prepared by autoclaving all the components together at 15 lb psi for 20 min.

The fungal version was prepared by adjusting the pH of all the components except the agar in 500 ml to

5.5 and autoclaving this separately from the agar in 500 ml water. This was to avoid acid hydrolysis of the agar which would have prevented it from setting. Just before pouring the complete medium was prepared by mixing the two followed by the addition of 2.5 ml filter sterilised chlorotetracycline-HCl (8 mg/ml) (Sigma) such that the final concentration was 20 µg/ml agar, to prevent the growth of any bacteria which could tolerate pH 5.5.

b) Specific

1) Laminarin Agar

Laminarin was incorporated into BARA at 10 g/l. In the fungal version it was autoclaved with the agar rather than the salts to avoid the possibility of acid hydrolysis. It was stable to autoclaving and about 3 days after preparation sufficient laminarin had precipitated out of solution such that the plates were opaque. Glucanase producing microorganisms were identified by haloes of clearing resulting from laminarin hydrolysis (Lilley and Bull, 1974) - see Plates 1a and 1b.

2) Christensen's (1946) Urea Agar (CUA)

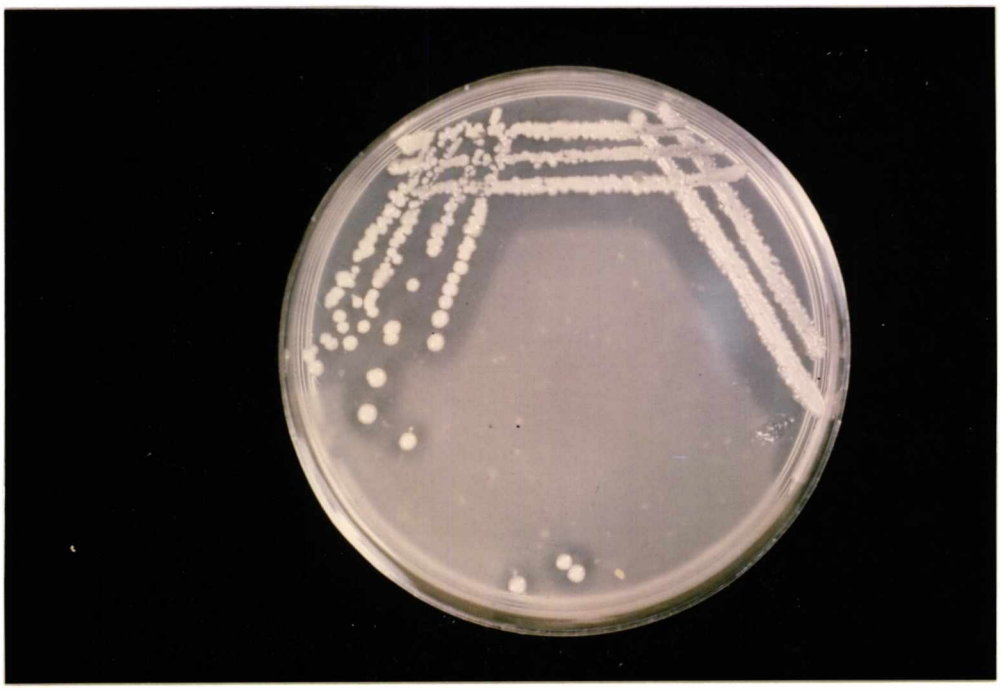
After soaking for 15 min, 9.6 g urea agar base (Oxoid) in 380 ml deionised water were autoclaved at 10 lb psi for 20 min. After cooling to around 55°C, 20 ml filter sterilised urea (40% w/v) were added and distributed throughout the agar by gentle

PLATE 1 Identification of glucanase producing microorganisms by zones of clearing on laminarin agar.

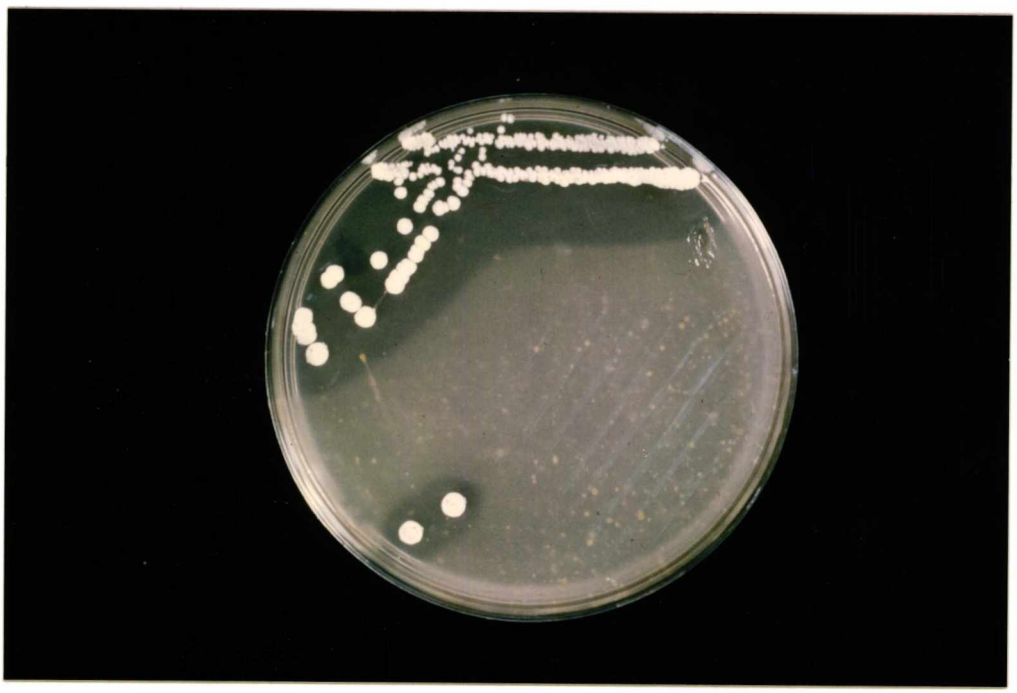
a) Gram negative bacterium,

b) Actinomycete.

1a



1b



agitation. Urea is thermolabile above 60°C and hence can not be autoclaved. The medium contains the indicator phenol red which changes colour from yellow to purple in response to the alkaline conditions resulting from urea hydrolysis. Thus ureolytic microorganisms were identified by zones of purple surrounding the colonies (see Plate 2).

2. Viable Counts of Soil Microorganisms

Duplicate samples (1 g) of air-dried soil were transferred to medical flats containing 100 ml sterile, deionised water and shaken at maximum speed on a Gallenkamp wrist action bench shaker for 15 min to dislodge microorganisms from the soil surfaces. The resulting suspension was then further diluted with deionised water in a series of 10 fold steps. Five 0.1 ml aliquots of three consecutive dilutions were spread onto the surface of agar plates as quickly as possible to reduce the risk of cell lysis in the hypotonic diluent. In an attempt to reduce microbial adsorption to pipette walls the suspensions to be transferred were sucked up and blown out of each pipette 3 times to saturate the adsorption sites of the glass surface.

Viable counts were tenfold lower on GSEA than on both NA and BARA (bacterial and fungal counts combined) (Table 15). BARA was chosen for all the subsequent work because of its ability to support the growth of actinomycetes (on the bacterial version) and its

PLATE 2 Colonies of a ureolytic fungus on Christensen's urea agar.

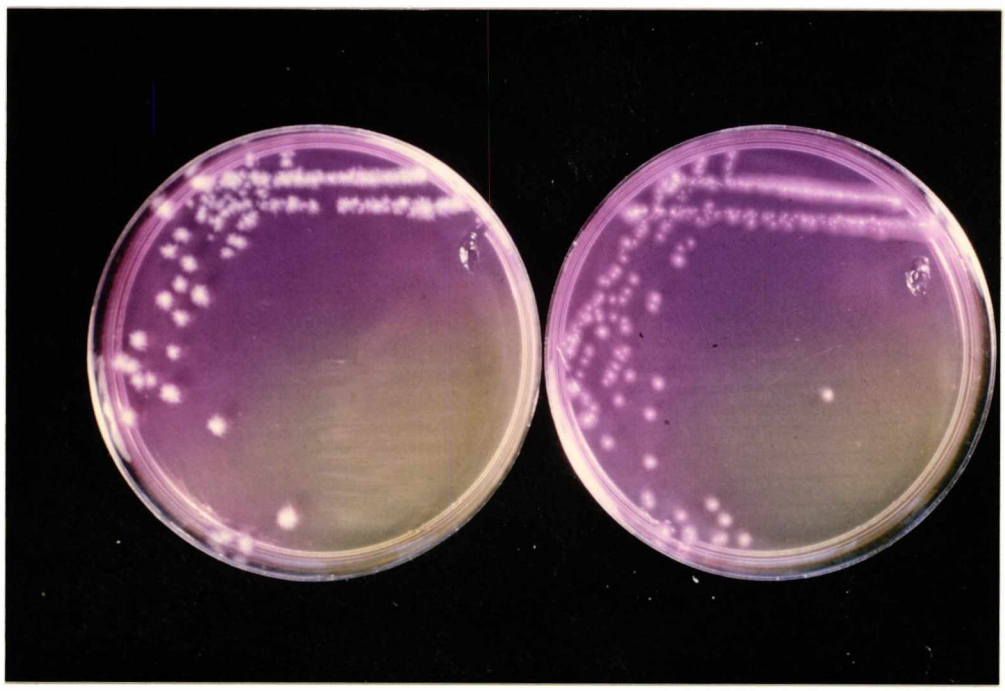


TABLE 15 Comparison of growth media for microbiological counts (April soil)

Agar	Viable count*/g	SD	Coefficient of variation
NA	8.0×10^7	$\pm 2.8 \times 10^7$	35%
GSEA	5.6×10^6	$\pm 3.5 \times 10^6$	63%
BARA	7.1×10^7	$\pm 3.3 \times 10^7$	46%

NA = nutrient agar, GSEA = glucose soil extract agar

BARA = Bunt and Rovira's agar, * = includes bacteria and fungi

SD = standard deviation.

TABLE 16 Effect of incubation time on microbial counts (April soil)

Microorganisms	Incubation time (counts/g)	
	3 days	7 days
Bacteria*	2.2×10^6	3.2×10^6
Actinomycetes	-	6.4×10^5
Fungi	1.5×10^6	4.3×10^6

* = excluding actinomycetes.

TABLE 17 Comparison of microbial counts in soil collected at different times of the year

Microorganisms	Sampling time (counts/g)		
	April	July	November
Bacteria*	3.2×10^6	6.4×10^6	7.2×10^6
Actinomycetes	6.4×10^5	1.5×10^6	5.4×10^5
Fungi	4.3×10^6	8.2×10^4	1.3×10^5

* = excluding actinomycetes

versatility. Very few fungi grew on GSEA and the first organisms to appear on NA often swamped it, giving the slower growers no chance to develop.

The plates were incubated at 25°C and counted after 7 days, since by day 3 not all the bacteria and fungi had appeared (Table 16) and the actinomycetes had not begun to sporulate and hence could not be identified on the basis of colony morphology. Ideally, a longer incubation time should have been used to detect any slow growing microorganisms but when this was attempted both Bunt and Rovira agars invariably became overgrown; the bacterial one by the swarming Bacillus cereus var. mycoides, thus making the colonies impossible to count. Any plates which were overgrown by day 7 were excluded from the counts.

The effect of sampling time (Table 17) and air-drying (Table 18) on viable counts will be evaluated in relation to soil enzyme activity in the Results and Discussion section.

To determine the percentage of the total microbial population that was ureolytic and capable of degrading laminarin (and hence producing glucanase), every colony from a BARA plate containing 30 to 40 colonies was replated onto individual CUA and laminarin agar plates or slopes (Table 19). When the ureolytic micro-organisms were transferred back to BARA the majority lost their ability to hydrolyse urea suggesting that it may be plasmid coded in these organisms. This was not the case with the laminarin degraders. When

TABLE 18 Comparison of microbial counts in air-dried and field wetness soil (April soil)

Microorganisms	Counts/g dry soil	
	Air-dried soil	Field-wetness soil
Bacteria*	4.7×10^6	3.2×10^8
Actinomycetes	3.0×10^6	7.0×10^7
Fungi	6.3×10^7	6.8×10^8

* = excluding actinomycetes

TABLE 19 The number of laminarin degrading and ureolytic microorganisms in November soil

Microorganisms	Total count/g	% Laminarin degraders	Percent ureolytic
Bacteria*	7.2×10^6	47	24
Actinomycetes	5.4×10^5	78	33
Fungi	1.3×10^5	63	75

* = excluding actinomycetes

TABLE 20 The number of laminarin degrading and ureolytic microorganisms in November soil capable of growing anaerobically

Microorganisms	
Total aerobic	7.9×10^6/g
Total anaerobic	3.7×10^5/g
% anaerobic laminarin degraders	4
% anaerobic ureolytic	20

growing on CUA the ureolytic microorganisms had to be sub-cultured every 3 days otherwise they lost their viability, probably as a result of the high pH generated during urea hydrolysis.

Viable counts of anaerobic microorganisms (Table 20) were performed using the same incubation conditions as for the aerobic counts with the exception that anaerobic jars, evacuated and flushed with hydrogen three times, were used. No obligately anaerobic ureolytic or laminarin-degrading microorganisms were isolated since they were all capable of growing aerobically, albeit somewhat slower in many cases.

D. Ecology

1. Soil Amendments

a) Pesticides

1) Formulations

Since one of the aims of this project was to determine whether field application rates of the pesticide formulations listed in Table 21 were likely to have any effect on soil glucanase and urease activities it was necessary to attempt to relate the manufacturer's recommended rates quoted in pints/acre, litres/hectare or kilograms/hectare to ppm for use in small scale laboratory experiments. The conversion depends on two basic assumptions and as a result is only an approximation (M.P. Greaves, 1976 - personal communication).

TABLE 21 Pesticide formulations

Formulation	Active Ingredient	Manufacturer	Mode of Application
2,4-D*	2,4-D	Shell	Solution
Avadex	Diallate	Monsanto	Stable emulsion
Roundup[1]	Glyphosate	Monsanto	Solution
Suffix	Benzoylprop ethyl	Shell	Stable emulsion
Malathion	Malathion	Murphy	Stable emulsion

* = triethanolamine salt
1 = isopropylamine salt

1) The pesticides are distributed in the top 5 cm of the soil.

2) The weight of a hectare of soil to a depth of 15 cm is approximately 2.2×10^6 kg.

Therefore an application rate of 1 kg/ha is equivalent to 1.4 ppm (assuming penetration to a depth of 5 cm).

The steps involved in calculating the volume of liquid formulation to be added per g dry soil, such that the final concentration was approximately equivalent to 5 times the recommended field rate are summarised in Table 22. The field rates quoted were those supplied by the manufacturers of the products. Five times recommended field rate was chosen because it was considered necessary to incorporate a realistic safety margin to account for human error in preparation,

TABLE 22 Pesticide formulation application rates

Pesticide	% a.i. w/v	Field rate				5 times field rate	
		Formulation *		a.i.		a.i.	Formulation
		Pints/ac	Litres/ha	kg/ha	ppm	ppm	nl/g
2,4-D	50	1	1.40	0.70	0.98	4.90	9.80
Diallate	40	3	4.21	1.68	2.36	11.79	29.47
Glyphosate	41	-	4.00	1.64	2.30	11.48	28.00
Benzoylprop ethyl	20	-	-	1.00	1.40	7.00	35.00
Malathion	50	3	4.21	2.11	2.95	14.74	29.47

a.i. = active ingredient,

* = see Appendix 3 for metric-imperial conversion factors

spray overlap, uneven distribution in soil and concentration effects. At the time this investigation was performed the Weed Research Organisation (WRO) and ICI were using field rate and 10 times field rate in their testing programmes, whereas the Ministry of Agriculture Fisheries and Food Agricultural Development and Advisory Service (ADAS) Wolverhampton were using 2.5 times the recommended rate in their experiments.

The pesticide formulation solutions or emulsions were freshly prepared for each experiment in large volumes of distilled water. They were applied to soil immediately in 0.5 ml (if no other soluble materials were to be added) or 0.25 ml water such as to bring the soil to 65% WHC. Before any volumes were pipetted the liquids were sucked into and blown out of the pipette 10 times in an attempt to saturate any pesticide adsorption sites on the pipette surface.

2) Analytical Grade Ingredients

With the exception of 2,4-D, analytical grade active ingredients of the formulations listed in Table 21 were applied to air-dried soil at 1000 ppm (soil basis) in a small volume of dried, distilled acetone (5 µl/g soil) as recommended by Lethbridge et al. (1976). They showed that concentrations of acetone and hexane above this level were potent inhibitors of at least one soil enzyme, urease.

On no account should pesticides be applied to soil in solvents which have effects on the activity under

test. It is not sufficient to perform solvent controls to take such effects into account because solvent and pesticide may have identical effects, and under such circumstances a pesticide would be recorded as having no effect. Lethbridge and Burns (1976) observed this phenomenon with the organophosphorus insecticide malathion, acetone or hexane (> 10 µl/g soil) and urease activity.

The solvent was allowed to evaporate for 15 min after which the soil was thoroughly shaken to evenly distribute the pesticides and brought to 65% WHC with sterile distilled water. Acetone controls were also set up to monitor inherent effects of the solvent on glucanase activity, but none were detected.

The 2,4-D was applied in 0.5 ml distilled water (up to 250 ppm soil basis) but when the amount required exceeded its solubility (500 and 1000 ppm), it was applied as a combination of finely powdered solid (see Methods and Materials, D.1.b) plus 250 ppm solution (see Results and Discussion, B.3.a.2).

Both formulations and active ingredients were investigated for inherent effects on urea stability and reducing sugar determination in the Nelson-Somogyi copper reduction method by incorporating them into glucose standard curves and substrate blank determinations. The only effect to be detected was that malathion formulation and active ingredient enhanced copper reduction. However, no such artificial enhancement of glucanase activity was detected in the

soil incubations suggesting that none of the insecticide was extracted from the soil during the assay procedure.

b) Fertilisers, Lime and Organic Amendments

The problems involved in relating pesticide formulation field application rates to small scale laboratory experiment concentrations also apply to the amendments listed in Table 23. These amendments were assumed to be distributed throughout the top 6 in (15.4 cm) of the soil (J.H. Williams, 1976 - personal communication). This is deeper than the pesticides because some of these are highly soluble and others are incorporated into the soil by cultivation. Assuming an acre of soil to a depth of 6 in weighs 2×10^6 lb (see Methods and Materials, D.1.a.1), then lb/ac can be approximated to ppm by multiplying by 0.5 - assuming penetration to a depth of 6 in (see Appendix 3 for metric-imperial conversion factors). In cases where the application rate was high (pig slurry, ground limestone, cellulose and glucose) the ppm conversion was rounded up to the nearest hundred. Field rates to be mimicked in the laboratory were selected from the top end of the concentration spectrum recommended by the suppliers.

The NPK components were supplied in the form of granules and these were crushed to a fine powder with a mortar and pestle to facilitate solublisation or mode of application in the case of single superphosphate which was only partially soluble. They were

TABLE 23 Fertilisers, lime and organic amendments

Amendment	Source	Field rate mimicked[8]	Equivalent Lab. Conc^{n} (ppm)	Mode of application
Nitram[1] (ammonium nitrate)	ADAS[4]	236 lb N/ac	118 N	Solution
Single super-phosphate[2]	ADAS[4]	236 lb P/ac	118 P	Powder
Muriate of potash[3] (potassium chloride)	ADAS[4]	236 lb K/ac	118 K	Solution
Urea	Fisons	100 kg N/ha	47 N	Solution
Pig slurry (8% w/v)	5	10,000 gallons/ac	4000	Freeze dried powder
Ground limestone	ADAS[6]	12 tonnes/ha	5400	Powder
Glucose	Fisons	-	700	Solution
Cellulose	7	-	700	Powder

1 = 34.5% N, 2 = 18% P_2O_5,

3 = 66% K_2O, 4 = Wye,

5 = Millfield Farm, Scredington, Sleaford, Lincs., 6 = Wolverhampton,

7 = Reeve Angel Scientific Ltd., 8 = see Appendix three.

investigated in a 1:1:1 combination, although they were applied to the soil separately.

The pig slurry dose of 10,000 gallons/ac was that being used on the farm from which the slurry came. Freeze-dried pig slurry was used because it did not prove possible to add the original slurry to small quantities of soil in a reproducible manner.

The cellulose application rate was based on two assumptions. Firstly, the amount of cereal stubble and roots ploughed back into the soil after a typical harvest is approximately 30 cwt dry matter/ac (= 3360 lb/ac) (M.J. Marks, 1976 - personal communication). Secondly, cereal straw is composed of about 40% cellulose (Waksman, 1952). Cellulose was chosen in preference to straw because it was considered likely to be turned over at a faster rate and hence effects might manifest themselves more rapidly. However, it is important to bear in mind that results obtained with cellulose may not hold for cereal straw, since the latter is composed of other compounds such as lignins, pentosans and hexosans, and much of its cellulose is not immediately available to microorganisms.

Glucose was investigated from a purely academic point of view, rather than with any applied aspect in mind, because it is a readily available carbon source and the constituent monomer of cellulose.

The insoluble amendments were added to 100 g quantities air-dried soil in a 500 ml glass-stoppered Erlenmeyer flask as powders and distributed by gentle

hand-shaking and rotation for 15 min. This bulk amended soil was then subdivided into 10 g fractions prior to incubation at 65% WHC. Whenever a pesticide formulation and another amendment were added to the same soil sample, the latter was added first followed almost immediately (within 30 min) by the former.

c) Effects of Amendments on Glucanase and Urease Activities in Soil

The amendments listed in Tables 21, 22 and 23 were added to duplicate 10 g quantities of air-dried soil in 100 ml Erlenmeyer flasks sealed with cotton wool bungs. The soil was brought to 65% WHC and maintained at that level for the duration of the experiment by adding 0.8 ml sterile distilled water every 7 days. The flasks were incubated at room temperature (21 ± 2°C) and at various time intervals 1.5 g samples wet soil (equivalent to 1 g dry soil) were removed for enzyme assays. After sampling all flasks were discarded rather than reincubated to avoid effects due to soil disruption (hence the small weight of soil used - 10 g).

d) Analysis of Pig Slurry

The freeze-dried pig slurry was so heterogeneous that the small sample required for C, H and N analysis in the Hewlett Packard F and M 185 automatic analyser (Table 24) would not have been representative of the whole powder. Attempts to homogenise the powder with

a mortar and pestle were unsuccessful so it was fractionated by sieving. For comparison the clear supernatant obtained from the original slurry, by centrifuging at 21,000x g in an MSE HS 18 refrigerated (4°C) centrifuge (6 x 250 ml rotar) for 20 min, followed by filtering through Whatman No. 1 filter paper, was evaporated to dryness at 120°C and included in the analysis. This fraction contained all the soluble materials of the slurry. Each value is the mean of two replicates. The percentage carbon was remarkably consistent throughout the fractions, whereas the percentage hydrogen and nitrogen increased as particle size decreased. The complete freeze-dried material was found to be 92% organic matter by combustion.

TABLE 24 C, H and N analysis of pig slurry

Freeze dried material	% C	% H	% N
850 µm	39.5	5.0	0.8
250 - 850 µm	40.6	6.3	1.4
250 µm	40.4	7.0	2.3
Mean	40.2	6.1	1.5
Supernatant fraction*	40.2	6.3	0.4

* = 2% w/v

The original slurry possessed both glucanase (0.18 μ moles reducing sugar as glucose equivalents/ 10 mg dry wt/h) and urease (5.6 μ moles ammonia evolved/ 10 mg dry wt/h) activity whereas the freeze-dried powder was devoid of these activities. Slurry which had been frozen but not dried under vacuum possessed only slightly less activity than the original slurry suggesting that the majority of the enzyme molecules had been denatured by the drying process rather than the freezing at - 40°C. In contrast, when jack bean urease was freeze-dried (Pettit et al., 1976) 58% of the activity was lost in the freezing process and a further 36% by the drying process.

Freeze-dried powder contained a negligable amount of reducing sugar and about 13 μ moles ammonia/10 mg but this did not interfere with the urease determinations since it was taken into account in the soil controls.

The microbial counts (Table 25) were performed in a similar manner to that described for soil (see Methods and Materials, C.2).

TABLE 25 Microorganisms in pig slurry

Microorganisms	Total/ g dry wt	% laminarin degraders	Percent ureolytic	
Bacteria	1.1×10^7	26	30	
Fungi	90	-	-	Original slurry
Bacteria	1.4×10^4	10	43	
Fungi	1.4×10^2	17	50	Freeze-dried powder

2. Flooded Soil

When the soil (10 g) in the 100 ml Erlenmeyer flask had been brought to 65% WHC with 5 ml pesticide formulation, a further 10 ml sterile distilled water were added to completely submerge the soil. The resulting slurry was swirled gently (so that no soil stuck to the side of the flask) to remove much of the air from the soil and the flasks were closed with rubber bungs and incubated at room temperature ($21 \pm 2^{o}C$). At various sampling times the flasks were swirled to get the soil into suspension and the slurry was poured into a Hilgard cup containing a Whatman No. 1 filter paper and allowed to drain for 1 h. Prior experimentation had shown that under such conditions 10 g soil retained 7 ml water, i.e. the 100% WHC of flooded soil was 0.7 ml/g compared to 0.75 ml/g for non-flooded soil. The most likely explanation for the reduced water holding capacity is the loss of structure observed in the flooded soil such that there was less space in which the water could reside. Soil enzyme assays were performed with 1.7 g wet soil ($\equiv$ 1 g dry soil) and the amount of water already present in the wet soil was taken into account.

3. Ammonia Volatilisation

Aliquots (1 g) of air-dried soil amended with NPK, urea and pig slurry were incubated at 65% WHC,

and maintained at this level by adding 0.1 ml sterile distilled water every 4 days, in the outer ring of a Conway microdiffusion dish covered with a ground-glass lid and containing 3 ml boric acid indicator in the central well. The indicator was back-titrated with 20 mM hydrochloric acid after 14 days. Each treatment was replicated 6 times.

PART THREE - RESULTS AND DISCUSSION

A. Enzymology

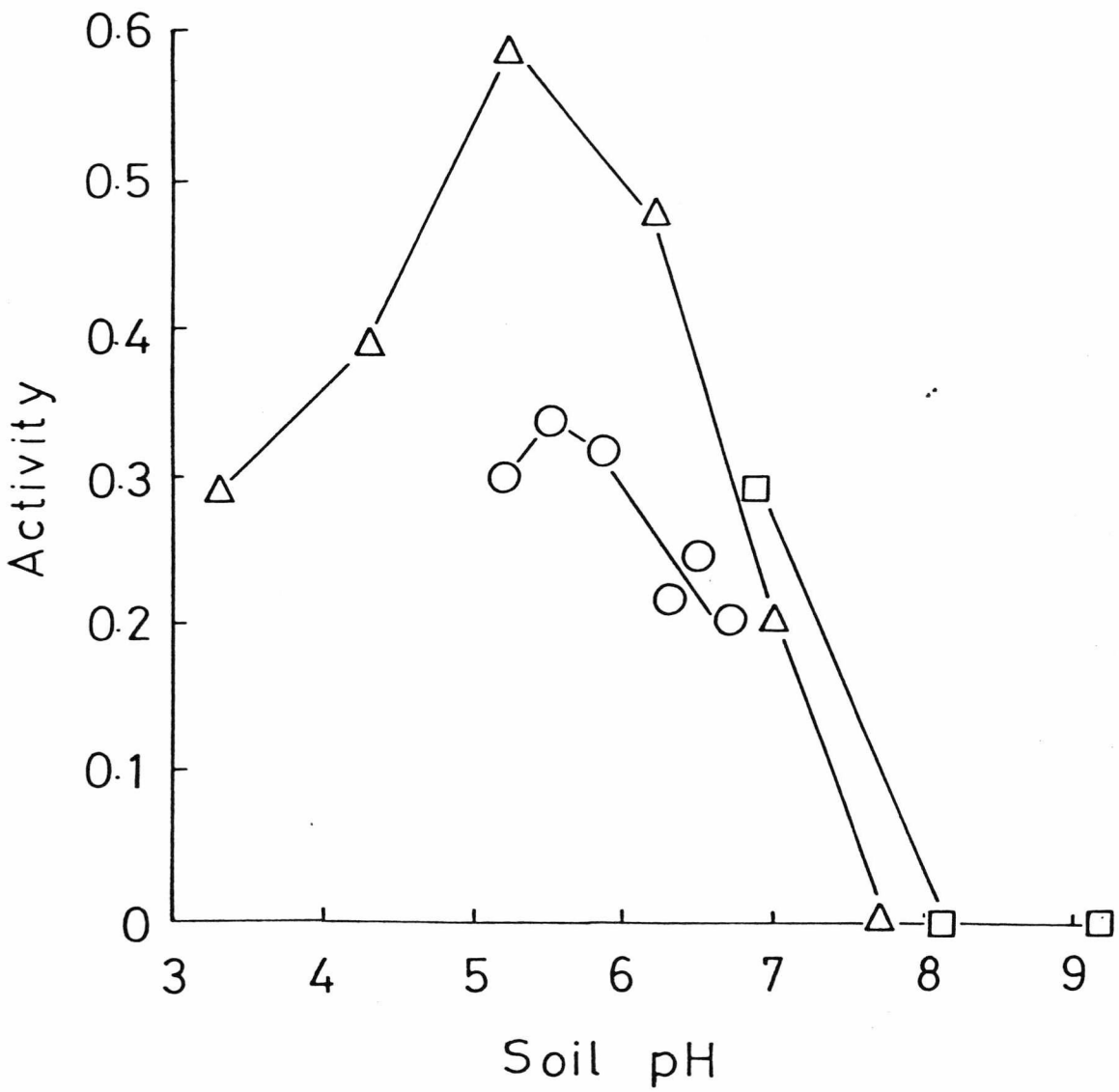
1. Characterisation of Soil Glucanase

a) Effect of pH on Activity

Ideally a pH curve should be constructed using only one buffer so that pH is the sole variable. However, when a wide pH range is required either side of neutrality the single buffer situation can only be achieved with modified universal buffer whose buffering range is between pH 2.4 and 11.8. Universal buffer was not used in this instance because it contains tris which had previously been shown to inhibit reducing sugar determination by the Nelson-Somogyi copper reduction method.

The effect of pH on enzyme activity was investigated over the range pH 3 to 10 using 0.1 M sodium acid maleate (pH range 5 to 7), 0.1 M glycine-sodium hydroxide (pH range 8 to 11) and 0.05 M citrate-phosphate (pH range 2.6 to 7) buffers (Figure 7). As recorded previously (see Methods and Materials, B.1.b.1), citrate-phosphate extracts organic matter from soil and would generally be unacceptable for the assay. It was decided that, in this instance, the benefits of its wide buffering range (which overlaps that of sodium acid maleate) outweighed the disadvantages of the high but consistent controls. The amount of organic matter extracted increased with pH and hence phosphate

FIGURE 7 Effect of pH on soil glucanase activity;
△ = citrate-phosphate buffer;
○ = sodium acid maleate buffer;
□ = glycine-sodium hydroxide buffer.
Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.



concentration. Glycine-sodium hydroxide also extracted organic matter due primarily to the sodium hydroxide.

Substrate blanks were set up at each pH to detect acid or alkaline hydrolysis of the laminarin. It was stable in acid pH (3 to 7), but above pH 7 the reducing sugar value of the substrate blank increased slowly with pH indicating that alkaline hydrolysis was taking place. This slow rate of laminarin breakdown was not sufficient to cause substrate limitation of the enzyme.

In Figure 7 activity is plotted against the final pH of the reaction mixture rather than buffer pH (Table 26). These two pHs are not always synonymous because of the buffering capacity of the soil itself, which will tend to modify the final pH of the mixture. This point was illustrated by glycine-sodium hydroxide; a rather poor buffer when in competition with Hamble soil at pH 5.4, but it was not alone in this regard. The pH of sodium acid maleate and citrate-phosphate at the limits of their buffering capacity was also drawn towards that of the soil, although not to the same extent as glycine-sodium hydroxide.

Glucanase activity was consistently higher in citrate-phosphate buffer than in sodium acid maleate but the optimum pH occurred in the same region with both; pH 5.2 (citrate-phosphate) and pH 5.4 (sodium acid maleate). A rapid decline in activity above pH 7 was observed. It is important to be aware that

Table 26. Buffer pH versus soil (pH 5.4)- buffer mixture pH.

Buffer	Buffer pH	Soil-buffer Mixture pH	
		0 h Incubation	17 h Incubation
Sodium acid maleate (0.1 M)	5.0	5.1	5.2
	5.4	5.4	5.4
	5.6	5.6	5.6
	5.8	5.8	5.8
	6.2	6.2	6.2
	6.6	6.6	6.6
	7.0	6.8	6.7
Citrate phosphate (0.05 M)	3.0	3.3	3.3
	4.0	4.2	4.3
	5.0	5.0	5.2
	6.0	6.1	6.2
	7.0	7.1	7.0
	8.0	7.8	7.7
Glycine-Sodium hydroxide (0.1 M)	8.0	6.9	6.8
	9.0	8.5	8.1
	10.0	9.4	9.2
	11.0	9.7	9.5

the pH values of soil-buffer slurries are bulk determinations and might not be the same as the micro-environment pH where the enzyme substrate interaction is occurring (McLaren, 1960; Bailey, White and Rothberg, 1968). The pH at the soil colloid surface is likely to be less than that of the gross soil-buffer pH due to adsorption and concentration of hydrogen ions. Having said all this it is still worth noting that microbial glucanases also have pH optima in the range pH 5 to 6 (Manners and Wilson, 1973; Nagasaki et al., 1976, 1977; Miyazaki et al., 1977) as does the enzyme from the surf clam Spisula solidissima (Lindley et al., 1976). However no generalisations can be made about their response to alkaline conditions. Chesters and Bull (1963b) recorded that the pH optima of fungal exo- and endoglucanases were 4.9 to 5.0 and 6.0 to 6.1 respectively.

Although it is interesting to make comparisons between soil enzymes and those from other sources they are of limited value unless they have been carried out using identical assay procedures. This comparison has been made by Pettit et al.(1976) with urease. Even then it is not an exact comparison of the same enzyme(s) because, as mentioned previously, a particular soil enzyme activity almost certainly reflects the action of a group of enzymes rather than just one. In the case of glucanase all the comparisons between the soil enzyme and those from other sources made in this and subsequent sections are based on assays using different

buffers, substrate concentrations, pH, temperatures and incubation times. All these factors will have a bearing on the characteristic under test and such comparisons should be viewed accordingly.

To test the hypothesis that the rapid decline in activity above pH 7 might mean that alkaline soils would possess low glucanase levels, an alkaline soil was assayed for activity. The soil was collected from the Devil's Basin on Wye Downs (Ashford, Kent) at a point where the chalk was only 3 cm below the surface. The soil (pH 7.4) was assayed in 0.1 M sodium acid maleate buffer (pH 5.4) and 0.1 M sodium phosphate buffer (pH 7.4) (Table 27). Some comparable values for Hamble soil are included. Rather than having very little glucanase activity, the alkaline soil possessed far more than the Hamble soil and this point admirably illustrates the dangers involved in attempting to extrapolate from one soil to another.

TABLE 27 Comparison of glucanase activity (μ moles reducing sugars as glucose equivalents/g soil/h) in an acidic and alkaline soil

Soil	Buffer	Buffer pH	Soil-buffer mixture pH 0 h	Soil-buffer mixture pH 17 h	Glucanase activity
Alkaline	Maleate	5.4	5.8	6.6	1.06
	Phosphate	7.4	7.3	7.3	0.90
Hamble	Maleate	6.6	6.6	6.6	0.25
	Citrate-phosphate	7.0	7.1	7.0	0.21

b) Kinetics

The effect of substrate concentration on glucanase activity was assessed over the range 0.07 to 6.7 mg/ml laminarin. Apparent Km and V max values were derived from computed least square analyses of the Lineweaver-Burk (Figure 8) and Eadie-Hofstee (Figure 9) plots and from the direct linear plot of Eisenthal and Cornish-Bowden (1974) on six separate occasions (Table 28). Km values are expressed as mg/ml rather than in molarity because the molecular weight of the substrate is changing throughout the assay period. The mean Km values of 0.23 mg/ml (Lineweaver-Burk), 0.21 mg/ml (Eadie-Hofstee) and 0.20 mg/ml (direct plot) are similar to those of purified glucanases from some micro-organisms such as Flavobacterium dormitator var. glucanolyticae - 0.26 mg/ml (Nagasaki et al., 1976) and Poria cocos - 0.22 mg/ml (Nagasaki et al., 1977) and the surf clam Spisula solidissima - 0.22 mg/ml (Lindley et al., 1976). In contrast, Miyazaki et al. (1977) reported a Km value of 0.48 mg/ml for Mucor hiemalis. However, comparisons of Km values for soil enzymes with those of purified enzymes from other sources are not strictly valid since the former are functioning in a heterogeneous environment, possibly in an adsorbed state. Under such circumstances Km might be affected by surface effects such as altered enzyme conformation, substrate and product adsorption and by the presence of inhibitors. In the case of phosphatase, when substrate adsorption to the soil

FIGURE 8 Lineweaver-Burk plot of soil glucanase activity. V = μ moles reducing sugars as glucose equivalents/g soil/h, S = mg/ml (laminarin).

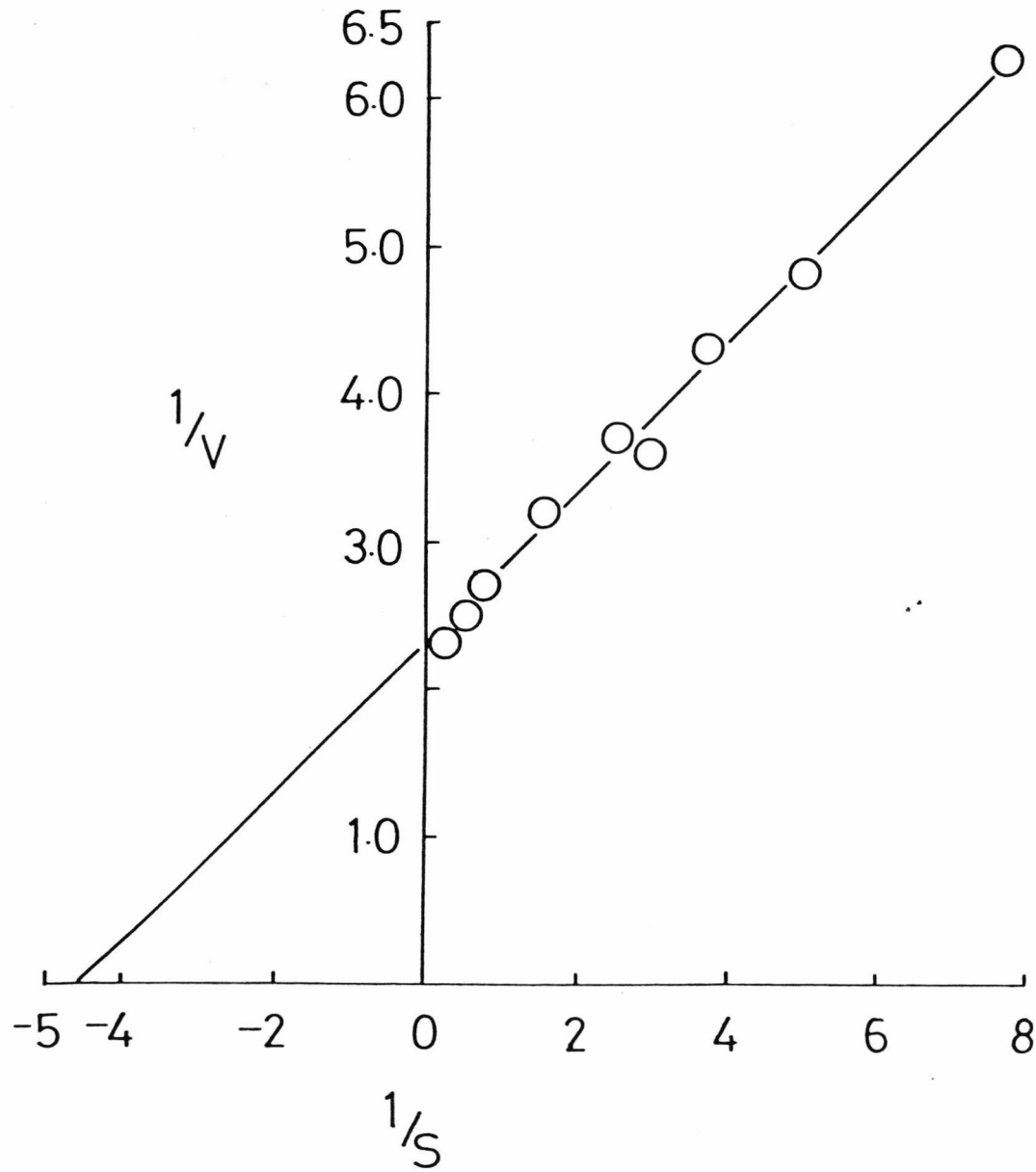


FIGURE 9 Eadie-Hofstee plot of soil glucanase activity. V = μ moles reducing sugars as glucose equivalents/g soil/h, S = mg/ml (laminarin).

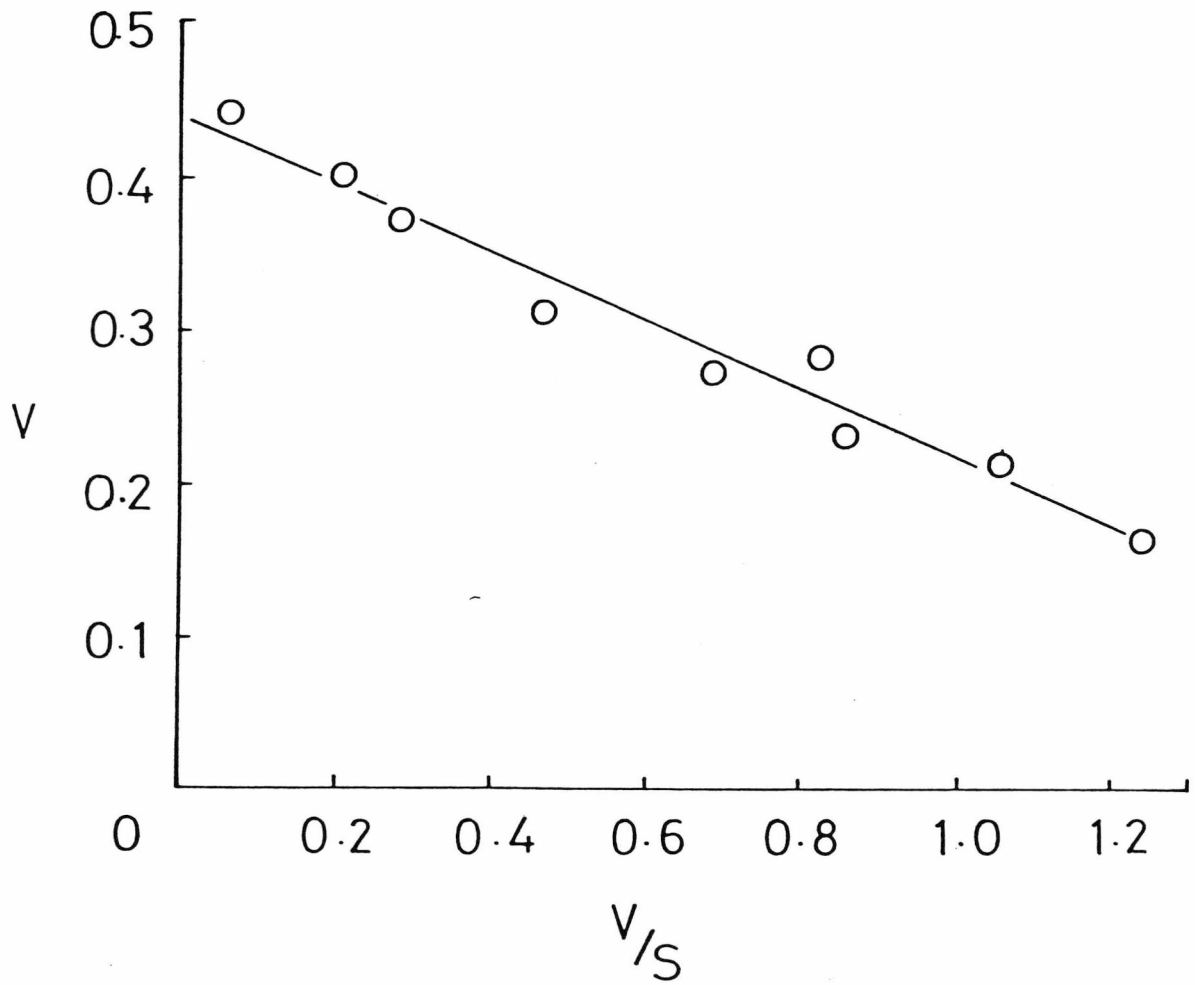


Table 28. Kinetics of soil glucanase.

Determination	Lineweaver-Burk		Eadie-Hofstee		Direct plot	
	Km (mg/ml)	V max*	Km (mg/ml)	V max*	Km (mg/ml)	V max*
1	0.27	0.37	0.28	0.38	0.29	0.39
2	0.17	0.31	0.15	0.29	0.10	0.26
3	0.22	0.39	0.16	0.35	0.17	0.35
4	0.22	0.44	0.22	0.44	0.23	0.45
5	0.35	0.60	0.27	0.53	0.27	0.52
6	0.16	0.35	0.17	0.37	0.16	0.36
Mean $\pm$ SD	0.23 ± 0.07	0.41 ± 0.10	0.21 ± 0.06	0.39 ± 0.08	0.20 ± 0.07	0.39 ± 0.09
Correlation Coefficient	0.96 - 1.00		0.90 - 0.99		NA	

* = μ moles reducing sugars as glucose equivalents/g soil/h.

NA = not applicable.

SD = standard deviation

matrix occurs the value of Km is much larger than that obtained when this adsorption is taken into account (Cervelli et al., 1973). These problems make it difficult to interpret the significance of Km values for soil enzymes and consequently such determinations are only of real value in the characterisation of a soil.

If it can be shown that the rate of breakdown of the enzyme-substrate complex to products is very slow compared to its formation from substrate then Km approximates to the dissociation constant of the enzyme-substrate complex. Under these circumstances the reciprocal of Km is a measure of the affinity or binding of substrate to enzyme. Assuming this to be the case for glucanase Nagasaki et al. (1976, 1977) showed that the affinity of the Poria cocos exoglucanase and Flavobacterium dormitator var. glucanolyticae endoglucanase increased with increasing chain length of the substrate.

Lethbridge, Bull and Burns (1978) have suggested that the direct linear plot may be the most useful form of analysis of soil enzyme kinetic data, especially when the correlation coefficients of the Lineweaver-Burk and Eadie-Hofstee plots are low. It has the advantage of being insensitive to errors because it is less dependent on the assumption of a normal distribution than the least squares methods (Markus, Hess, Ottaway and Cornish-Bowden, 1976). The purpose of the Eadie-Hofstee plot was to detect deviations from

typical Michaelis Menten kinetics (Walter, 1974), but none were found.

c) Effect of Assay Temperature on Activity

The effect of assay temperature on glucanase activity was investigated over the range 4 to 65°C (Figure 10) and the optimum temperature was found to fall between 50 and 65°C. Typical temperature optima for other glucanases range from 50 to 60°C irrespective of source (Reese and Mandels, 1959; Miyazaki et al., 1977; Nagasaki et al., 1976, 1977) although Chesters and Bull (1963b) reported that the temperature optimum for some fungal glucanases was 37°C. The pitfalls of comparing results obtained under different assay conditions have already been discussed (see Results and Discussion, A.1.a). This point is admirably illustrated by the fact that glucanase denaturation in some fungi is a function of pH (Reese and Mandels, 1959). As pH increased the temperature at which denaturation started decreased.

The activation energy (Ea) of the enzyme catalysed reaction calculated from the Arrhenius plot (Figure 11) was 11.8 k cals/mole (49 kJ/mole) compared to 6.2 k cals/mole (26 kJ/mole) for the Poria cocos enzyme (Nagasaki et al., 1977) Q_{10} over the range 4 to 50°C was 1.93.

d) Stoichiometry

Many authors, unwisely in my opinion, extrapolate

FIGURE 10 Effect of assay temperature on soil glucanase activity. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.

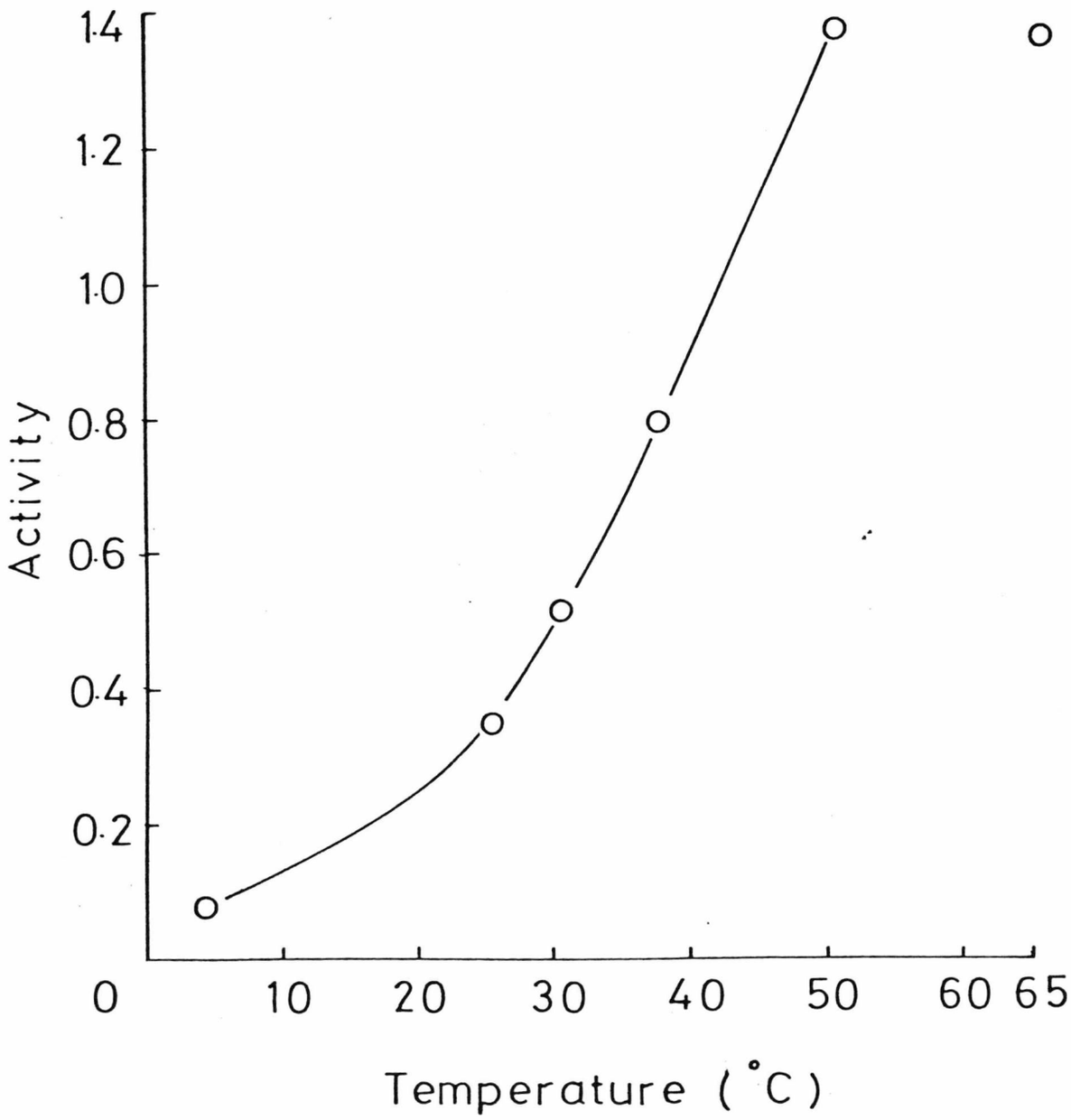


FIGURE 11 Arrhenius plot of soil glucanase activity. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.

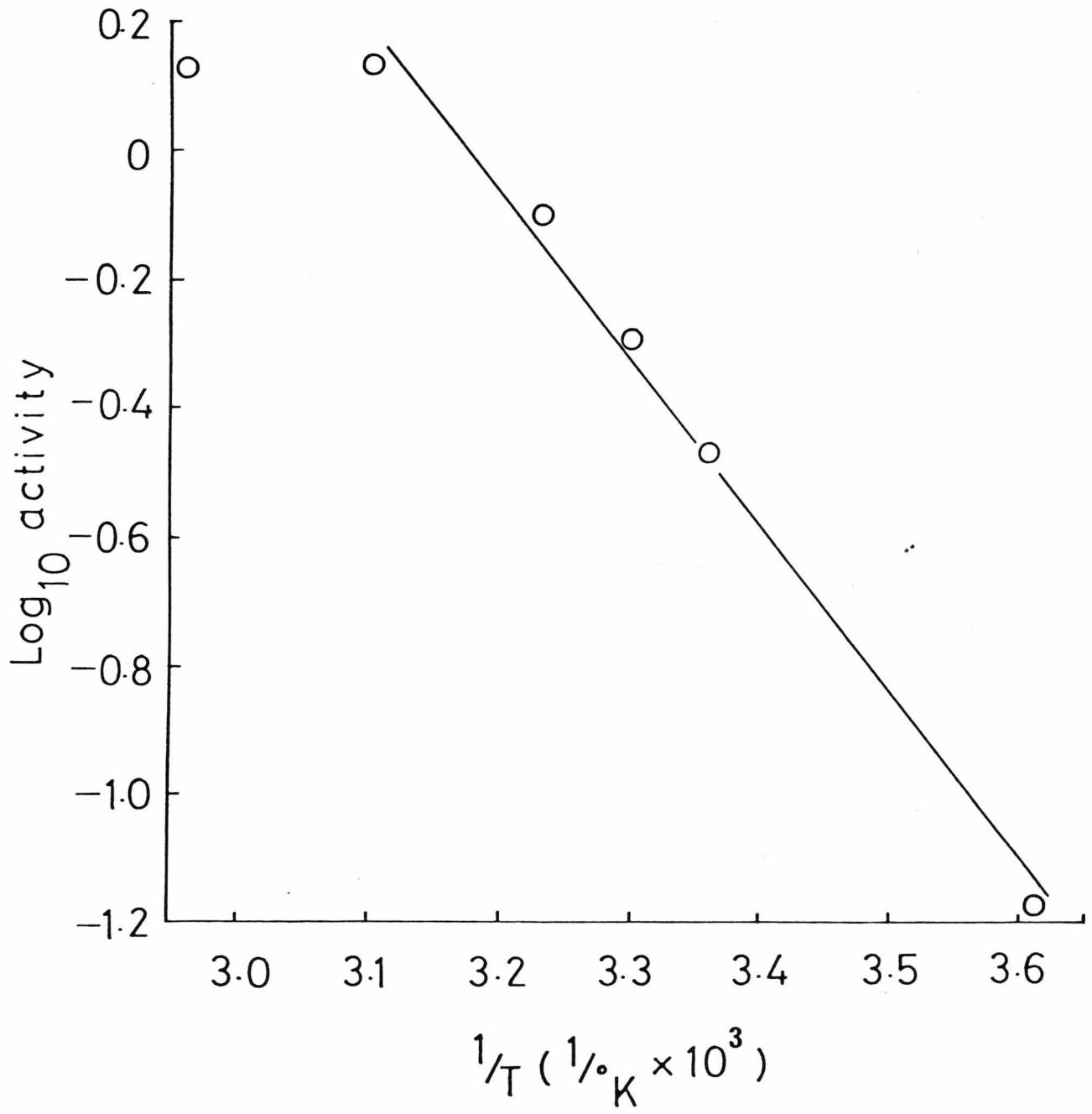
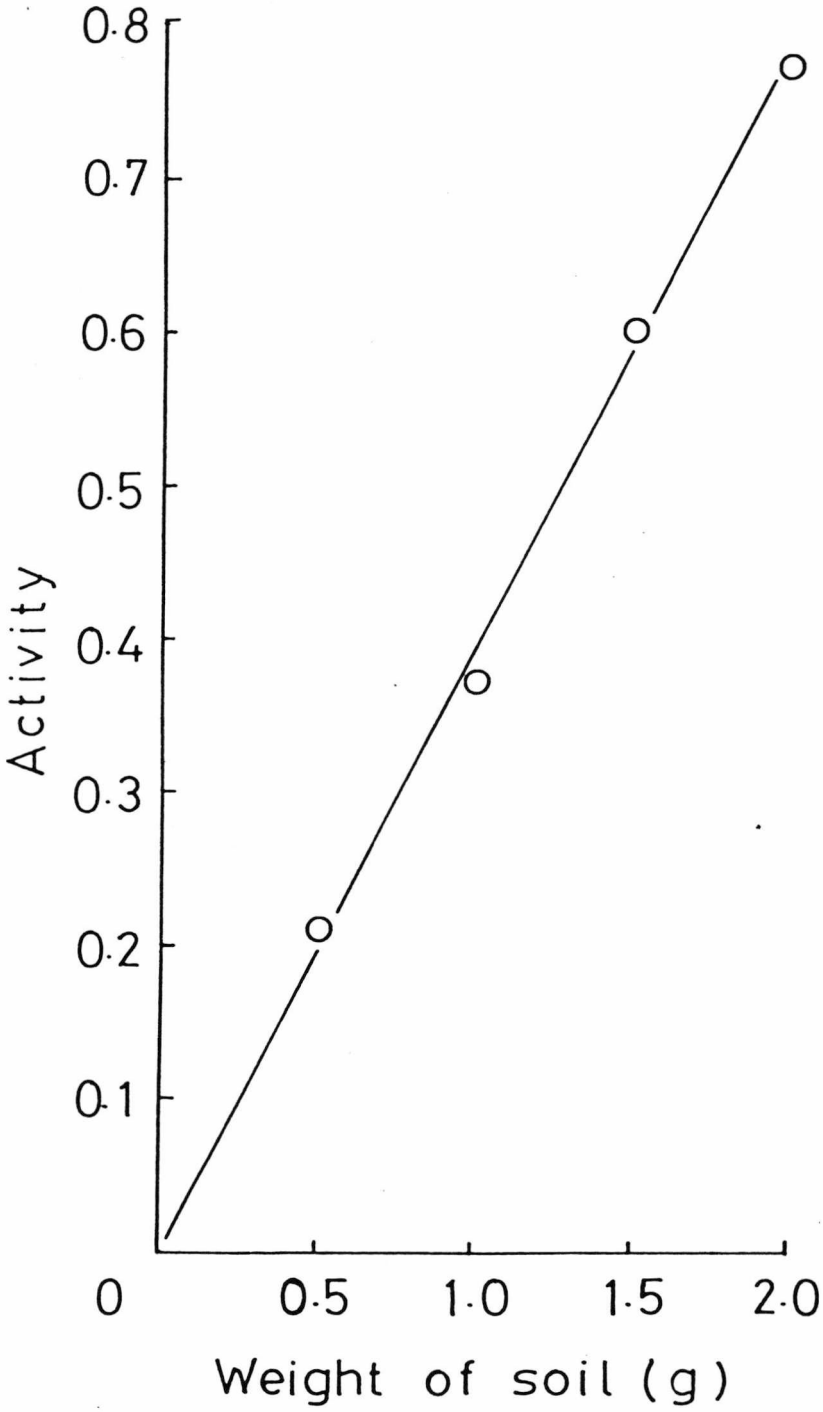


FIGURE 12 Stoichiometry of soil glucanase. Activity is expressed in μ moles reducing sugars as glucose equivalents/h.



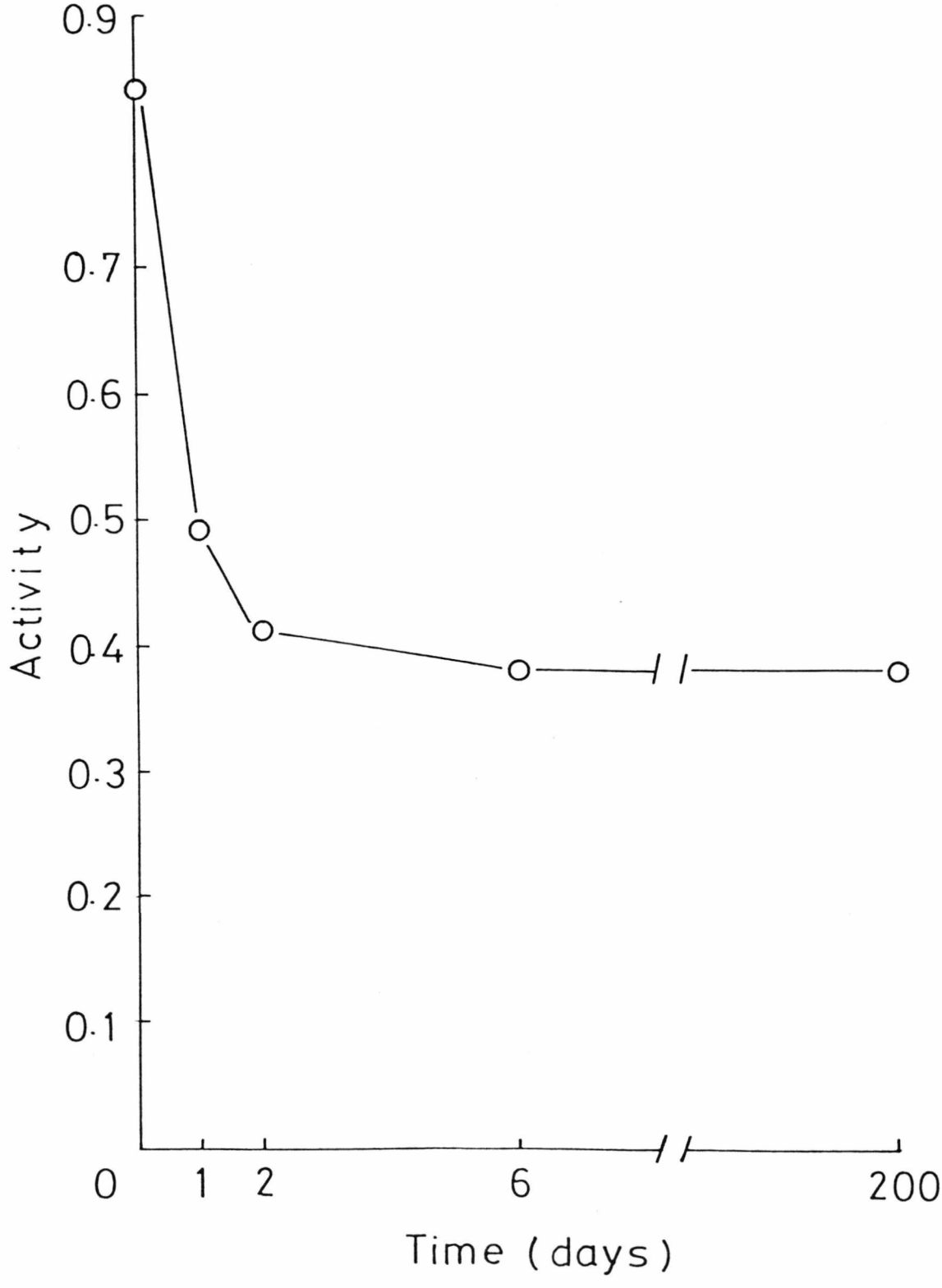
from results obtained using one quantity of soil to another without first examining the stoichiometry of the reaction. The glucanase activity of 0.5, 1.0, 1.5 and 2.0 g quantities of soil was measured (Figure 12) and the results show that the volume of soil did not impede the diffusion of substrate to the enzyme; in other words activity doubled as weight of soil doubled.

e) Effect of Air-Drying and Storage

After hand crumbling and sieving as described previously (see Methods and Materials, A), a fresh soil sample at field-wetness was assayed for glucanase activity within 60 minutes of collection. The remainder of the sample was air-dried and assayed at regular intervals for 7 months. When the first two days assays were performed the field water holding capacity was unknown (it was calculated by air-drying to constant weight), so 1.3 g wet soil were used and the results were subsequently recalculated on the basis of the amount of dry soil in 1.3 g field-wetness soil.

Air-drying was complete by day 2 and reduced activity by 50%, but thereafter enzyme levels remained constant (Figure 13). The activity of the same soil examined after 7 months storage had not declined any further. The lost activity could not be regained by rewetting the soil. It seems reasonable to propose that the physical stress of air-drying denatured much of the extracellular unbound (unprotected) enzyme free

FIGURE 13 Effect of air-drying on soil glucanase activity. Activity is expressed in μ moles reducing sugars as glucose equivalents/g dry soil/h.



in the soil solution. Reduction of enzyme activity upon air-drying has also been reported for urease, protease and arylsulphatase (Speir and Ross, 1975).

In another experiment, fresh soil was again collected and divided into two. One half was hand crumbled, sieved and sealed in polyethylene bags at 4°C, while the other was sealed in polyethylene bags at 4°C without crumbling or sieving. Neither fraction was air-dried. Samples were withdrawn from each bag at regular intervals and assayed for glucanase activity. The amount of water in the wet soil was taken into account as described above. After 63 days the sieved and unsieved fractions were each subdivided, one half being incubated at 4°C and the other at 25°C, again in sealed polyethylene bags. The four fractions were then monitored for glucanase activity. At each sampling time air-dried soil was also assayed.

Although glucanase activity fluctuated from day to day in both wet and dry soil (Figure 14) there was no consistent upward or downward trend. Neither sieving nor storage temperature had any effect on enzyme activity in field-wetness soil (Table 29). Within the limits of the day to day variation both field-wetness and air-dried soil have constant but differing glucanase activities, the former being approximately twice as active as the latter. Thus either soil will give experimental reproducibility but if one is interested in getting as close to the field activity as possible then field-wetness soil must be

FIGURE 14 Effect of laboratory storage on soil glucanase activity; ○ = air-dried soil stored at room temperature (21 ± 2°C); △ = field-wetness soil stored at 4°C. Activity is expressed in μ moles reducing sugars as glucose equivalents/g dry soil/h.

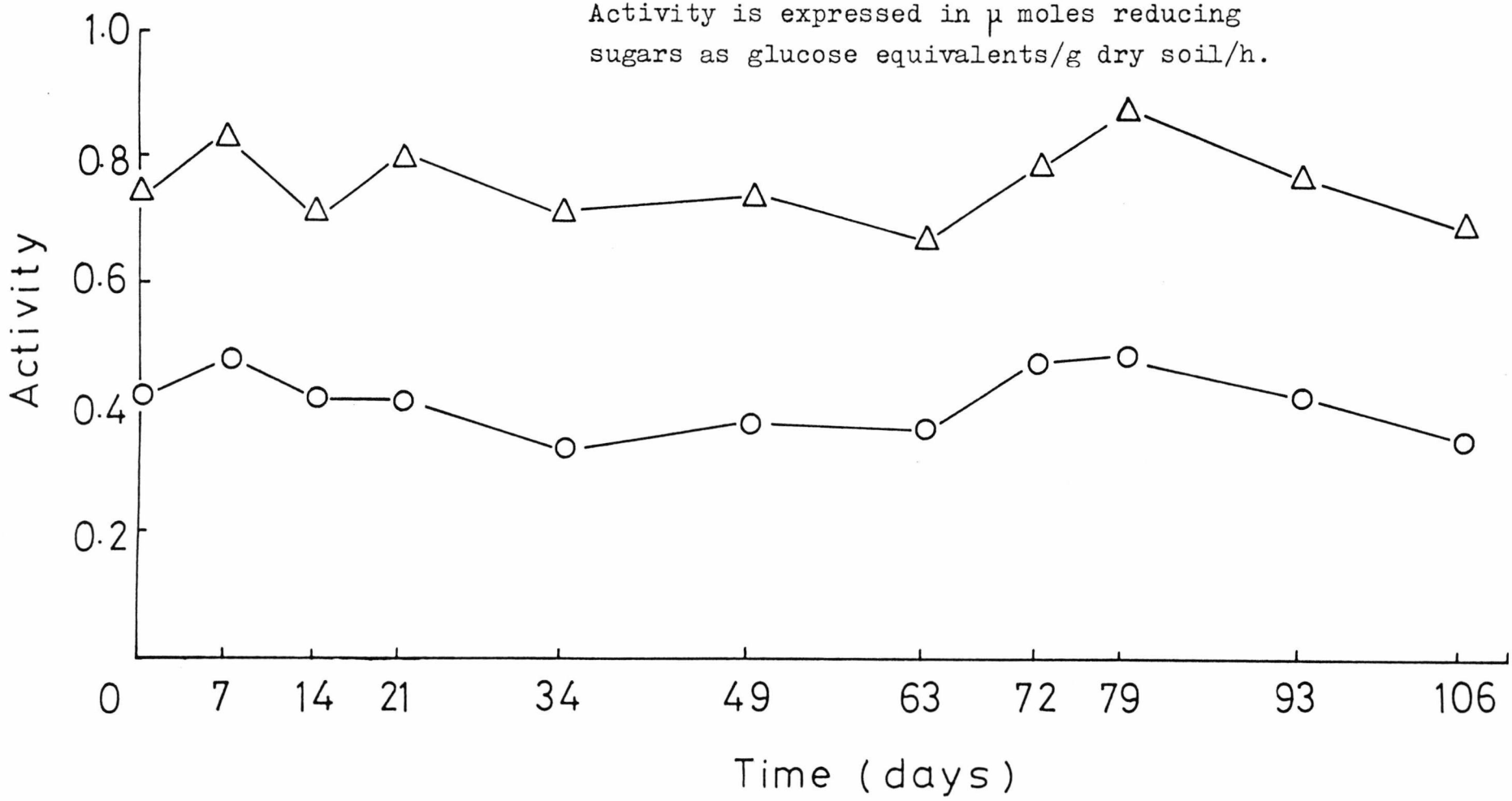


Table 29. Effect of sieving and storage temperature on glucanase activity in field-wetness soil. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.

Time (days)	Glucanase activity			
	Sieved		Non-sieved	
	4°C	25°C	4°C	25°C
0	0.74	ND	0.73	ND
7	0.82	ND	0.91	ND
14	0.70	ND	0.69	ND
21	0.79	ND	0.82	ND
34	0.70	ND	0.71	ND
49	0.73	ND	0.70	ND
63	0.66	ND	0.59	ND
72	0.78	0.78	0.77	0.75
79	0.87	0.90	0.86	0.86
93	0.76	0.77	0.68	0.78
106	0.68	0.64	0.66	0.65

ND = no determination

used. The majority of work in this project involved air-dried soil because early on it was discovered that when field wetness soil was stored in the laboratory, on occasions it became covered with white fungal mycelium, even at 4°C. This did not occur when air-dried soil was re-wetted, possibly because the air-drying process drastically reduced this fungal population or released a fungal inhibitor.

The extractable reducing sugar concentration (estimated in the soil control) of field-wetness soil was very low (0 to 60 µg/g soil) and stayed this way so long as it remained wet. On air-drying this value increased to 60 to 100 µg/g soil, indicating the release of reducing sugars from fractured cells and possibly humic material during the drying process. When the air-dried soil was rewetted to 65% WHC these reducing sugars disappeared rapidly (within 2 days), presumably by microbial metabolism, and the reducing sugar level returned to that of the field-wetness soil.

f) Long-Term Temperature Stability

Samples of air-dried soil (1 g) were remoistened to 65% WHC with 0.5 ml sodium azide solution (2 mg/ml) as a microbial inhibitor (Gibson and Burns, 1977), incubated at -22, 4, 25, 50 and 80°C in screw-capped universal bottles and assayed for glucanase activity at intervals up to 36 days (Table 30). The water present in the soil was taken into account when the assays were performed by adding 1.5 ml sodium azide

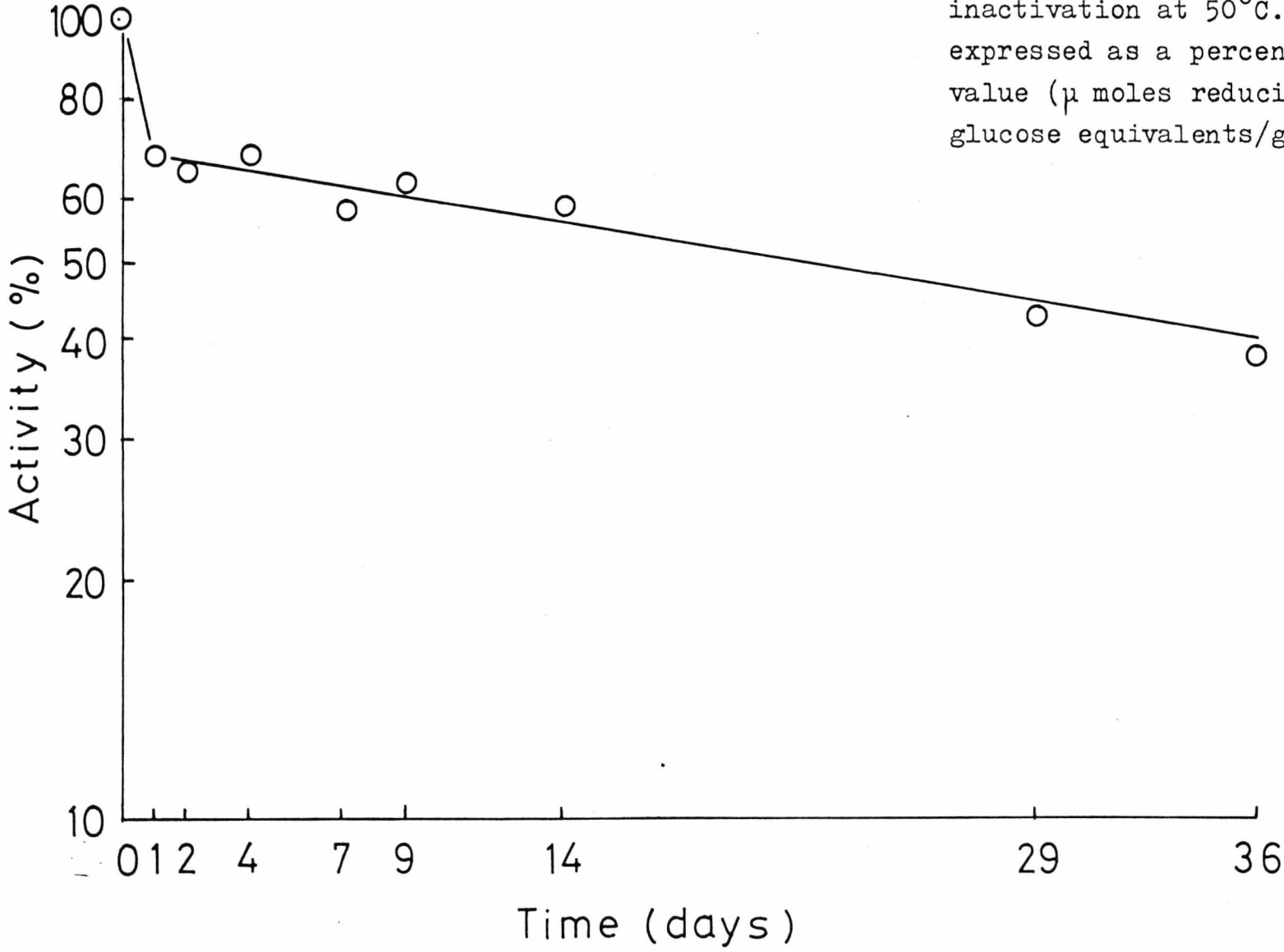
Table 30. Response of glucanase to long-term storage of wet soil at a range of temperatures. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.

Time (days)	Glucanase activity					
	Air-dried soil (25°C)	Wet soil				
		-22°C	4°C	25°C	50°C	80°C
1	0.53	0.52	0.56	0.54	0.38	0
2	0.53	0.52	0.50	0.53	0.33	0
4	0.50	0.51	0.53	0.47	0.36	0
7	0.47	0.44	0.46	0.43	0.27	0
9	0.52	0.52	0.50	0.54	0.32	0
14	0.35	0.37	0.36	0.35	0.21	0.03
29	0.40	0.37	0.37	0.39	0.16	0.01
36	0.51	0.49	0.53	0.55	0.20	0

solution as opposed to 2 ml. The function of the sodium azide was to prevent the possibility of extracellular enzyme production by proliferating microorganisms. It was considered necessary to replenish the sodium azide at the time of assaying to replace that broken down by chemical degradation in the soil during the incubation (Ketchersid and Merkle, 1976). Air-dried soil, which has constant glucanase activity (see Results and Discussion, A.1.e), was assayed at the same time to see whether sodium azide treatment had any effect on activity - but no consistent effect was detected.

At 50°C there was an initial rapid loss of glucanase activity (about 30%) in the first 24 h followed by a much slower rate of inactivation, but even after 36 days 40% of the original activity remained. This is illustrated in the semi-log plot of enzyme inactivation (Figure 15). If we assume that air-drying removed the extracellular unbound enzyme, i.e. that free in the soil solution (see Results and Discussions, A.1.e), this biphasic time course suggests the presence of at least two extracellular bound glucanase fractions each responding differently to heat stress. The first 30% that is lost rapidly may represent a poorly protected fraction, perhaps entrapped within the fungal cell wall matrices, whereas the gradual reduction in activity from then onwards is indicative of a highly protected fraction, perhaps located somewhere in the organo-mineral complex.

FIGURE 15 Semi-log plot of soil glucanase inactivation at 50°C. Activity is expressed as a percentage of the 4°C value (μ moles reducing sugars as glucose equivalents/g soil/h).



Similar responses at 50°C have been observed with soil phosphatase and arylsulphatase (Pettit *et al.*, 1977) and at 45°C with urease (Pettit *et al.*, 1976). To illustrate just how stable soil glucanase is compared to cell free purified preparations from microorganisms; when the *Poria cocos* enzyme was subjected to 50°C for 10 min it lost 95% of its original activity (Nagasaki *et al.*, 1977) and under the same conditions the *Mucor hiemalis* enzyme lost 25% (Miyazaki *et al.*, 1977).

At 80°C soil glucanase was inactivated within the first 24 h and comparable rapid inactivation at 70 and 75°C has been reported for urease (Pettit *et al.*, 1976) and arylsulphatase (Pettit *et al.*, 1977); phosphatase however survived for 5 days at 75°C (Pettit *et al.*, 1977).

Soil maintained at -22, 4 and 25°C showed no consistent decline in glucanase activity during the experimental period. In the same soil Pettit *et al.*, (1977) showed that phosphatase activity was unaffected at -20 and 4°C but had declined by 20% after 28 days at 25°C and that arylsulphatase activity decreased even at 4°C, 30% having been lost after 28 days. Urease too was slowly inactivated at 4°C (Pettit *et al.*, 1976).

Pettit *et al.* (1977) proposed the order of stability in stored wet soil as phosphatase > urease > arylsulphatase. The glucanase research suggests that it is less stable than phosphatase but more stable than urease.

g) Effect of Gamma-Irradiation on Activity

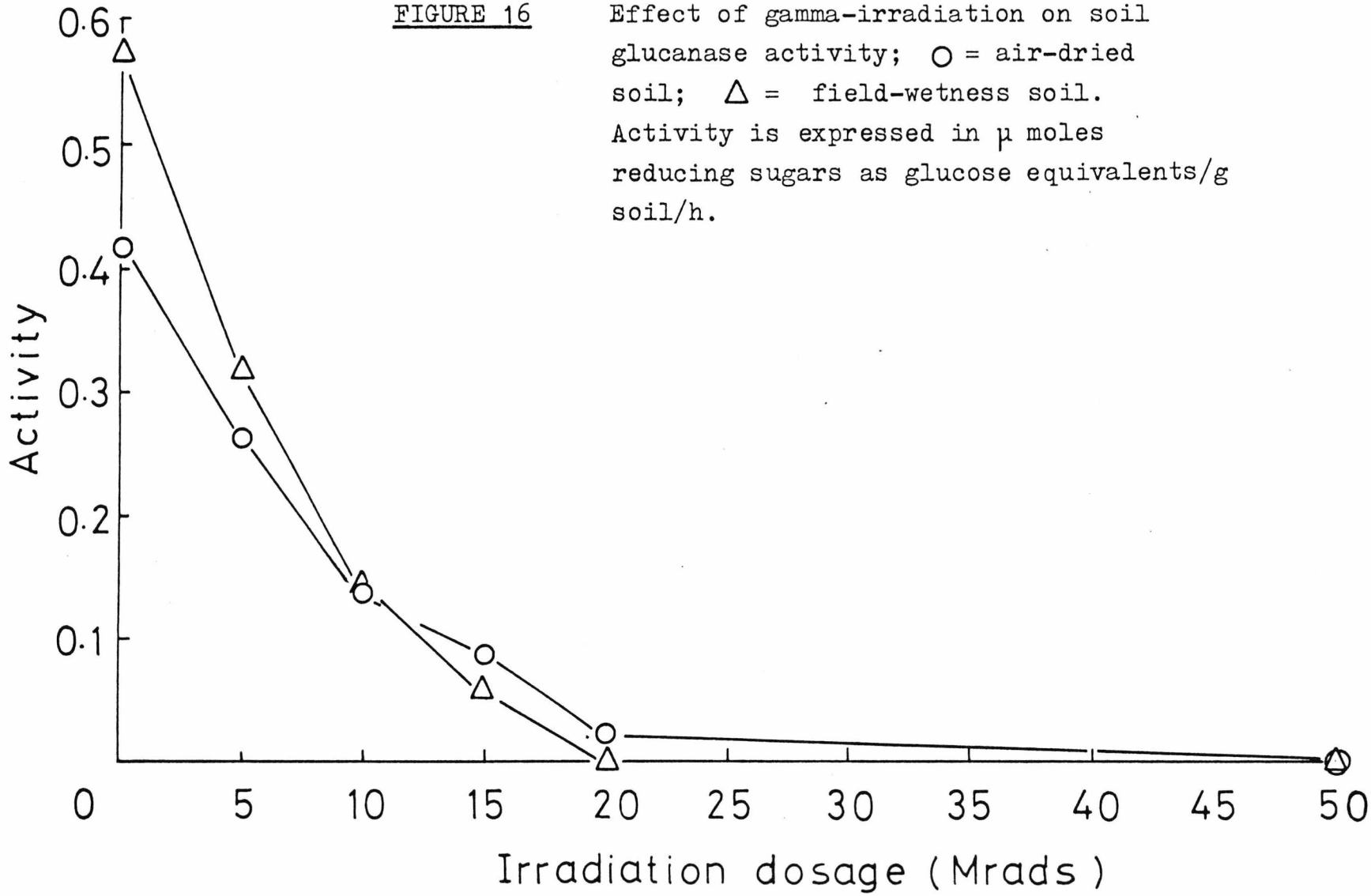
Air-dried and field-wetness (29% WHC) soil samples (25 g) were sealed in polyethylene bags and subjected to 5, 10, 15, 20 and 50 Mrad doses of gamma-irradiation at approximately 4 Mrad/h at the Atomic Energy Research Establishment, Harwell, Fuel Pond Assembly. Prior and subsequent to irradiation the soils were stored at 4°C. The samples were checked for sterility by scattering a small quantity of the soil (amout 0.1 g) over the surface of a Bunt and Rovira's agar plate and incubated at 25°C for 2 weeks. All the samples were sterile. Non-irradiated samples were also sealed and refrigerated. The amount of water already present in the wet soil was taken into account when the assays were performed.

The effect of gamma-irradiation on glucanase activity is summarised in Figure 16. Field-wetness soil is known to be twice as active as air-dried soil (see Results and Discussion, A.1.e) but in this instance when the unirradiated controls were compared the wet soil was only 1.4 times as active as the air-dried soil, presumably because it had dried out somewhat on its travels and hence lost some activity. This extra activity in the wet soil was rapidly lost on irradiation such that at 10 Mrad both soils had the same activity. From this dose onwards the glucanase was slightly more susceptible to inactivation by irradiation in the wet soil.

Skujins, Braal and McLaren (1962) have described

FIGURE 16

Effect of gamma-irradiation on soil glucanase activity; ○ = air-dried soil; △ = field-wetness soil. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.



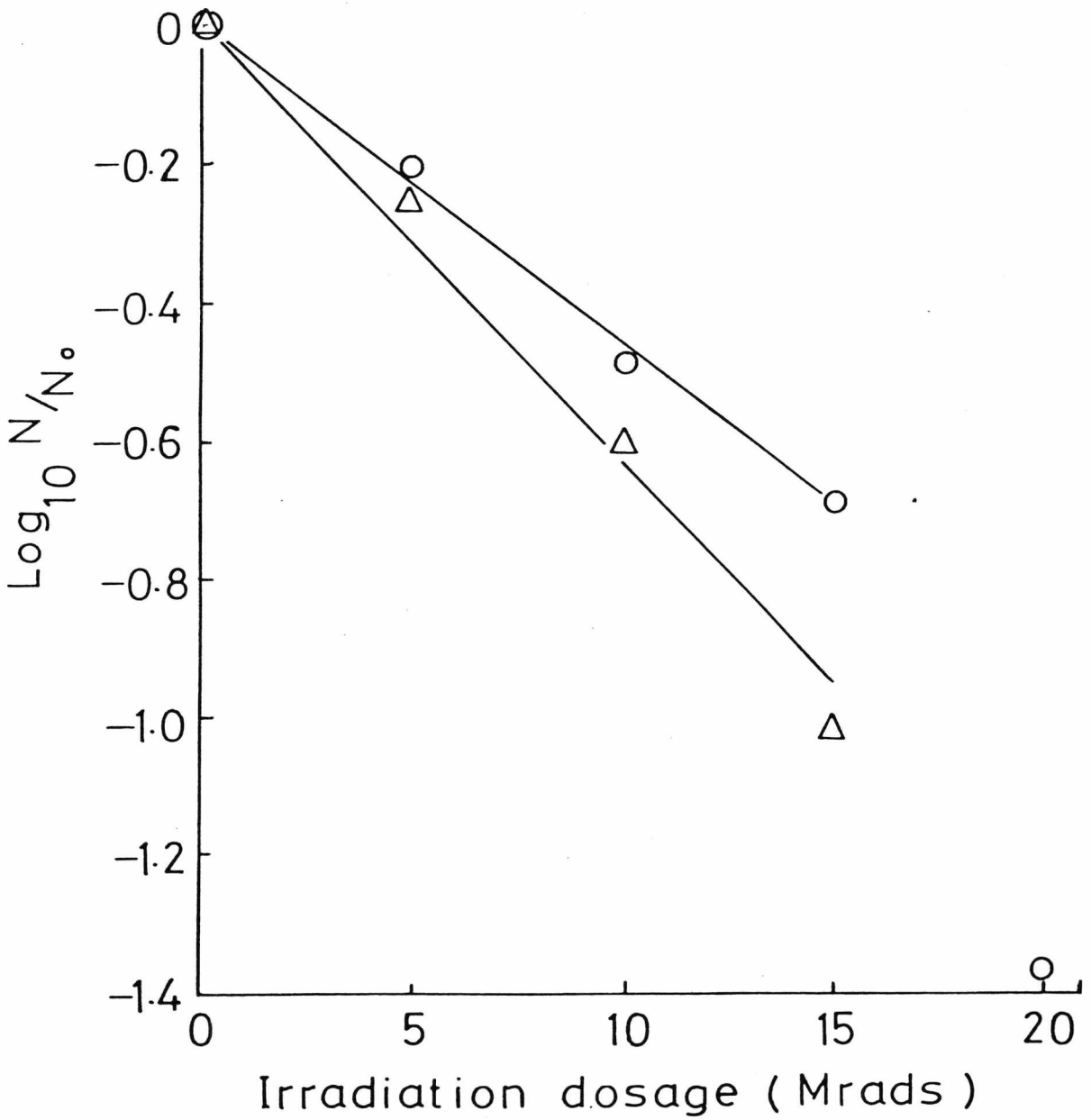
an enzyme inactivation coefficient (k) [2], where N = activity

$$\log_{10}(N/N_0) = - (k/2.303)\ D \quad .....[2]$$

at irradiation dose D and N_0 = activity of non-irradiated soil. A plot of $\log_{10}(N/N_0)$ versus D gives a straight line whose slope is -k/2.303 (Figure 17). The smaller the inactivation coefficient the more stable the enzyme to irradiation. The values of k for glucanase in wet and dry soil were 0.15 and 0.11 respectively. Phosphatase (Ramirez-Martinez and McLaren, 1966; Burns et al., 1978), arylsulphatase and urease (Burns et al., 1978) have also been reported to be more susceptible to inactivation by irradiation in wet than in dry soil. It is well known that the radiosensitivity of micro-organisms and enzymes usually increases in wet soil (Cawse, 1975), due partly to the reactive free radicals (OH, H and HO_2) produced when water is ionised (Becking, 1971).

In Hamble soil the order of irradiation stability for these four enzymes was the same in both wet and dry soil, viz. phosphatase > glucanase > urease > arylsulphatase and was identical to the one derived from long-term storage of re-wetted air-dried soil at a range of temperatures (see Results and Discussion, A.1.f). The reasons for the differential stability of the four enzymes are not resolved by these observations

FIGURE 17 Inactivation of soil glucanase by gamma-irradiation; O = air-dried soil; Δ = field-wetness soil. N = activity at irradiation dose D and N_o = activity of non-irradiated soil. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.



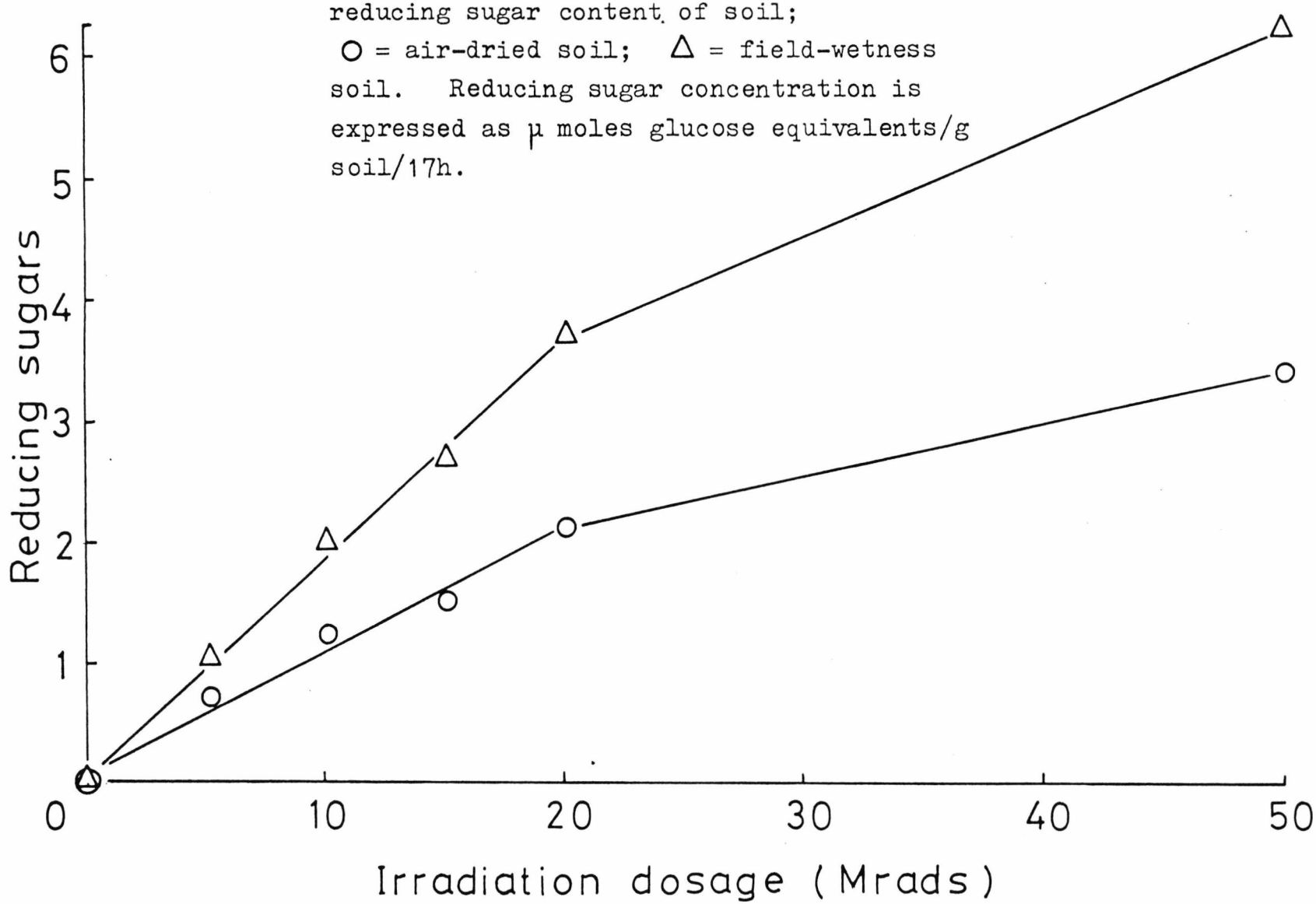
but it is pertinent to propose that one or a combination of the following factors might be involved:

1) The inherent structural and chemical differences between the enzymes.
2) The predominant cellular location of the enzyme. Urease and arylsulphatase function intracellularly, whereas glucanase is an extracellular enzyme and phosphatase is periplasmic.
3) The site of enzyme action, be it free in solution or in a membrane bound state.
4) The protection afforded by the location of the extracellular fractions. Burns et al. (1972a) have suggested that urease with its soluble low molecular weight substrate may function from within the soil organo-mineral complex.

It was noticeable that the reducing sugar concentration of the soil control (no laminarin) increased with increasing dose of irradiation (Figure 18), suggesting that reducing sugars had been released from the humic material and microbial biomass by the high energy radiation. Gamma rays are known to cause the hydrolysis of polysaccharides, proteins, lipids and nucleic acids (Becking, 1971). The increase which was more pronounced in the wet soil was linear up to 20 Mrad.

Incidentally, gamma irradiation is often presented as a less harsh method of sterilising soil than

FIGURE 18 Effect of gamma irradiation on extractable reducing sugar content of soil; ○ = air-dried soil; △ = field-wetness soil. Reducing sugar concentration is expressed as μ moles glucose equivalents/g soil/17h.



autoclaving. This may well be the case at low radiation levels but the soils which received 20 and 50 Mrad exhibited a marked hydrophobic tendency and a considerable decline in aggregate size, possibly due to the depolymerisation of the organic materials which hold them together (Griffiths and Burns, 1968). Thus the observed decrease in glucanase activity may not only be due to denaturation by the high energy irradiation but also the release of organic molecules which inhibit the enzyme.

h) Identification of Laminarin Breakdown Products in Soil

In an attempt to determine whether soil glucanase activity was primarily exo- or endohydrolytic, or a mixture of them both, the assay supernatant was analysed by descending paper chromatography using an n-butanol-pyridine-water-benzene (5:3:3:1) solvent system which is known to separate mixtures of glucose, laminaridextrins and laminarin (Bull, 1962).

Samples (20 µl) were applied to Whatman No.1 chromatography paper by spotting from 20 µl microcaps. Hot air from a hair drier was used to prevent the spots from spreading too much by evaporating the water rapidly and the necessary control was carried out to make sure that the heat did not break down the laminarin or any laminaridextrins that might be formed. The chromatograms were equilibrated with solvent overnight and run for 18 h at room temperature (21 $\pm$ 2°C) after

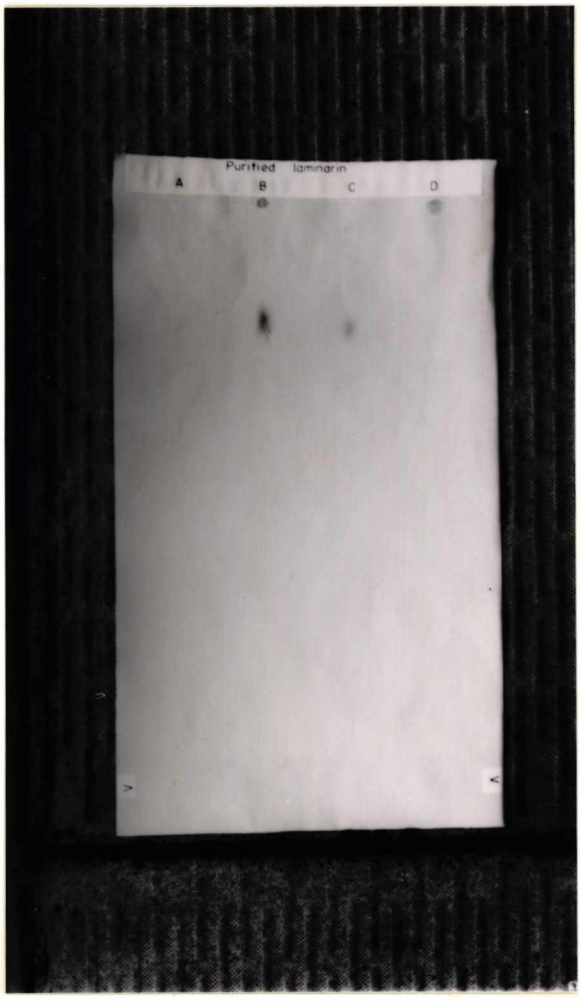
which time they were hung up to dry. They were then pulled through a tray of 1 g silver nitrate in 2 ml distilled water diluted with 200 ml acetone (Trevelyan, Procter and Harrison, 1950). After drying, they were sprayed with a mixture of 5 g sodium hydroxide in 10 ml distilled water diluted with 200 ml industrial methylated spirit, in a fumecupboard. Reducing sugars appeared immediately as black spots which were fixed by dragging the chromatograms through 0.1 M sodium thiosulphate. This latter treatment also had the advantage of cleaning up the brown background so it became white.

In addition to the assay supernatants, glucose (100 µg/ml) and laminarin (6.7 mg/ml) standards were run every time. Laminaridextrin standards were not commercially available. After 17 h incubation of either purified (Plate 3a) or crude (Plate 3b) substrate with the soil only two reducing spots were detected in the assay supernatants and these corresponded to laminarin (which did not migrate from the origin) and glucose. An identical result was obtained when the assay supernatant was sampled at 1, 2, 4, 7, 10 and 13 h, the glucose spot becoming more intense as the concentration increased. The absence of detectable amounts of laminaridextrins even early on in the assay suggests that the exohydrolase is the predominant glucanase in this soil. This is a little surprising as one would expect both the exo- and endo- enzymes to be present in soil, unless the latter are unstable. The

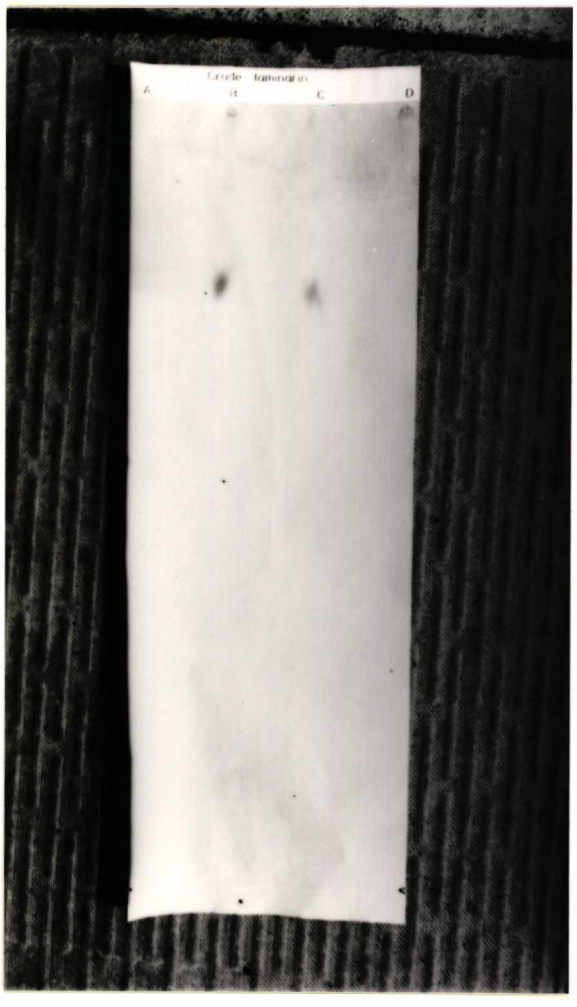
PLATE 3 Identification of the products resulting from the action of soil glucanase on laminarin using descending paper chromatography.

A = soil control, B = assay,
C = glucose standard,
D = laminarin standard.

a) Purified laminarin,
b) Crude laminarin.



3a



3b

result could be verified by measuring glucose in the supernatant using the glucose oxidase method and comparing this value to the total reducing sugar concentration as determined by the Nelson-Somogyi copper reduction method. Using such methodology Benefield (1971) showed that there was no significant difference between the glucose and reducing sugar concentrations in soil cellulase assays, thus indicating that glucose was the major breakdown product of cellulose by the soil enzyme under those conditions. Dragan-Bularda and Kiss (1972) chromatographed the filtrates of a brown forest soil treated with a bacterial dextran, a branched polysaccharide consisting of α-glucose residues in 1,6 and 1,3 linkage. They detected minute quantities of intermediary reducing oligosaccharides along with glucose, but in another soil (a chernozem) they could only detect glucose. They attributed the absence of reducing oligosaccharides to the activity of α-glucosidase. Since various β-glucosidase activities have been demonstrated in soil (Kiss et al., 1975; Skujins, 1976) it is possible that the β-glucosidases laminaribiase and laminaritriase etc. are responsible for the absence of laminaridextrins in laminarin treated soil. But this is unlikely to be the complete explanation, since even if these enzymes were present one would still expect to detect some laminaridextrins early on in the incubation - unless these β-glucosidases were extremely active compared to the endoglucanases.

To verify that laminaridextrins were not being broken down to glucose by the 20 min heating period in the 100°C water bath used to stop the reaction, non-heated supernatants were chromatographed. Yet again glucose was the only detectable product.

Drying the spots with heat from a hair drier did not affect the results. No reducing sugars were detected in the soil control (no substrate). The crude laminarin standard contained a small amount of glucose, but the purified substrate was glucose free.

i) Seasonal Variation in Activity

Soil samples collected in April, July and November 1976 had very similar glucanase activities (Table 63) suggesting that the accumulated enzyme was little affected by seasonal variations, cultivation and cropping. In contrast the urease activities of the July and November samples (20 to 30 μ moles ammonia evolved/g soil/h) were more than twice that in the April sample (5 to 9 μ moles ammonia evolved/g soil/h) but by June 1977 activity had returned to the lower level (Pettit, 1978). The temporarily elevated urease levels between July and November 1976 may have resulted from the extraordinarily hot summer of that year, since no seasonal fluctuation had been observed in previous years.

B. Ecology

1. Effects of Pesticide Formulations on Glucanase and Urease Activities in Soil

a) Unamended Soil

1) Air-dried Soil

None of the pesticide formulations tested had any consistent, significant (see footnote) effect on glucanase (Table 31) or urease (Table 32) activities in air-dried soil re-wetted and maintained at 65% WHC for 42 days. The experiment was terminated at this stage on the assumption that any effects would have been manifest by this time. This will almost certainly be the case for the active ingredients 2,4-D and malathion, since the time required for the complete disappearance of these chemicals from this soil is 16 days for 19.8 ppm 2,4-D and 14 days for 19.5 ppm malathion (Gibson, 1977; Gibson and Burns, 1977). However this latter work was performed with analytical grade pesticides and it must be borne in mind that the active ingredient may not behave in the same manner in the formulation. One can not be

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Statistical Note In Tables 31 to 67 an asterisk indicates a value which is significantly different from its corresponding control at the 5% testing level (student's t test). Thus, in experiments where two variables were under test and hence two different controls set up, i.e. where a pesticide was being investigated in combination with another treatment (flooding, NPK fertiliser, urea, pig slurry, lime, glucose, cellulose, acetone) the control value for the pesticide treated soil is always regarded as the amended control (e.g. NPK control, urea control etc.) rather than the unamended control.

Table 31. Effect of five pesticide formulations on glucanase activity in air-dried soil.

Time (days)	Glucanase activity (μ moles reducing sugars [a]/g soil/h)					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	0.36	0.33	0.33	0.33	0.32*	0.33
1	0.41	0.40	0.39	0.39	0.42	0.42
2	0.49	0.48	0.50	0.51	0.47	0.51
5	0.33	0.34	0.32	0.32	0.33	0.33
9	0.38	0.38	0.37	0.38	0.38	0.38
16	0.36	0.33	0.35	0.34	0.34	0.36
23	0.37	0.36	0.37	0.35	0.36	0.36
30	0.32	0.33	0.32	0.31	0.33	0.32
37	0.44	0.47	0.45	0.49*	0.47	0.48*
42	0.36	0.39	0.36	0.38	0.38	0.39

a = as glucose equivalents

* = significantly different (P = 0.05) from the control value.

Table 32. Effect of five pesticide formulations on urease activity in air-dried soil.

Time (days)	Urease activity (μ moles ammonia evolved/g soil/h					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop Ethyl	Malathion
0	25.9	25.7	25.9	27.8	25.7	26.4
1	23.3	23.4	24.2	25.9	24.7	26.2
2	21.8	21.5	22.7	22.9	22.7	22.4
5	25.3	24.1	24.7	24.2	25.5	24.0
9	24.0	23.5	24.2	26.9	25.8	25.6
16	25.8	24.8	24.3	25.9	25.5	25.8
23	28.4	27.3	28.6	27.6	29.0	27.7
30	26.1	25.8	26.8	27.0	26.6	26.0
37	26.0	26.2	25.7	28.2	28.3	26.0
42	25.7	25.7	25.1	26.7	26.6	26.8

sure about no effects with the other three active ingredients because Gibson (1977) has shown that only 50% 19.8 ppm diallate had disappeared 70 days after application. Benzoylprop ethyl and glyphosate disappearance have not been followed in this soil. Extrapolation from breakdown rates in other soils suggests that not all the glyphosate would have disappeared by the end of the experiment (Nomura and Hilton, 1977; Rueppel et al., 1977). So few studies on benzoylprop ethyl degradation in soil have been reported (Beynon et al., 1974) that no predictions about persistence can be made.

Unfortunately, many of these breakdown studies only monitor disappearance of the active ingredient and since there is little or no information on any intermediary breakdown products, or on the rates of disappearance of formulation additives, longer term effects can not be ruled out categorically. This point illustrates one of the drawbacks of working with pesticide formulations as opposed to the active ingredient; by definition the former are composed of a number of chemicals. While the structure of a commercially available pesticide is readily available this is not the case for all formulation additives, some of which are closely guarded secrets. Therefore, when working with formulations the biologist finds himself testing not only a mixture of compounds but often an undefined one at that. However, this should not be used as an excuse to work solely with active

ingredients, since it is the formulation which is used in agriculture and it is just as important to be aware of any effects due to formulation additives as it is to the pesticide per se. Indeed some formulations may possess greater quantities of additives (solvents and emulsifiers) than active ingredient (only 20% w/v of the Suffix formulation is the active herbicide benzoyl-prop ethyl) and hence there is at least a possibility that the non-pesticide components present a greater ecological threat. For example, Stanlake and Clarke (1975) have shown that the aromatic petroleum distillates present in a malathion formulation had a greater effect on the viability of pure cultures of bacteria isolated from soil than did the malathion itself. In addition there may be synergistic effects involving the active ingredient and the formulation additives. Only when effects have been observed with the formulation is it time to investigate its individual components to facilitate an in depth understanding of such effects.

2) Field-Wetness Soil

Field-wetness soil contains glucanase (see Results and Discussion, A.1.e) and urease (Pettit, 1978) components which are sensitive to air-drying; possibly that fraction which survives air-drying is better protected. The unstable fraction may also be more sensitive to applications of pesticide formulations. The microbial counts were approximately 10 to 100 times higher in field-wetness than air-dried soil (see

Methods and Materials, Table 18) so that pesticide additions might be metabolised more rapidly in the former. This increased rate of breakdown might alleviate or reduce inhibitory effects, or cause a surge in enzyme levels resulting from an increase in the microbial population degrading the formulation components (Cervelli et al., 1978).

After 42 days none of the pesticide formulations had any consistent, significant effect on glucanase (Table 33) and urease (Table 34) activities in field-wetness soil maintained at 65% WHC.

The soil used in this experiment was collected in June 1977 when the high urease levels noted in July and November of 1976 (20 to 30 μ moles ammonia evolved/g dry soil/h) had returned to those of April 1976 (5 to 9 μ moles evolved/g dry soil/h) (see Results and Discussion, A.1.i). This observation explains why the urease activity of the field-wetness soil used in this experiment was low. The expected value for July and November 1976 field-wetness soil would have been about 50 μ moles ammonia evolved/g dry soil/h.

The concentration of 2,4-D formulation used was 9.8 nl/g soil (4.9 ppm ai w/w) which approximates to a field rate of 3.5 kg ai/ha. Zinchenko, Osinskaya and Prokudina (1969) have reported increased urease levels in the field following 2,4-D application at 2 kg/ha (about 2.8 ppm). However there is no indication as to whether this concentration relates to

Table 33. Effect of five pesticide formulations on glucanase activity in field-wetness soil.

Time (days)	Glucanase activity (μ moles reducing sugars[a]/g soil/h					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	0.81	0.76	0.74*	0.80	0.79	0.77
3	0.68	0.69	0.70	0.68	0.71	0.68
7	0.66	0.69	0.69	0.70	0.67	0.68
14	0.71	0.66	0.71	0.68	0.70	0.71
28	0.75	0.78	0.77	0.76	0.78	0.78
42	0.87	0.88	0.90	0.90	0.94	0.92

a = as glucose equivalents.

* = significantly different (P = 0.05) from the control value.

Table 34. Effect of five pesticide formulations on urease activity in field-wetness soil.

Time (days)	Urease activity (μ moles ammonia evolved/g soil/h)					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	18.9	19.6	19.1	18.9	18.6	19.2
3	19.6	19.2	20.0	19.7	19.0	19.1
7	18.2	19.4	17.6	18.5	19.9	17.8
14	19.3	20.5	19.9	20.4	19.5	18.9
28	20.2	19.8	19.3	19.7	19.5	18.8
42	19.0	18.8	19.7	19.4	19.6	19.2

formulation or active ingredient. In contrast, Zinchenko and Osinskaya (1969) observed urease inhibition by 20 ppm 2,4-D in the laboratory. These results have been extracted from other reviews (see Introduction, Table 8) and unfortunately the original papers were not available for consultation, so it is invalid to discuss these results further.

Verstraete and Voets (1974) observed that Avadex (a.i. diallate) applied at 1.4 kg/ha in combination with 5 kg/ha Pyramin (a.i. pyrazone) and 1.2 kg/ha Betanol (a.i. phenmedipham) reduced urease levels in the field. They were only interested in sugarbeet pesticide treatment systems which are actually used by the farmer and did not carry out the necessary controls to determine whether this inhibition was due to one or a specific combination of these three herbicide formulations.

The pesticide formulations were tested for inherent effects on urease using purified enzyme from jack beans (Sigma, Type 3) and Bacillus pasteurii (Sigma, Type X), but none were detected (Table 35). A similar enzyme activity to pesticide formulation ratio (on an activity to weight basis) to that in soil was used. Thus the same amount of formulation that was added to 1 g soil was added to a Conway Microdiffusion Dish containing sufficient purified urease to give an activity in the region of 25 μ moles ammonia evolved/h.

TABLE 35 Effect of five pesticide formulations on jack bean and Bacillus pasteurii urease activity

Pesticide	Urease activity (μ moles ammonia evolved/h)	
	Jack bean	Bacillus pasteurii
Control	33.1	23.9
2,4-D	34.6	23.3
Diallate	30.5	22.6
Glyphosate	33.4	23.7
Benzoylprop ethyl	35.2	24.4
Malathion	31.5	23.7

3) Multiple Malathion Applications

During the persistently hot summer of 1976 some farmers sprayed malathion at monthly intervals, in an attempt to keep the rampant aphids under control. With this in mind the glucanase and urease activities of soil which had been treated with three successive doses of malathion formulation at 28 day intervals for up to 90 days were monitored but no significant effects were detected with either enzyme (Table 36). The second and third doses were added on day 28 and 56 respectively in the 0.8 ml distilled water required to return the soil to 65% WHC.

Table 36. Effect of repeated applications of malathion formulation on glucanase and urease activities in soil.

Time (days)	Glucanase activity[1]		Urease activity[2]	
	Control	Malathion	Control	Malathion
0[a]	0.31	0.33	25.1	24.9
27	0.36	0.40	24.3	24.3
28[b]	0.38	0.34	25.6	23.8
29	0.42	0.39	24.7	23.6
30	0.41	0.44	24.3	23.9
34	0.39	0.41	25.2	23.2
36	ND	ND	24.6	24.5
42	0.43	0.45	28.1	27.3
55	0.41	0.40	30.6	30.1
56[c]	0.49	0.51	22.2	25.9
57	0.41	0.42	30.6	28.7
58	0.41	0.39	28.7	27.9
63	0.36	0.36	23.9	26.6
67	0.41	0.39	23.0	24.3
71	0.46	0.44	23.1	22.3
80	0.41	0.42	23.3	23.4
90	0.45	0.44	24.2	25.0

1 = μ moles reducing sugars as glucose equivalents/g soil/h.

2 = μ moles ammonia evolved/g soil/h.

ND = no determination.

a = first dose; b = second dose; c = third dose.

4) Flooded Soil

During the wet spring of 1977 the field from which the soil was collected suffered periods of flooding, some lasting up to 3 weeks. On these occasions areas of the field were submerged under a few centimetres of water.

Soil oxygen levels are inversely related to water content and as a result poor aeration is usually associated with improper drainage, the problem being worse in fine (clay) than in coarse (sand) textured soils, because small pores have greater tenacity for water. If the dissolved oxygen present in water is rapidly consumed by actively respiring microorganisms its subsequent replacement by diffusion from air is a slow process. The flooding of a soil is known to alter pesticide disappearance rates by affecting adsorption, volatility and microbial metabolism (Burns, 1975). Such changes may affect the response of soil enzymes to pesticide treatments, however no consistent, significant changes in glucanase (Table 37) or urease (Table 38) levels were detected when pesticide formulation treated soil was maintained flooded for up to 36 days.

Flooding itself also had no effect on the activity of either soil enzyme, not an altogether unexpected result considering that the assays measure turnover due to accumulated enzyme and not microbial utilisation.

When urea hydrolysis was monitored in waterlogged soils Wang, Tseng and Puh (1966) observed a 50%

Table 37. Effect of five pesticide formulations on glucanase activity in flooded soil.

	Glucanase activity (μ moles reducing sugars[a]/g soil/h).								
	Time (days)								
Pesticide	1	2	7	9	14	16	23	29	36
65% WHC control	0.29	0.31	0.49	0.31	0.32	0.29	0.38	0.39	0.43
Flooded control	0.29	0.34	0.47	0.31	0.31	0.31	0.39	0.44*	0.45
2,4-D	0.28	0.35	0.46	0.29	0.33	0.29	0.39	0.41	0.43
Diallate	0.29	0.32	0.45	0.29	0.34	0.31	0.38	0.42	0.45
Glyphosate	0.25*	0.34	0.44	0.34	0.34	0.30	0.40	0.41	0.43
Benzoylprop ethyl	0.29	0.35	0.47	0.27*	0.34	0.31	0.38	0.42	0.45
Malathion	0.29	0.34	0.44	0.27*	0.33	0.28*	0.38	0.41	0.42

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

Table 38. Effect of five pesticide formulations on urease activity in flooded soil.

	Urease activity (μ moles ammonia evolved/g soil/h)								
	Time (days)								
Pesticide	1	2	7	9	14	16	23	29	36
65% WHC control	24.2	26.8	23.2	24.2	25.1	25.8	25.5	24.6	27.6
Flooded control	26.1	27.4	23.9	25.3	25.3	25.6	29.3	26.0	29.0
2,4-D	27.2	28.8	25.2	26.0	26.7	27.0	29.1	29.7*	29.7
Diallate	27.8	28.9	26.8	27.2	29.1*	27.4	31.8	28.0	31.0
Glyphosate	28.0	28.5	25.9	26.6	28.3	27.5	30.4	28.5	29.3
Benzoylprop ethyl	27.8	29.2	25.4	26.1	26.8	27.2	29.3	27.8	29.9
Malathion	28.3	29.7	27.4	25.8	27.1	26.6	29.0	28.4	29.8

* = significantly different (P = 0.05) from the corresponding control value.

reduction compared to field moisture soil, whereas Delaune and Patrick (1970) could detect no effect. These dissimilar responses presumably reflect a difference in the contribution of microbial metabolism and accumulated urease activity to urea turnover in the two soils. The effect of water holding capacity on the urease assay has been investigated by Pel'tser (1972), who observed a bell shaped response - activity decreasing above 75% WHC, and Zantua and Bremner (1978) who in contrast recorded no effect. Pel'tser's (1972) result strongly suggests that his long term assay (100 h), carried out in the absence of an inhibitor, was measuring microbial degradation of urea besides accumulated urease activity.

Ventura and Yoshida (1977) observed that ammonia volatilisation from added urea was 3.6 times greater in flooded soil than in field-wetness soil. They attributed this to the increase in soil pH from 6.6 to 7.1 after flooding. In direct contrast however, Delaune and Patrick (1970) found that volatilisation was reduced in flooded soil compared to that at $\frac{1}{3}$ bar moisture and concluded that the sheer bulk of flood water had slowed down ammonia volatilisation because of its high solubility.

Since the urease assay employed in this project did not depend on passive ammonia volatilisation but upon its displacement from the soil colloids by potassium ions, it is unlikely that volatilisation rate would have interfered with urease determinations.

In addition, the assay itself was performed under flooded conditions.

b) Amended Soil

Very few agricultural soils are treated solely with pesticides, they are also amended with fertilisers, lime and manures and in addition there are discontinuous inputs of organic matter from plant debris and root exudates. This section reports the findings from soil treated with some of these amendments alone and in paired combinations with pesticide formulations.

1) NPK Fertiliser

Soil amended with an artificially prepared NPK fertiliser at the rate 118 ppm on an N, P and K basis showed no consistent, significant changes in glucanase (Table 39) or urease (Table 40) activities. The source of the N, P and K were Nitram (ammonium nitrate), muriate of potash (potassium chloride) and superphosphate (mono- di- and tricalcium phosphates).

Zantua and Bremner (1978) have also noted that urease failed to respond to these same chemicals when applied individually to soil at 500 ppm on an N, P or K basis. Vasilenko (1962) however reported that 5000 ppm K as potassium chloride elevated urease levels by 38% after 36 days incubation. From then onwards the activity of the amended soil decreased and by day 137 was the same as the control. Although the

Table 39. Effect of five pesticide formulations on glucanase activity in soil amended with NPK fertiliser.

	Glucanase activity (μ moles reducing sugars[a]/g soil/h)						
	Time (days)						
Pesticide	0	7	14	28	42	56	70
Unamended control	0.29	0.33	0.39	0.43	0.44	0.46	0.41
NPK control	0.31	0.34	0.42	0.44	0.45	0.40*	0.41
2,4-D	0.29	0.32	0.38	0.46	0.43	0.44*	0.42
Diallate	0.30	0.31	0.40	0.45	0.43	0.43	0.39
Glyphosate	0.30	0.33	0.35*	0.44	0.47	0.43	0.40
Benzoylprop ethyl	0.28	0.31	0.39	0.44	0.43	0.40	0.40
Malathion	0.32	0.33	0.40	0.45	0.45	0.42	0.41

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

Table 40. Effect of five pesticide formulations on urease activity in soil amended with NPK fertiliser.

	Urease activity (μ moles ammonia evolved/g soil/h)						
	Time (days)						
Pesticide	0	7	14	28	42	56	70
Unamended control	25.2	27.3	28.7	28.9	28.8	31.6	28.0
NPK control	24.2	27.9	29.6	30.2	28.0	28.7	30.0
2,4-D	24.8	27.3	30.0	30.9	31.0	30.4	28.9
Diallate	24.5	28.3	29.8	30.0	30.9	28.8	29.9
Glyphosate	25.1	27.4	30.4	30.8	31.1	31.2	28.4
Benzoylprop ethyl	25.1	25.6	28.3	28.3	31.6	30.5	30.8
Malathion	26.1	27.1	28.6	31.0	31.1	28.7	31.1

majority of the reports suggest that urease is unaffected by inorganic N, P, K or S carriers, Vasilenko (1962) is not alone in his claim to have stimulated soil urease activity by such additions (Conrad, 1942a; Bhavanandan and Fernando, 1970). The conclusions of Bhavanandan and Fernando (1970) should be viewed with caution because the variation in urease activity in their field experiment was very large and they do not quote any control values. It is difficult to understand why these mineral additions should stimulate urease activity in the absence of a metabolisable carbon source, unless such substrates are present in the soil but cannot be used for reason of N, P, K or S limitation. Alternatively, the ions involved might be specific activators of the enzyme. No inhibitory effects of inorganic N, P, K or S carriers on urease have been reported.

No consistent, significant effects on either enzyme were detected when the NPK amended soil was treated with the pesticide formulations (Tables 39 and 40).

2) Urea

Addition of 47 ppm urea nitrogen (100 ppm urea) had no significant effect on glucanase (Table 41) or urease (Table 42) levels in soil, nor did it induce either enzyme to respond to additions of the pesticide formulations. In the context of urease this experiment has yielded valuable information on long-term soil

Table 41. Effect of five pesticide formulations on glucanase activity in soil amended with urea.

Pesticide	Glucanase activity (μ moles reducing sugars[a]/g soil/h) Time (days) 0	3	9	15	30	50	70
Unamended control	0.37	0.36	0.40	0.41	0.43	0.35	0.37
Urea control	0.38	0.36	0.42	0.40	0.46	0.35	0.39
2,4-D	0.38	0.36	0.42	0.43	0.46	0.36	0.37
Diallate	0.34*	0.37	0.39	0.42	0.44	0.37	0.39
Glyphosate	0.39	0.36	0.42	0.41	0.43	0.37	0.38
Benzoylprop ethyl	0.37	0.40	0.43	0.43	0.43	0.36	0.37
Malathion	0.40	0.37	0.41	0.42	0.45	0.36	0.42

a = as glucose equivalents.

* = significantly different (P = 0. 05) from the corresponding control value.

Table 42. Effect of five pesticide formulations on urease activity in soil amended with urea.

Pesticide	Urease activity (μ moles ammonia evolved/g soil/h) Time (days) 0	1	3	6	8	9	13	15	30	50	70
Unamended control	29.7	29.0	28.4	26.5	25.9	30.3	20.4	29.8	26.1	27.4	33.1
Urea control	28.5	28.3	28.4	25.3	24.7	29.4	22.3	30.5	26.0	25.6	32.6
2,4-D	28.6	ND	29.7	ND	ND	29.6	ND	29.9	26.7	28.6	32.9
Diallate	30.7	ND	29.5	ND	ND	30.4	ND	30.1	26.5	27.1	34.1
Glyphosate	32.1	ND	29.2	ND	ND	31.7	ND	28.7	24.9	27.8	32.2
Benzoylprop ethyl	27.9	ND	29.1	ND	ND	30.4	ND	30.4	24.7	26.8	32.8
Malathion	30.0	ND	29.7	ND	ND	27.6	ND	28.8	27.3	27.1	32.0

ND = no determination.

enzyme-substrate interactions. That urease levels were not increased by the addition of urea suggests that all the substrate was rapidly broken down by the extracellular enzyme before the existing ureolytic microbial population was induced to increase and/or synthesise more enzyme. Under the urease assay conditions the enzyme in 1 g soil converts about 12.5 μ moles of urea to ammonia every hour. In this experiment each 1 g of soil received 1.67 μ moles of urea and whereas the enzyme will not be so active in the 65% WHC incubation (substrate limitation, unbuffered system, a considerable way from optimum pH) these figures show that the hypothesis put forward is not an unreasonable one.

Urea disappearance from soil was not monitored directly, but the amount of ammonia evolved from the enzyme assay soil control indicated that all of the urea had been converted to ammonia within 24 h of being added (Table 57). Indeed 87% was hydrolysed in the time (about 2 h) between adding the urea and stopping the day zero assay.

Even much higher doses of urea (233 ppm N and 467 ppm N) which persisted in the soil for a slightly longer period (2 to 3 days), did not increase urease activity (Table 43).

TABLE 43 Effect of urea on soil urease activity

Time (days)	Urease activity[a]		
	Urea N concentration (ppm)		
	0	233	467
0	23.7	25.0	24.4
3	20.7	20.6	20.8
7	24.8	23.5	25.4
12	27.8	25.8	28.7

a = μ moles ammonia evolved/g soil/h

These results confirm previous investigations by Conrad (1942a), Tanabe and Ishizawa (1969), Lloyd and Sheaffe (1973) and Zantua and Bremner (1976). There are however three reports of urease stimulation by urea (Paulson and Kurtz, 1969b; Bhavanandan and Fernando, 1970; Namdeo and Dube, 1973a, b, c) and one of varying response with time (Vasilenko, 1962). Paulson and Kurtz based their conclusion, that urea stimulates urease activity in soil, on the finding that the activity was higher in soil treated with urea and glucose than soil treated with glucose and ammonium sulphate. Unfortunately they did not perform the correct controls of incubating soil with urea, ammonium sulphate and glucose individually and hence their work does not permit any valid conclusions to be made concerning the effect of urea on soil urease levels.

Previous criticisms of Bhavanandan and Fernando (1970) also apply here. In a field experiment (the results of which have been published on no less than three occasions!) Namdeo and Dube (1973a, b, c) observed that urease activity increased when urea was applied to a grassland sward at a concentration of 200 kg/ha urea N, but that it was unaffected by half the dose. When added in combination with the herbicides dalapon and paraquat, which themselves stimulated activity, the resulting increase in urease levels was less than that predicted and they concluded that some form of 'biochemical antagonism' (sic) had taken place between the urea and these pesticides, but it was impossible to tell which one had reduced the stimulating capacity of the other. In the opinion of this author, Vasilenko (1962) placed too much emphasis on the fluctuating urease levels in his long-term 'semi-field' experiments (air-dried soil treated in the laboratory was returned to the field for incubation) and it would be safer to reinterpret such results in the manner of Lloyd and Sheaffe (1973) as being indicative of no effect.

Although they did not measure urease activity, Roberge and Knowles (1967) showed that the number of ureolytic microorganisms in a black spruce humus increased following application of 3500 ppm urea N and this change was paralleled by the total count so that the percentage of ureolytic microorganisms remained constant. This non-specific stimulation of the microflora was complete after 3 days, by which time urea

hydrolysis was complete.

3) Pig Slurry

After a short lag period of less than 4 days, soil amended with 4000 ppm freeze-dried pig slurry possessed 20 to 30% more glucanase activity than the unamended control (**Table** 44) and these elevated enzyme levels persisted for the duration of the experiment (70 days). None of the pesticide formulations had any consistent, significant effect on this stimulation.

This increase in glucanase activity may have been due to proliferation of the zymogenous glucanase producing microflora of the soil, using the pig slurry as a carbon and nitrogen source, or colonisation of the soil by allochthonous glucanase producing micro-organisms which were already present in the freeze-dried pig slurry. The latter explanation is considered unlikely when the microbial counts of the pig slurry are examined (see Methods and Materials, Table 25). These indicate that the approximate numbers of glucanase producing bacteria and fungi per gram of freeze-dried powder were 1400 and 24 respectively. Considering that the rate of application was 4 mg/g dry soil, this means that on average each 10 g soil received 56 bacteria and one fungal propagule capable of degrading laminarin. It is doubtful whether such a small number of micro-organisms could cause such a consistent effect as the one observed.

In an attempt to understand this interaction in

Table 44. Effect of five pesticide formulations on glucanase activity in soil amended with pig slurry.

Pesticide	Glucanase activity (μ moles reducing sugar[a]/g soil/h) Time (days) 0	4	7	14	28	42	56	70
Unamended control	0.28	0.31	0.34	0.35	0.42	0.45	0.40	0.43
Pig slurry control	0.29	0.39*	0.44*	0.44*	0.53*	0.54*	0.52*	0.56*
2,4-D	0.29	0.38	0.47	0.46	0.52	0.55	0.56	0.53
Diallate	0.31	0.39	0.43	0.50	0.52	0.53	0.54	0.52
Glyphosate	0.31	0.41	0.46	0.44	0.53	0.56	0.58	0.55
Benzoylprop ethyl	0.31	0.41	0.44	0.49*	0.55	0.52	0.50	0.49*
Malathion	0.31	0.37	0.45	0.50	0.56	0.54	0.58	0.55

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

greater detail the pig slurry was subjected to a number of treatments and fractionations before being added to the soil in the presence and absence of 1000 ppm (soil basis) of the microbial inhibitor sodium azide (Table 45). Glucanase assays were performed after 14 days. That no increase in glucanase activity was observed in the presence of sodium azide is strongly indicative of microbial proliferation being the cause of the enhanced glucanase levels. Both the aqueous extracts of the freeze-dried powder and the untreated pig slurry supernatant stimulated activity, but not to the same extent as the freeze-dried powder, suggesting that both soluble and insoluble components of the pig slurry were being metabolised by the glucanase producing microflora. Concrete proof that the increased glucanase levels were not due to proliferation within the soil of glucanase producing microorganisms present in the pig slurry, came from the fact that the activity was still enhanced in the soil treated with sterile, freeze-dried powder (autoclaved) or supernatant (autoclaved and bacteriologically filtered). Indeed, autoclaving the freeze-dried pig slurry led to an even greater enhancement of activity, and since there was no such difference between autoclaved and non-autoclaved slurry supernatant it appears that autoclaving made some of the insoluble material more susceptible to microbial utilisation - perhaps by destroying its structure and increasing its degree of solublisation. That autoclaved samples never caused less stimulation

Table 45. Effect of pig slurry on glucanase activity in soil.

Type of pig slurry	Glucanase[1] activity
Unamended soil	0.36
Freeze-dried powder	0.51*
Freeze-dried powder + sodium azide	0.35
Autoclaved freeze-dried powder	0.59*
Water extract of freeze-dried powder	0.46*
Untreated supernatant	0.42*
Untreated supernatant + sodium azide	0.34
Autoclaved supernatant	0.42*
Bacteriologically filtered supernatant	0.43*

1 = μ moles reducing sugars as glucose equivalents/g soil/h.

* = significantly different (P = 0.05) from the unamended control value.

of activity than their corresponding non-autoclaved samples, is indicative that no inhibitory compounds were released or formed during autoclaving.

Observations with glucose and cellulose additions (see Results and Discussion, B.1. b.5,6) suggest that these enhanced glucanase levels will eventually return to those of the non-amended control once all the relevant substrates, which are stimulating the glucanase producing microflora, have been consumed.

The possibility of controlling plant pathogenic fungi by adding materials (laminarin, chitin, Laminaria fronds and ground lobster shells) to soil which stimulate glucanase and chitinase producing microflora has been discussed in the introduction (Mitchell and Alexander, 1962; Mitchell, 1963). The response of soil glucanase to pig slurry applied at a rate similar to that recommended in agriculture suggests that pig slurry (and perhaps other animal slurries or manures) might be a useful tool in this context. Obviously many more pig slurry samples and other manures or slurries will have to be investigated, using the original material (as opposed to a freeze-dried preparation), in a field situation, for direct effects on the plant pathogenic fungi, to determine the feasability of this hypothesis. Animal slurries and manures have the advantage of being cheaper and more readily available than the materials used by Mitchell and Alexander (1962) and Mitchell (1963).

In the case of urease, pig slurry did not enhance activity. On the contrary, from day 42 onwards the urease levels in the amended soil were significantly less than those in the control soil (Table 46). No consistent difference in the pH of amended and unamended soil was detected at any stage of the incubation, suggesting that this decrease in activity which ranged from 10 to 21% was not due to a pH shift (at least on the macroscale) resulting from microbial degradation of the pig slurry. The pH of the freeze-dried powder resuspended in the ratio 8 mg powder to 1 ml water (the same as occurs in the soil incubations) was 7.0. (The pH of the original undiluted slurry was 7.5). Balasubramanian, Siddaramappa and Rangaswami (1972) reported that 3641 ppm unspecified farmyard manure similarly had no effect on soil pH over the 60 day incubation period.

This slow decrease in urease levels, following a long lag period, suggests that an inhibitory compound was being released or produced from pig slurry as a result of its microbial transformation. It is unlikely that the freeze-drying process produced this inhibitor, since if this had been the case inhibition at a much earlier stage in the experiment would have been expected. The freeze-dried pig slurry was shown to have no inherent effect on jack bean or Bacillus pasteurii urease.

There have been several investigations into the effects of manures and composts on soil urease activity

Table 46. Effect of five pesticide formulations on urease activity in soil amended with pig slurry.

	Urease activity (μ moles ammonia evolved/g soil/h)										
	Time (days)										
Pesticide	0	1	3	7	8	13	14	28	42	56	70
Unamended control	22.8	29.0	28.4	23.3	25.9	20.4	24.1	26.8	24.5	26.1	25.7
Pig slurry control	23.3	24.5*	27.2	20.8	23.2	18.3	22.5	24.7	22.1*	20.5*	21.6*
2,4-D	21.4	ND	ND	22.2	ND	ND	23.8	24.9	21.0	21.2	23.2
Diallate	22.6	ND	ND	22.5	ND	ND	21.3	22.9	22.2	22.9	21.4
Glyphosate	23.4	ND	ND	23.0	ND	ND	22.8	25.5	22.2	22.7	21.3
Benzoylprop ethyl	22.3	ND	ND	22.1	ND	ND	21.9	23.3	23.5	21.6	22.0
Malathion	22.5	ND	ND	21.1	ND	ND	21.3	25.0	22.5	21.8	21.9

ND = no determination.

* = significantly different (P = 0.05) from the corresponding control value.

(see Introduction, Table 10) but none have substantiated the findings reported here. All but one of the reports concerned enhancement of activity for varying periods of time, followed, in some cases (Balasubramanian et al., 1972; Zantua and Bremner, 1976, 1978), by a return to the original enzyme level. Khan (1970) on the other hand reported that an unspecified manure had no effect on urease activity in the field.

Addition of the pesticide formulations to the pig slurry treated soil resulted in no detectable change in response of the enzyme to the organic amendment (Table 46).

4) Lime

Treatment of soil with 5400 ppm ground limestone resulted in a slight (7 to 18%) but consistent and significant reduction in glucanase activity which, after manifesting itself at day 7, remained for the duration of the experiment (Table 47). The purpose of the liming treatment was to elevate the soil pH from 5.4 to 6.5; in fact it raised it to about pH 6.7 (Figure 19). The lime exerted its effect rapidly so that there was no further pH increase after day 7. Glucanase inhibition began once the soil had stabilised at pH 6.7. The reduction in activity was not simply due to a change in the gross pH of the assay mixture, since the buffer was able to counteract the increased pH in the treated soil so that the assays were still

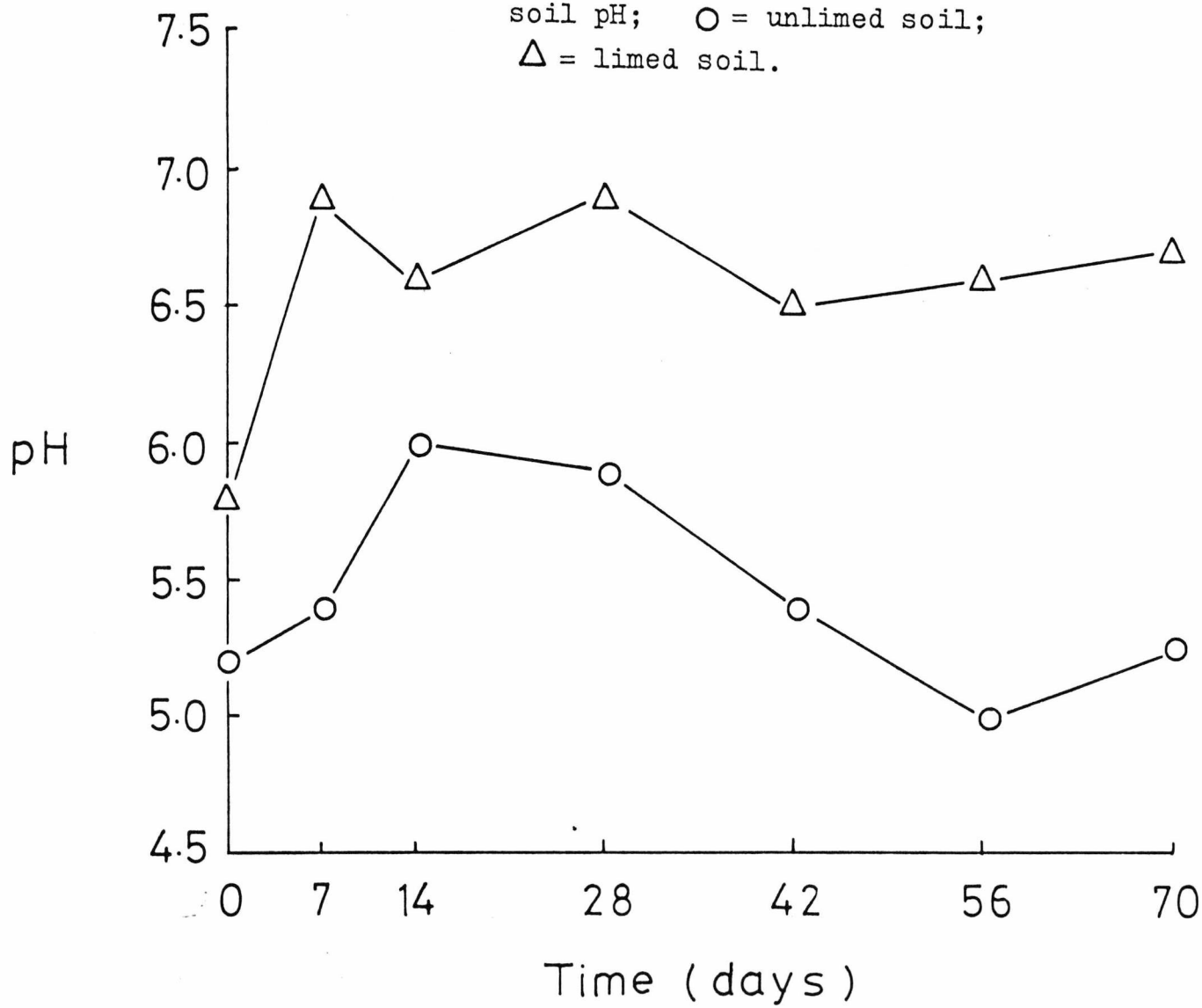
Table 47. Effect of five pesticide formulations on glucanase activity in limed soil.

Pesticide	Glucanase activity (μ moles reducing sugars[a]/g soil/h) Time (days) 0	7	14	28	42	56	70
Unamended control	0.30	0.34	0.45	0.40	0.45	0.42	0.44
Limed control	0.28	0.30*	0.37*	0.37*	0.41*	0.37*	0.38*
2,4-D	0.28	0.32	0.40	0.34	0.41	0.38	0.40
Diallate	0.30	0.30	0.39	0.37	0.39	0.37	0.37
Glyphosate	0.31	0.32	0.41	0.37	0.40	0.38	0.39
Benzoylprop ethyl	0.27	0.31	0.39	0.35	0.45*	0.36	0.40
Malathion	0.30	0.31	0.40	0.36	0.43	0.35	0.38

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

FIGURE 19 Effect of 5400 ppm ground limestone on soil pH; ○ = unlimed soil; △ = limed soil.



being performed at pH 5.4. However pH effects at the microenvironment level can not be ruled out. One possible explanation is that the increased soil pH reduced the glucanase producing fungal population of the soil directly (the fungal counts had to be performed on Bunt and Rovira's agar at pH 5.5, since very few grew at pH 7, that used in bacterial counts), or indirectly by stimulating a mycolytic bacterial population which grew better at pH 6.7 than 5.4. An alternative explanation is that the glucanase was inhibited by calcium ions although they appear to have no effect on the microbial enzyme (Nagasaki et al., 1976, 1977; Miyazaki et al. 1977).

One other interesting point to emerge from Figure 19 is that the pH of the unamended soil increased initially from pH 5.2 to 6.0 during the first 2 weeks, but there after slowly returned to the starting value. This suggests that alkaline products are produced on re-wetting air-dried soil. One such product is known to be ammonia (see Results and Discussion, B.3.b.2) and the slow decline in pH after day 14 may represent its conversion to nitrous and nitric acids in the nitrification process.

The effects of some pesticides, particularly the ionic ones, on microorganisms are known to be pH dependent. Zetterberg, Busk, Elovson, Starec-Nordenhammer and Ryttman (1977) have shown that Saccharomyces cerevisiae and Salmonella typhimurium were more susceptible to 2,4-D at low pH. The lower the pH the

more of the 2,4-D is in its undissociated form and they proposed that the microbial cells could only take up the undissociated 2,4-D. Despite the pH change of the soil, no response of glucanase to any of the pesticide formulations was detected.

Urease was unaffected by the liming treatment alone or in combination with the pesticide formulations (Table 48). Other workers have reported that urease was inhibited, (Volk, 1966; Pel'tser, 1972) stimulated (Verstraete and Voets, 1977) and unaffected (Zantua and Bremner, 1978) by calcium carbonate. The findings of Verstraete and Voets (1977) cannot be compared directly to the rest because they used sugar lime and it is likely that the stimulation of activity observed was due to the sugars in this mixture rather than the limestone. Zantua and Bremner's (1978) exercise seems to have been rather futile since they worked with a soil whose pH was 6.7 even before liming.

Since all the observed effects were made in unbuffered systems it is likely that they were simply due to a pH change in the assay. With this in mind the effect of ground limestone on glucanase and urease activity after 12 days was investigated, using buffered and unbuffered assays (Table 49). Lime had a greater effect on glucanase when an unbuffered assay was used because the assay pH was increased from 5.4 to 6.7 (see glucanase pH-activity profile in Results and Discussion A.1.a). The urease activities of both limed and unlimed soils in buffered or unbuffered

Table 48. Effect of five pesticide formulations on urease activity in limed soil.

Pesticide	Urease activity (μ moles ammonia evolved/g soil/h) Time (days) 0	7	14	28	42	56	70
Unamended control	23.1	24.2	25.2	27.0	26.9	27.2	27.4
Limed control	24.5	25.2	28.7*	27.1	28.3	26.7	25.7
2,4-D	25.0	24.0	27.5	28.1	29.1	26.8	27.5
Diallate	25.2	24.6	27.0	27.0	29.7	27.7	28.9
Glyphosate	25.7	26.1	27.8	28.6	28.9	31.0*	29.3
Benzoylprop ethyl	25.0	25.4	27.2	29.3	29.3	28.9	30.4*
Malathion	26.1	24.4	26.8	28.0	29.3	26.4	27.5

* = significantly different (P = 0.05) from the corresponding control value.

Table 49. Effect of lime on glucanase and urease activities in soil using buffered and unbuffered assays.

	Glucanase				Urease			
	Buffered		Unbuffered		Buffered		Unbuffered	
	Activity[1]	Final assay pH	Activity[1]	Final assay pH	Activity[2]	Final assay pH	Activity[2]	Final assay pH
Unamended soil	0.44	5.4	0.48	5.9	28.0	7.0	26.0	5.8
Limed soil	0.37*	5.4	0.31*	6.7	27.8	7.0	28.9	6.7

1 = μ moles reducing sugars as glucose equivalents/g soil/h.

2 = μ moles ammonia evolved/g soil/h.

* = significantly different (P = 0.05) from the unamended control value.

assays were all very similar and these results can be explained on the basis of pH. The limestone increased the soil pH from 5.4 to 6.7 which is close to the optimum pH for urease activity (pH 7). However in the unlimed soil the pH of the unbuffered assay mixture was 5.8 compared to 7.0 for the buffered assay, but Pettit et al. (1976) have shown that urease activity varies little over the pH range 5 to 8.

Shortly after this experiment had been performed the field where the soil had been collected was treated with quick lime (calcium oxide) at a rate of 2 tons/acre (≡ 2240 ppm) which is approximately equivalent to 4480 ppm ground limestone on the basis of neutralising value. Thus the field treatment was similar to that used in the laboratory (5400 ppm). Comparison of glucanase activity in pre- and post-limed soil (4 weeks) from the field revealed no significant reduction in glucanase activity.

It is interesting to note that wherever comparisons between calcium carbonate and the other liming materials, calcium hydroxide and calcium oxide, which have a greater neutralising value than limestone, have been carried out (Pel'tser, 1972; Zantua and Bremner, 1978) the latter have been more effective at altering soil urease levels. Despite the fact that these liming materials were applied in approximately the correct ratios based on their neutralising values (i.e. twice as much calcium carbonate as calcium hydroxide and calcium oxide) the hydroxide and

oxide were much more effective at changing soil pH). Since those comparisons were based on unbuffered assays it is likely that such changes were due in part at least to the effect of pH on enzyme activity per se. The only way to determine other liming effects, as in the case of glucanase, is to employ buffers to return the limed soil to its original pH. In that way only one variable is being investigated at one time.

5) Glucose

Within 24 h of its application, 700 ppm glucose had enhanced both the glucanase (Table 50) and urease (Table 51) activities of the soil by 18% and 23% respectively. In the case of glucanase the increase was only transient and by day 8 there was no difference between the activities of the amended and unamended soil. The elevated urease levels however persisted a little longer and it was not until day 35 that the activity of the amended soil had returned to that of the control.

The soil control in the glucanase assay showed that all the added glucose had disappeared within the first 24 h (Table 53). Since glucanase, urease (Table 52) and glucose (Table 53) levels all remained constant in glucose-amended soil pretreated with sodium azide (1000 ppm), it is reasonable to propose that enhanced activities in the absence of azide have resulted from stimulation of the glucanase producing

Table 50. Effect of five pesticide formulations on glucanase activity in soil amended with glucose.

Pesticide	Glucanase activity (μ moles reducing sugars[a]/g soil/h) Time (days) 0	1	2	4	8	14
Unamended control	0.34	0.33	0.35	0.33	0.45	0.41
Glucose control	0.33	0.39*	0.39*	0.38*	0.43	0.43
2,4-D	0.34	0.42	0.40	0.39	0.43	0.44
Diallate	0.33	0.40	0.37	0.42	0.45	0.42
Glyphosate	0.35	0.41	0.40	0.40	0.40	0.44
Benzoylprop ethyl	0.34	0.41	0.39	0.40	0.42	0.43
Malathion	0.33	0.42	0.42	0.38	0.45	0.42

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

Table 51. Effect of five pesticide formulations on urease activity in soil amended with glucose.

	Urease activity (μ moles ammonia evolved/g soil/h)								
	Time (days)								
Pesticide	0	1	2	4	8	14	23	35	44
Unamended control	28.1	28.4	26.2	25.9	22.2	25.3	29.5	24.7	26.9
Glucose control	27.7	35.0*	29.6*	30.3*	27.7*	29.1*	35.4*	22.7	26.8
2,4-D	28.7	33.0	30.1	29.7	27.5	29.3	36.2	25.0	25.4
Diallate	28.4	33.6	31.9	29.1	28.9	28.0	36.4	25.4	27.1
Glyphosate	29.0	32.5	30.6	32.0	28.1	30.4	35.5	23.6	27.0
Benzoylprop ethyl	29.3	37.5	29.6	30.4	26.7	30.4	35.0	26.2	26.9
Malathion	28.4	36.3	29.8	30.6	26.0	30.6	34.3	24.7	26.3

* = significantly different (P = 0.05) from the corresponding control value.

and ureolytic microflora. This also explains why there was no further increase in the enzyme levels once the glucose had disappeared. The fact that in both cases all the newly synthesised enzyme was eventually denatured suggests that either the soil's capacity for protection of enzyme was saturated or that the conditions in the soil were not conducive to stabilisation.

It has been suggested that accumulated soil enzymes are in a continual state of turnover, even though this may be a slow process for the more protected fractions (Burns, 1977). If this is the case then the steady state levels in long-term incubations of untreated moist soil must be maintained by replenishment of the accumulated fraction that is inactivated, otherwise activity would slowly decline with time. The question of where this replenishing enzyme comes from will be discussed later on in this section in terms of pesticide disruption. A direct consequence of all this is that if unamended moist soil has the capacity to stabilise enzyme entering the accumulated pool, then the newly synthesised enzyme in glucose amended soil does not fail to become stabilised because of incorrect conditions, but rather that the soil's capacity for enzyme protection is saturated. If the constant state of enzyme turnover hypothesis is incorrect and the steady state levels are instead due entirely to the long-term stability of accumulated soil enzymes, in the same way as the humic matter which protects them,

then the above argument does not hold and it is impossible to deduce why newly synthesised enzyme is not stabilised. However it is difficult to conceive that none of the accumulated enzyme would lose its catalytic ability over long periods of time in such a harsh environment as the soil.

Regardless of the mechanisms involved in stabilisation the difference in persistence of the newly synthesised glucanase and urease fractions may be explained in terms of their original spatial relationship to their parent cells. Glucanase is an extracellular enzyme, so it follows that any new enzyme will be free in the soil solution and very prone to proteolytic attack and physico-chemical denaturation. Consequently when microbial growth and enzyme synthesis stop , as a result of the glucose being exhausted, the newly synthesised enzyme is rapidly destroyed and activity returns to its original steady state level. The intracellular location of urease, on the other hand, may afford some short-term protection and despite the cessation of microbial growth activity may remain elevated for a short period.

There is total agreement in the literature that glucose stimulates soil urease activity (see Introduction, Table 10). Of the three investigations involving time studies, two reported that elevated enzyme levels returned (or were in the process of doing so) to the value of the unamended soil (Laugeson, 1972; Zantua and Bremner, 1976, 1978). In his semi-field

Table 52. Effect of glucose on glucanase and urease activities in soil.

Glucose (ppm)	Glucanase activity[a]		Urease activity[b]	
	Day 1	Day 2	Day 1	Day 2
Air-dried soil				
0	0.31	0.28	24.0	26.6
700	0.37*	0.35*	28.3*	30.6*
700 + NaN_3	0.30	0.29	23.0	27.4
2500	0.51*	0.42*	31.8*	35.0*
2500 + N	ND	0.52*	ND	44.6*
5000	0.49*	0.41*	32.5*	37.7*
5000 + N	ND	0.56*	ND	42.0*
Field-wetness soil				
0	0.76	0.84	15.7	ND
700	0.75	0.80	18.7*	ND

a = μ moles reducing sugars as glucose equivalents/g soil/h.

b = μ moles ammonia evolved/g soil/h.

ND = no determination.

* = significantly different (P = 0.05) from the unamended control value.

experiment Vasilenko (1962) observed that the three fold enhancement of urease activity following the addition of 50,000 ppm glucose persisted for at least 5½ months.

Addition of the pesticide formulations to the glucose amended soil had no significant effect on the response of the two enzymes to the carbon source.

The short-term effect of larger glucose concentrations (2500 ppm and 5000 ppm) on the activity of these two enzymes (Table 52) in the presence and absence of available nitrogen (118 ppm N as ammonium nitrate in Nitram) was monitored, along with glucose disappearance (Table 53), over a period of two days. Strictly speaking extrapolations between day one and two determinations should be made with caution as they were not the same experiment, i.e. they were not started on the same day.

TABLE 53 The disappearance of glucose from soil

Glucose concentration (ppm w/w)		
Added	Remaining	
	Day 1	Day 2
Air-dry soil		
700	0	0
700 + NaN_3	718	728
2500	1302	1020
2500 + N	ND	120
5000	3558	3630
5000 + N	ND	2160
Field-wetness soil		
700	0	0

ND = no determination

As long as the soil was not nitrogen limited, i.e. that there was sufficient nitrogen present for glucose utilisation to take place, then the activity of both enzymes increased with increasing glucose concentration. Thus 5000 ppm glucose on its own caused very little or no more stimulation than did 2500 ppm glucose on its own but when 118 ppm N was added along with the 5000 ppm glucose then there was a further increase in activity as more glucose was utilised. The results suggest that in the absence of an exogenous nitrogen source 1 g dry soil could 'dispose' of about 1.4 mg glucose before nitrogen limitation set in. Similar observations have been made by Tanabe and Ishizawa (1969), Musa and Mukhtar (1969) and Laugeson (1972). In contrast Zantua and Bremner (1976, 1978) have reported that combinations of 2000 ppm glucose and 50 ppm nitrogen (ammonium sulphate or carbonate) brought about much smaller increases in urease activity than did 2000 ppm glucose on its own. The nitrogen sources themselves had no effect on urease activity. Not surprisingly they made no attempt to explain this curious result.

In field-wetness soil 700 ppm glucose enhanced urease levels by approximately the same amount (19%) as it had done on air-dried soil but had no effect on the glucanase levels (Table 52). This suggests that when the soil was air-dried and the number of micro-organisms reduced (see Methods and Materials, Table 18), the ratio of ureolytic to non-ureolytic organisms remained constant, whilst the ratio of glucanase producers to non producers was increased, i.e. the glucanase

producing microorganisms tended to survive the air-drying better than the others. Thus in wet soil the glucanase producers were swamped by the non-producers and could not compete very efficiently for the glucose. In dry soil where they constituted a larger percentage of the total population they were able to use some of the glucose before it was consumed by the non-glucanase producers. It is unlikely that the lack of response by glucanase was due to the catabolic repression of the enzyme by glucose observed by Lilley and Bull (1974) with their thermophilic streptomycete and Santos et al. (1978) with Penicillium italicum, since if this was the case a similar result would have been expected in air-dried soil. In any case the glucose disappeared so rapidly that it is doubtful whether any catabolite repression would manifest itself in the long-term at the macro-level.

6) Cellulose

After a 4 day lag period, soil amended with 700 ppm cellulose possessed more glucanase activity than unamended soil, a difference maintained up to day 56, but by day 70 the level had returned to that of the control (Table 54). The stimulation of activity was only slight (8 to 17%) but was consistent and reproducible. Since glucose itself is known to stimulate glucanase activity it is reasonable to propose that this enhanced activity resulted from an increase in the glucanase producing microflora using the glucose

Table 54. Effect of five pesticide formulations on glucanase activity in soil amended with cellulose.

Pesticide	Glucanase activity (μ moles reducing sugars[a]/g soil/h) Time (days) 0	4	7	14	28	42	56	70
Unamended control	0.27	0.34	0.30	0.43	0.48	0.40	0.44	0.38
Cellulose control	0.28	0.33	0.35*	0.47*	0.52*	0.46*	0.51*	0.37
2,4-D	0.27	0.32	0.34	0.48	0.52	0.46	0.48	0.36
Diallate	0.27	0.34	0.33	0.48	0.52	0.47	0.49	0.40
Glyphosate	0.28	0.33	0.35	0.47	0.52	0.46	0.53	0.40
Benzoylprop ethyl	0.28	0.33	0.34	0.44	0.52	0.46	0.49	0.39
Malathion	0.28	0.32	0.35	0.46	0.54	0.45	0.50	0.40

a = as glucose equivalents.

* = significantly different (P = 0.05) from the corresponding control value.

released from cellulose. This hypothesis could be tested using sodium azide treated soil but the experiment was not performed. This long-term stimulation may reflect the slow release of glucose from cellulose by extracellular cellulases, both associated and unassociated with microbial proliferation, resulting in its complete disappearance by day 70. Glucose released from cellulose and residual cellulose were not detected in the soil control of the assay at any stage, presumably because the former is rapidly metabolised by the soil microflora (see Results and Discussion, B.1.b.5) and never has a chance to accumulate and that the latter is insoluble.

Despite the fact that the cellulose and glucose additions were equivalent (700 ppm), the enhanced glucanase levels persisted much longer in the cellulose than glucose amended soil and this might indicate that glucanase producing microorganisms can compete more effectively for the glucose under such conditions than when it is added all at once, thus helping them to maintain their increased population for a longer period of time.

The presence of the pesticide formulations did not interfere with the enhancement of glucanase activity by cellulose.

Urease activity was unaffected by cellulose in the absence or presence of the pesticide formulations (Table 55) but Zantua and Bremner (1978) found that it responded to 5000 ppm cellulose, in the absence of an

Table 55. Effect of five pesticide formulations on urease activity in soil amended with cellulose.

Pesticide	Urease activity (μ moles ammonia evolved/g soil/h) Time (days) 0	7	14	28	42	56	70
Unamended control	24.7	24.4	25.7	27.0	27.1	25.7	26.6
Cellulose control	24.5	25.0	25.9	26.3	27.0	25.5	25.7
2,4-D	24.2	26.2	26.8	27.7	27.5	27.8	25.7
Diallate	24.5	26.8	25.5	28.0	28.7	27.3	28.5
Glyphosate	24.2	24.9	26.7	28.7	30.2	27.2	26.6
Benzoylprop ethyl	24.7	25.3	26.4	28.4	27.5	27.8	26.8
Malathion	24.2	27.0	26.8	27.7	26.6	26.8	26.4

exogenous nitrogen source, in a similar yet more dramatic fashion to the one described for glucanase above. Five days after the application of cellulose, the urease levels had increased three-fold, but from this point onwards activity slowly decreased and by day 60 had returned to that of the control value.

Had time permitted it would have been interesting to investigate a range of cellulose concentrations in the presence and absence of a nitrogen source to see whether varying the C : N ratio alters the response of these two enzymes, since cellulose turnover in soil is reported to be very dependent on the C : N ratio (Imshenetsky, 1968).

2. Disappearance and Volatilisation of Ammonia From Soil

None of the pesticide formulations had any consistent, significant effect on the turnover of ammonia added to soil as NPK fertiliser (ammonium nitrate) (Table 56) and pig slurry (Table 58) or the ammonia released upon urea hydrolysis (Table 57). The ammonia was estimated in the urease assay soil control and the background level of unamended soil was taken into account.

Ammonia volatilisation could only be detected in soil treated with pig slurry and even then it was low (7% of the total amount added over 14 days). This almost complete absence of ammonia volatilisation was possibly a function of the acidic pH (5.4) of the soil. The lower the pH the more tenaciously adsorbed will be

Table 56. Effect of five pesticide formulations on the disappearance of NPK ammonia.

Time (days)	Ammonia evolved (μ moles/g soil)					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	4.0	4.0	4.0	4.0	4.2	4.2
1	3.8	4.0	3.6	3.8	3.7	3.7
3	3.3	3.4	3.7	3.5	3.5	3.4
6	3.2	3.0	3.1	3.1	3.0	3.5
8	2.6	2.5	2.6	2.5	2.6	2.5
13	0	0	0	0	0	0

Table 57. Effect of five pesticide formulations on the disappearance of ammonia released from urea.

Time (days)	Ammonia evolved (μ moles/g soil)					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	2.9	2.8	2.9	2.9	2.5	3.4*
1	3.3	3.3	3.5	3.2	3.4	3.4
3	3.1	3.6*	3.2	3.4	3.0	3.1
6	3.0	3.5	3.1	2.8	3.0	2.9
8	2.4	2.2	1.9*	2.4	2.5	2.5
13	0	0	0.2	0	0.1	0.3

* significantly different (P = 0.05) from the control value.

Table 58. Effect of five pesticide formulations on the disappearance of pig slurry ammonia.

Time (days)	Ammonia evolved (μ moles/g soil)					
	Control	2,4-D	Diallate	Glyphosate	Benzoylprop ethyl	Malathion
0	5.5	5.1	5.6	6.2*	4.9	4.6*
1	4.3	3.9	4.9	3.9	4.0	4.6
3	4.5	4.7	4.4	3.9	5.1	4.8
7	3.9	4.1	3.8	4.6	4.2	4.2
13	0.8	1.2*	0.9	0.7	0.6*	1.3*
28	0	0	0.1	0.1	0	0

* = significantly different (P = 0.05) from the control value.

the ammonia to the negatively charged soil colloids. Wang et al. (1966) observed that volatilisation of ammonia produced during urea hydrolysis decreased with soil pH and was not detectable below pH 5.8. So it appears that the processes responsible for the loss of the majority of the added ammonia were immobilisation in microbial biomass and nitrification - during which the ammonia was converted to nitrite and nitrate.

3. Response of Soil Glucanase and Urease to Chemical Stress

Applications of various pesticide formulations at 5 times the recommended field rates had no effect on the steady state or temporarily enhanced levels of either glucanase or urease activity. The object of this particular piece of research was to attempt to induce changes in the steady state enzyme levels using high concentrations (compared to field rates) of the active ingredient. The hope was that any disruptions would aid our understanding of interactions within the microenvironment and give an insight to the genesis of soil enzymes. In addition, although there is abundant information on the stability of soil enzymes to physical stress, little is known about their reaction to chemical stress.

a) Glucanase

1) Benzoylprop ethyl, Diallate, Glyphosate and Malathion

Glucanase activity responded to 1000 ppm applications of benzoylprop ethyl (Figure 20), diallate (Figure 21) and malathion (Figure 22) in a similar fashion but this need not mean that the pesticides have identical effects. After a lag period of at least 10 days glucanase activity declined slowly for the remainder of the experiment such that by day **111** it had been reduced by 50% (benzoylprop ethyl), 41% (diallate) and 56% (malathion) and there was no sign of recovery, suggesting that the lost activity was due to permanent enzyme inactivation rather than temporary inhibition. None of the pesticides had any effect on soil pH.

The presence of a lag period might suggest that the active inhibitory molecule is a breakdown product. However, this need not necessarily be the case because all these chemicals are only sparingly soluble in water, so it might take a while for them to penetrate to the site of the enzyme in sufficient concentration to exert their effects.

The fact that three vastly different types of molecules caused a similar response might indicate that it resulted not from direct chemical interaction but from an inhibitory effect on the soil glucanase producing microflora such that further synthesis or subsequent release of enzymes was prevented. Under such circumstances one would expect the activity of the

FIGURE 20 Effect of 1000 ppm benzoylprop ethyl on soil glucanase activity. Activity is expressed as a percentage of the solvent (acetone) control (see Table 59)

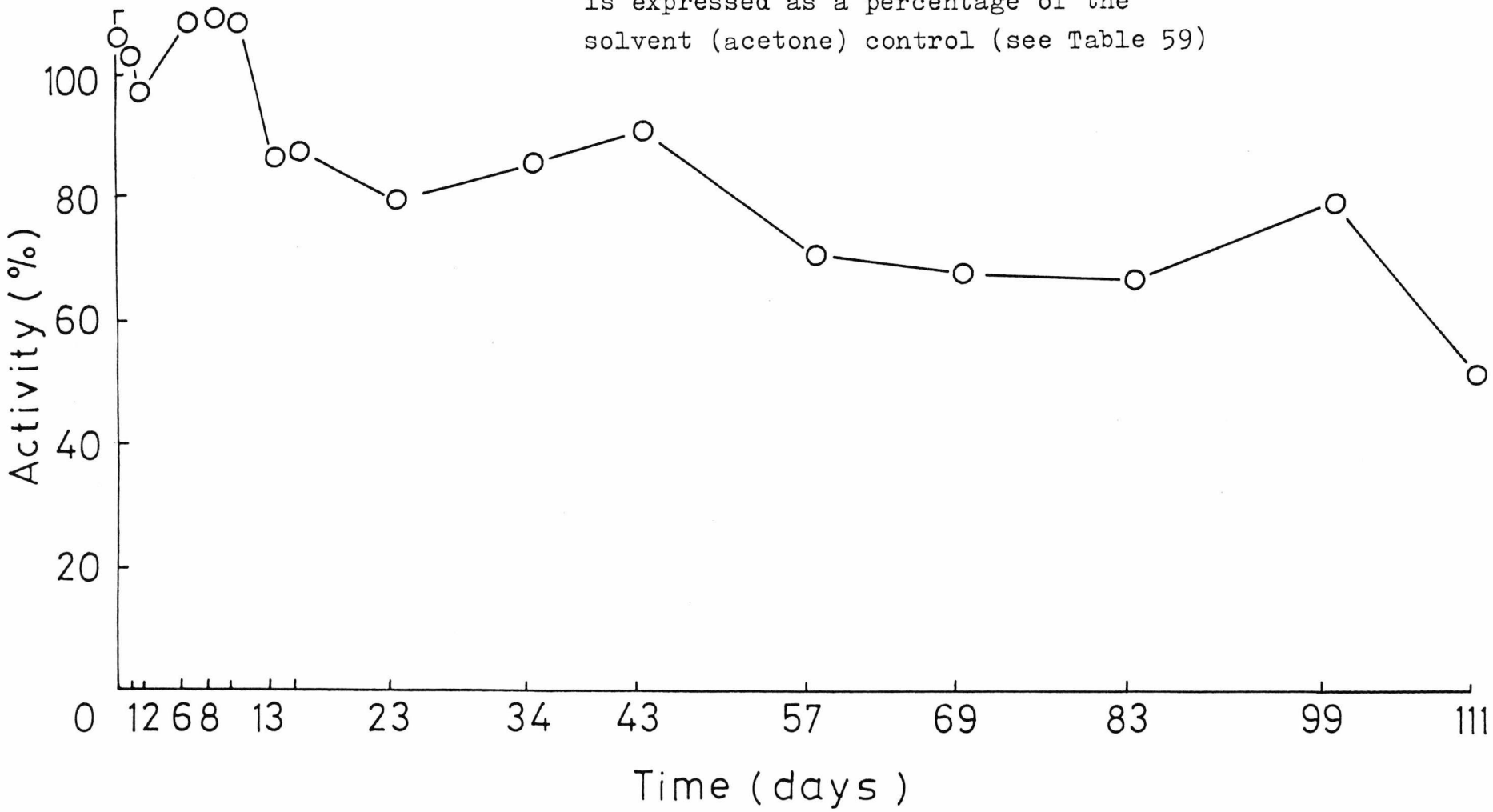


FIGURE 21 Effect of 1000 ppm diallate on soil glucanase activity. Activity is expressed as a percentage of the solvent (acetone) control (see Table 59).

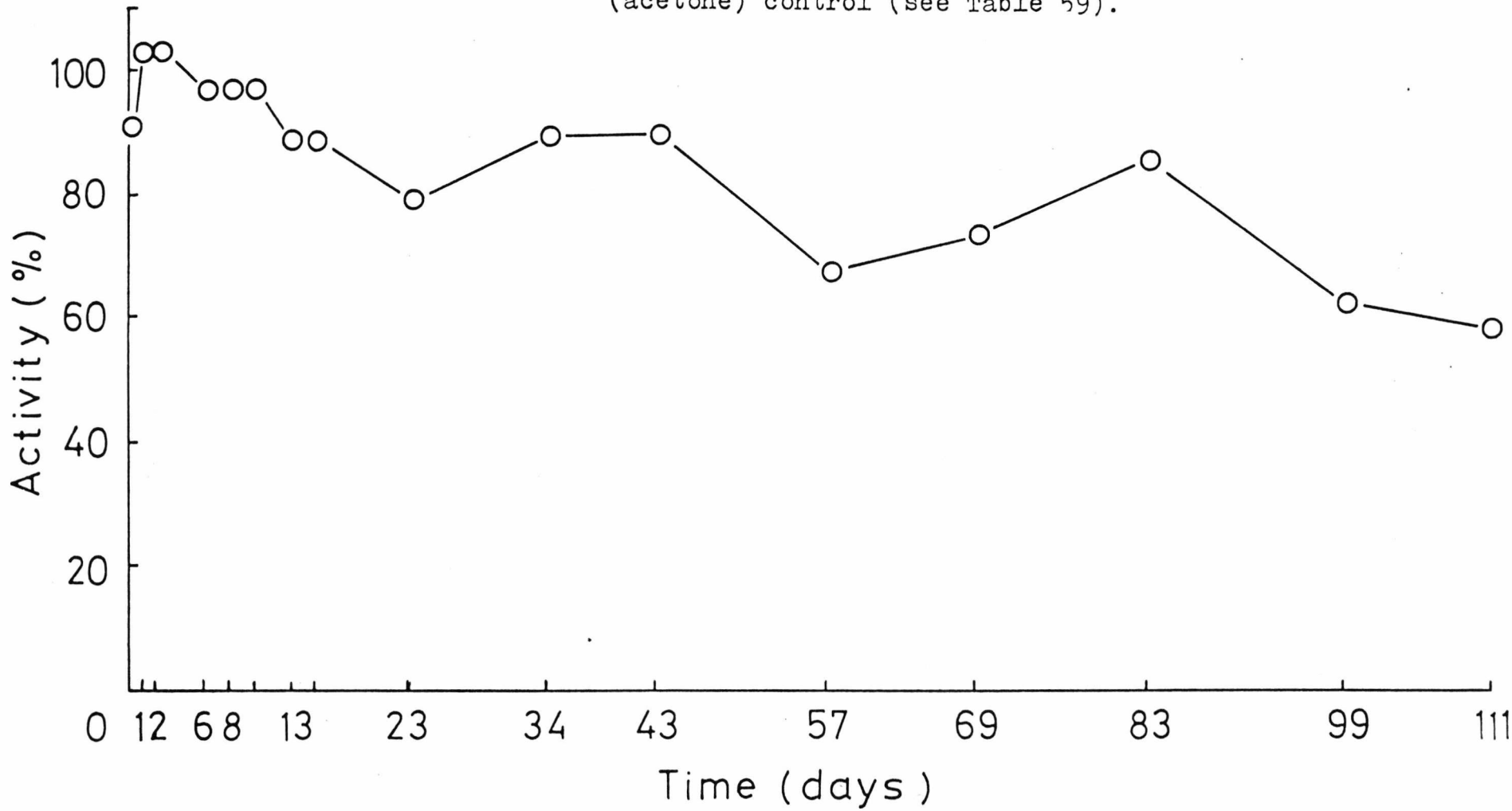
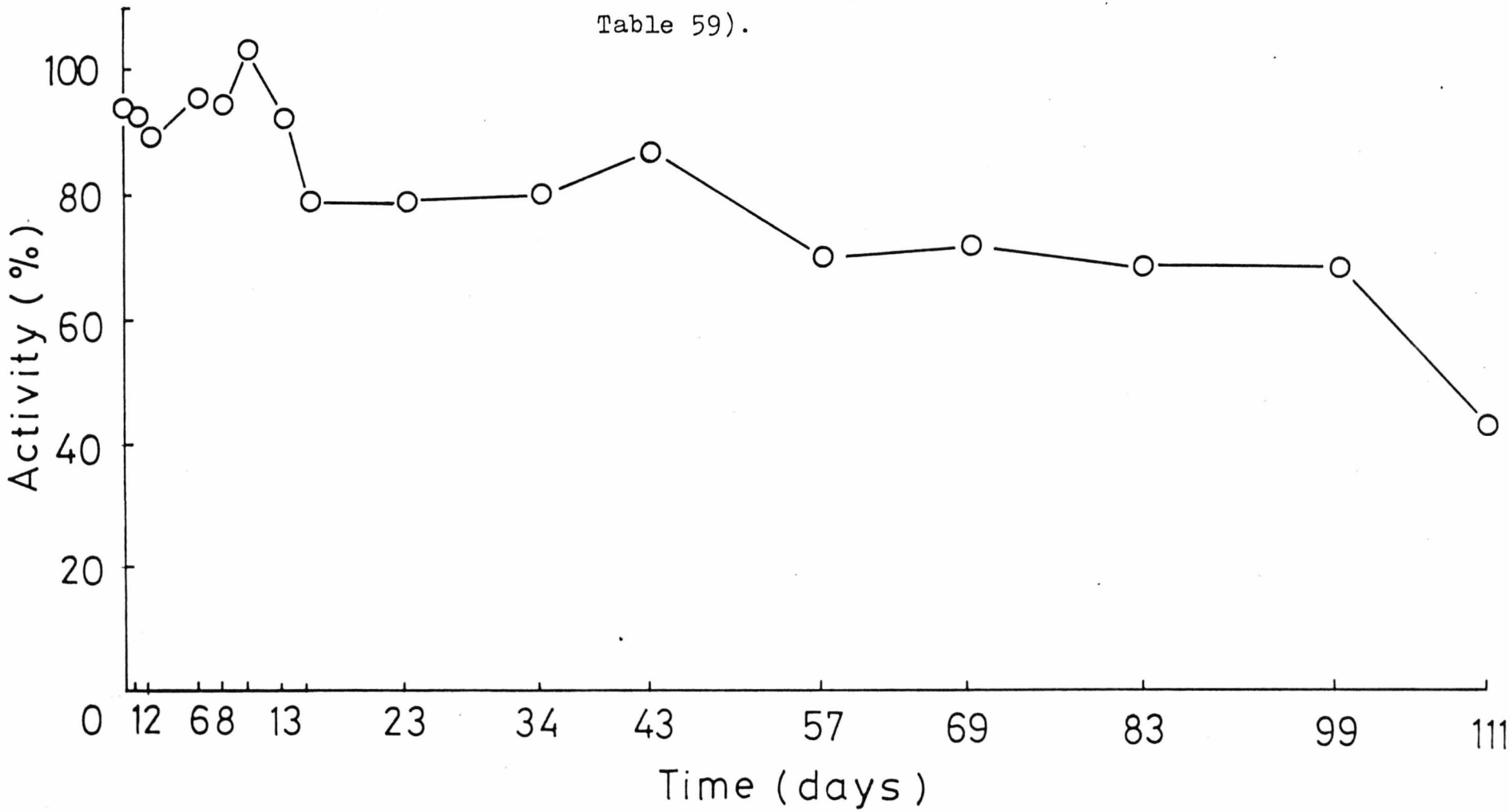


FIGURE 22 Effect of 1000 ppm malathion on soil glucanase activity. Activity is expressed as a percentage of the solvent (acetone) control (see Table 59).



accumulated enzyme to decrease slowly, since there will be no replenishment of that lost by inactivation (Cervelli et al., 1978). This hypothesis depends upon an important assumption: in long-term incubations of unamended wet soil the steady state accumulated enzyme levels that have been observed result from replenishment of the denatured enzyme molecules. This replacement enzyme must either be newly synthesised or newly arrived in the accumulated pool. This latter fraction has been present all the time, but has just not contributed to the accumulated activity in the assay because its location prevented enzyme substrate interaction. Activity of this type results from release of enzymes from ruptured cells and cell fragments and could occur at any time. The question of enzyme synthesis in unamended soil however raises some interesting points. It undoubtedly occurs during the initial burst of microbial activity observed when air-dried soil is re-wetted. This activity is manifested by an increase in ammonia production (see Results and Discussion, B.3.b.2) and a reduction in extractable reducing sugar concentration (see Results and Discussion, A.1.e), but this only lasts for a few days and once all the substrates released by air-drying have been consumed the soil returns to its typically nutrient poor status (Gray and Williams, 1971). But even under these conditions it is apparent that some enzyme synthesis occurs, (Dawes, 1976; Gray 1976) particularly of those concerned with mobilisation of

reserve materials and maintenance of cellular integrity, i.e. those involved in endogenous metabolism. However there are no specific reports of glucanase or urease synthesis under these conditions and whilst the possibility exists for glucanase, glucans being reserve materials in some algae and in terms of cell lysis to reduce competition, it seems extremely unlikely for urease.

If a reduction in microbial numbers is the explanation for reductions in enzyme activity, then once the inhibitory compound has disappeared it should be possible to return the enzyme levels to their original value by adding a readily available carbon and nitrogen source to stimulate the surviving microbial population.

Yet another possibility, to account for decreased enzyme levels is that the pesticide might have been responsible for the release of protected enzyme which was subsequently inactivated.

Malathion, along with two other organophosphorus insecticides accothion and thimet, is known to inhibit another soil enzyme, namely urease, at concentrations ranging from 50 to 1000 ppm (Lethbridge and Burns, 1976) but in this case the effect was more immediate and dramatic. After 60 days, when less than 10 ppm of the original 1000 ppm remained the enzyme activity had been halved and showed no signs of recovery.

Glyphosate (1000 ppm) had no consistent, significant effect on glucanase activity up to 99 days and neither did the 5 µl acetone in which the pesticides were applied (Table 59).

Table 59. Effect of 1000 ppm glyphosate on soil glucanase activity.

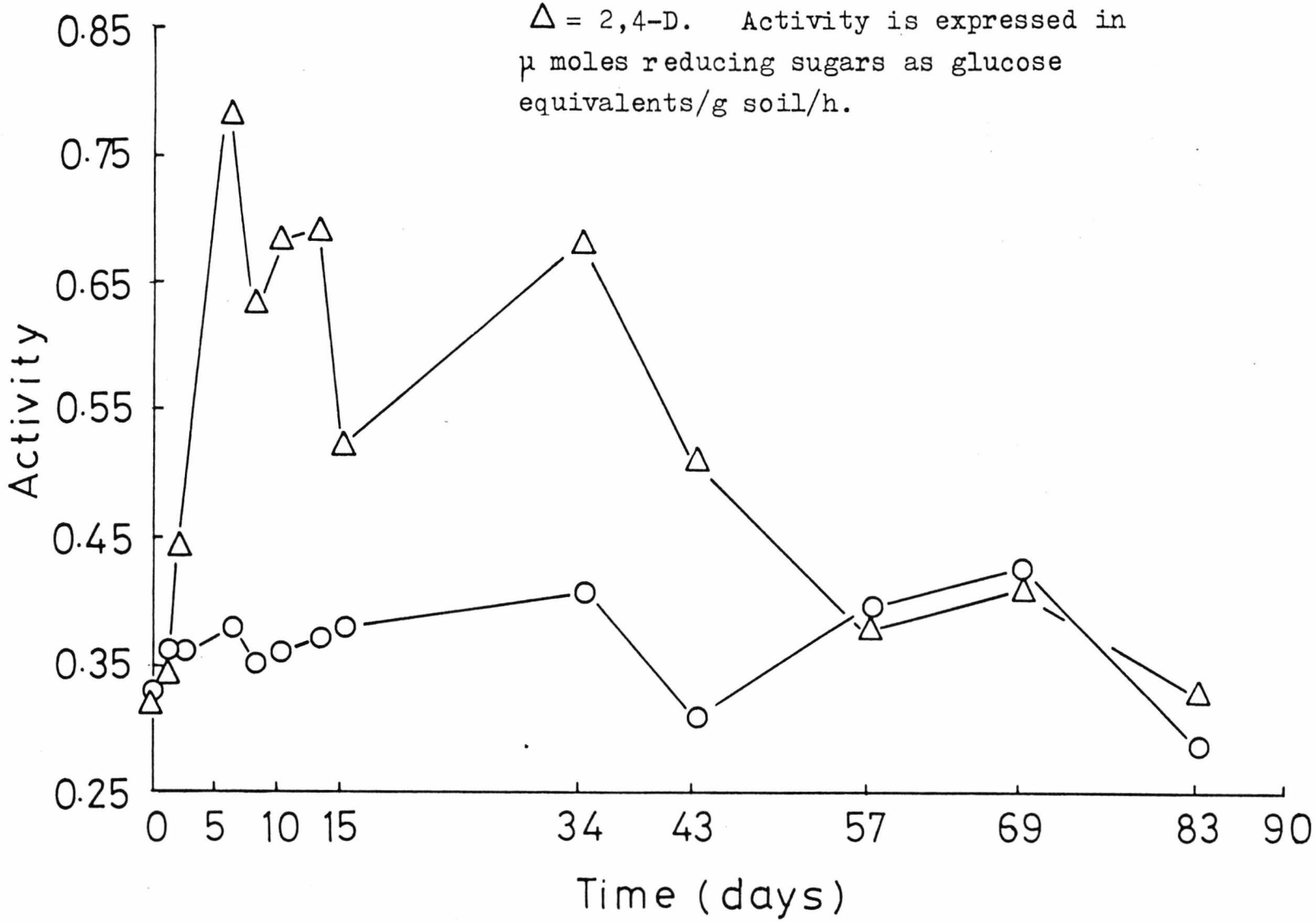
Time (days)	Glucanase activity (μ moles reducing sugars[a]/g soil/h) Unamended control	Solvent control	Glyphosate
0	0.30	0.33	0.30
1	0.34	0.36	0.34
2	0.37	0.36	0.40*
6	0.36	0.38	0.41
8	0.35	0.35	0.33
10	0.38	0.36	0.39
13	0.39	0.37	0.40
15	0.36	0.38	0.38
23	0.43	0.43	0.45
34	0.42	0.41	0.39
43	0.30	0.31	0.32
57	0.35	0.35	0.35
69	0.38	0.43*	0.40
83	0.32	0.29	0.34*
99	0.34	0.32	0.36
111	0.35	0.34	ND

a = as glucose equivalents.

ND = no determination.

* = significantly different (P = 0.05) from the corresponding control value.

FIGURE 23 Effect of 1000 ppm 2,4-D on glucanase activity in April soil; ○ = control; △ = 2,4-D. Activity is expressed in μ moles reducing sugars as glucose equivalents/g soil/h.



2) 2,4-D

After a short lag period of 24 to 48 h, treatment of April soil with 1000 ppm 2,4-D resulted in a rapid increase in glucanase activity such that by day 6 it had doubled (Figure 23). This elevation then gradually decreased and by day 57 the stimulation effect had completely disappeared.

A preliminary survey of the effect of 2,4-D (25 to 1000 ppm) on this enhancement of glucanase activity revealed stimulation over the range 100 to 1000 ppm, with 100 ppm causing a smaller increase than the equally effective 250, 500 and 1000 ppm treatments (Table 60).

TABLE 60 Effect of 2,4-D on glucanase activity (April soil)

2,4-D (ppm)	Glucanase activity[a] Time (days) 0	3	8	14
0	0.38	0.35	0.33	0.40
25	0.40	0.31	0.34	0.38
50	0.37	0.38	0.33	0.39
100	0.37	0.49*	0.46*	0.55*
250	0.36	0.52*	0.64*	0.70*
500	0.41	0.57*	0.65*	0.69*
1000	0.39	0.57*	0.60*	0.74*

a = μ moles reducing sugars as glucose equivalents/g soil/h.

* = significantly different (P = 0.05) from the unamended control value.

The maximum solubility of the 2,4-D in water was found to be around 0.6 mg/ml which means that the maximum amount of 2,4-D in solution at any one time in the 65% WHC soil incubation experiments was about 300 ppm (w/w with respect to the soil). To treat the soil with 500 and 1000 ppm, sufficiently finely powdered 2,4-D was shaken with the air-dried soil (see Methods and Materials, D.1.a.2) to produce concentrations of 250 and 750 ppm respectively, which were finally brought to 500 and 1000 ppm by the addition of 250 ppm 2,4-D in water. This method ensured compatability between the different doses early on in the experiment. Presumably in the case of the 500 and 1000 ppm treatments an equilibrium situation arose, whereby as soon as any 2,4-D was broken down it was replaced in solution until all the herbicide had dissolved.

There was no stimulation of glucanase after 6 days when 250 ppm 2,4-D was added to soil in the presence of 1000 ppm sodium azide - which is known to prevent the microbial breakdown of the herbicide (Audus, 1964; Gibson, 1977) (Table 61). This strongly suggests that the enhanced enzyme levels observed in the absence of sodium azide were due to the stimulation of a 2,4-D degrading, glucanase-producing microbial population. Once the 2,4-D had been depleted activity returned to its original steady state value, because there was no way of stabilising this newly produced enzyme, in a situation analagous to the glucose one (see Results and Discussion, B.1.b.5). The fact that 1000 ppm

TABLE 61 Effect of sodium azide on the stimulation of glucanase activity by 250 ppm, 2,4-D

Soil treatment	Glucanase activity (μ moles reducing sugars[a]/g soil/h)
Unamended control	0.39
NaN_3	0.41
2,4-D	0.71*
2,4-D + NaN_3	0.40

a = as glucose equivalents

* = significantly different (P = 0.05) from the control value

2,4-D led to a greater and more prolonged increase in glucanase activity than did 700 ppm glucose indicates that a large percentage of the 2,4-D-degrading microorganisms also produce glucanase, so there is less competition between glucanase and non-glucanase producers for this carbon source than for glucose which can be used as a growth substrate by almost all microorganisms.

In contrast to this dramatic response in April soil, 2,4-D had no effect on glucanase activity in November soil unless a concentration of 1000 ppm was used and even then the stimulation was ephemeral and quite modest (Table 62).

TABLE 62 Effect of 2,4-D on glucanase activity (November soil)

2,4-D (ppm)	Glucanase activity[a] Time (days) 2	4	8	11	17	36	51
0	0.39	0.37	0.38	0.41	0.43	0.43	0.40
25	0.37	0.38	0.40	0.41	0.42	0.42	0.39
50	0.38	0.38	0.41	0.42	0.47	0.42	0.37
100	0.36	0.40	0.40	0.43	0.48*	0.43	0.41
250	0.39	0.38	0.40	0.40	0.47	0.43	0.41
500	0.39	0.39	0.42	0.41	0.46	0.41	0.38
1000	0.40	0.44*	0.43*	0.45*	0.47	0.37*	0.38

a = μ moles reducing sugars as glucose equivalents/g soil/h

* = significantly different (P = 0.05) from the corresponding control value

The 10 to 19% increase had disappeared by day 17. The fact that 500 ppm had no effect when 1000 ppm did is difficult to explain since initially the amount of 2,4-D in solution from these two treatments would be approximately the same (250 to 300 ppm).

A comparison of the glucanase activity and its response to 1000 ppm 2,4-D in soils collected on three occasions during 1976 (April, July and November) showed that accumulated enzyme levels did not vary between sampling times (see Results and Discussion, A.1.i),

yet the response to 2,4-D was much greater in soil collected during April and July than during November (Table 63).

TABLE 63 Effect of sampling time on the stimulation of soil glucanase activity by 1000 ppm 2,4-D after 6 days

Sampling time	Glucanase activity[1]	
	Control	2,4-D
April	0.36	0.63*
July	0.34	0.57*
November	0.36	0.44*

1 = μ moles reducing sugars as glucose equivalents/g soil/h

* = significantly different (P = 0.05) from the control value

This suggests that during the year there had been a reduction in the number of glucanase producing micro-organisms capable of degrading 2,4-D. This reduction could have been brought about by: 1) an application of Gramoxone (a.i. paraquat), the only pesticide used in that period, 2) the baking of the soil during the long hot summer such that the surface was like concrete, or 3) a regular seasonal variation brought about by the cultivation pattern of the field. Although counts of glucanase producing microorganisms were not performed

on the three samples, total counts suggested that whereas the numbers of bacteria and actinomycetes remained reasonably constant, there were considerably less fungi in the July sample than in the April one, but by November they had started to recover, although they were still below the April population size (see Methods and Materials, Table 17). The maximum reduction of fungal numbers did not coincide with the reduced enzyme response to 2,4-D, but since the fungal counts in November soil were still 33 times less than in April soil, reduction of fungal numbers may be involved in the changed response of glucanase to 2,4-D. There were plenty of glucanase producing microorganisms in November soil (see Methods and Materials, Table 19), but whether they were capable of degrading 2,4-D is another matter altogether.

2,4-D has also been reported to stimulate the activities of dehydrogenase (Lenhard, 1959; Klein, Loh and Goulding, 1971), catalase (Zinchenko et al., 1969) and invertase (Zinchenko et al., 1969).

b) Urease

1) Benzoylprop ethyl, Diallate and Glyphosate

Urease activity was unaffected by 1000 ppm applications of benzoylprop ethyl, diallate and glyphosate and by the 5 µl acetone in which the herbicides were applied (Table 64).

Table 64. Effect of 1000 ppm benzoylprop ethyl, diallate and glyphosate on soil urease activity.

Time (days)	Urease activity (μ moles ammonia evolved/g soil/h)				
	Unamended control	Acetone control	Benzoylprop ethyl	Diallate	Glyphosate
0	27.7	27.8	26.5	26.8	30.0
1	26.0	25.2	26.3	25.8	25.4
2	27.3	27.5	26.6	27.3	26.8
4	32.9	33.1	29.7	33.9	31.8
8	29.7	29.2	28.4	29.2	28.7
14	22.3	21.6	22.5	26.2*	23.3
21	24.9	25.4	25.8	22.4	24.4
35	26.5	25.8	28.9	27.4	27.7
44	27.4	28.9	26.9	30.5	26.6
51	27.0	26.6	27.3	28.6	30.5*
63	28.2	28.0	27.9	26.2	28.4
70	25.5	26.7	28.6	27.0	26.8
84	23.7	23.1	22.7	23.4	22.4

* = significantly different (P = 0.05) from the corresponding control value.

2) 2,4-D

Approximately one week after treating soil with 2,4-D concentrations ranging from 25 to 1000 ppm, urease activity started to decline in the presence of 250, 500 and 1000 ppm herbicide (Table 65). This decrease in activity continued until around day 24 when the enzyme levels appeared to plateau. This was certainly the case for 250 and 500 ppm but the 1000 ppm response was rather erratic after this time, activity fluctuating between 23 and 46% of the unamended soil. Reduction in urease activity was proportional to 2,4-D concentration such that the final reductions in activity (estimating the plateaux) were 20% (250 ppm), 35% (500 ppm) and 65% (1000 ppm).

This is a similar response to the ones observed between glucanase and benzoylprop ethyl, diallate and malathion, i.e. a lag period of equivalent length with no recovery, with the exception that at the higher concentrations it was more pronounced and the effect levelled off rather than being a continuous one; however in the case of glucanase the experiment may not have been continued long enough for the plateau to be reached. If 2,4-D was having a similar effect on urease its greater magnitude might be a result of its increased water solubility over benzoylprop ethyl, diallate and malathion. All the arguments and possible explanations for the glucanase effects apply to urease so there is no need to reiterate them here, but there are some additional possibilities in this case.

Table 65. Effect of 2,4-D on urease activity (November soil).

Time (days)	Urease activity[a] of control	Urease activity of 2,4-D amended soil (as a % of the control) 25 ppm	50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm
0	22.4	104	104	102	111	102	90
1	27.7	104	104	101	93	97	96
2	27.8	99	99	103	109	106	99
4	25.8	101	103	99	103	100	87
6	22.8	96	102	95	109	104	89
8	27.3	97	98	96	96	92	71*
11	33.3	102	98	92	84*	82*	63*
13	26.3	98	100	103	78*	80*	68*
17	32.5	101	101	96	82*	71*	58*
24	35.4	108	109	95	82*	65*	36*
36	31.6	96	103	94	75*	64*	45*
51	25.9	110	105	100	88*	76*	46*
70	32.0	98	103	100	86*	65*	23*
84	35.0	ND	ND	ND	78*	60*	38*
100	33.5	ND	ND	ND	86*	ND	29*

a = μ moles ammonia evolved/g soil/h. ND = no determination

* = significantly different (P = 0.05) from the control value.

The soil control of the urease assay revealed a number of interesting points about ammonia levels not associated with urease activity (Table 66). When air-dried soil was re-wetted there was a burst of ammonia production reaching a maximum after 2 days and returning to the basal level of about 1 μ mole/g soil round about day 8. This flush of ammonia was reduced by half in sodium azide treated soil suggesting that some of the ammonia was produced as a result of the microbial breakdown of protein made available by the air-drying process (it did not occur in the field-wetness soil). The rest may have been due to the release of ammonia itself from cells ruptured in the air-drying process or to protein degradation by chemical catalysis or accumulated proteolytic enzyme activity.

This initial burst of ammonia production was retarded by the 2,4-D concentrations which eventually reduced urease activity (Table 66). From this result it seemed possible that the decline in urease levels may not have been a true reduction in activity, but due to an inhibition of ammonia evolution from soil. In other words, the enzyme was producing ammonia from urea at the normal rate but that the release of this ammonia from the soil at the end of the assay by potassium carbonate was being retarded by the 2,4-D. This hypothesis was tested by adding a known amount of ammonia as ammonium hydroxide (corresponding to that produced in the urease assay - 30 μ moles) to 250 ppm 2,4-D amended soil at various time intervals and then

Table 66. Effect of 2,4-D on ammonia evolution from soil.

Time (days)	Ammonia evolution (μ moles/g soil) 2,4-D concentration (ppm w/w) 0	25	50	100	250	500	1000
0	1.2	1.1	1.2	1.2	1.1	1.0*	0.9*
1	2.3	2.5	2.2	2.0	1.7*	1.6*	1.2*
2	3.0	3.4	3.3	3.5*	2.7	2.0*	1.6*
4	1.9	2.6*	3.0*	3.7*	4.3*	3.4*	3.2*
6	2.1	2.6*	2.7*	3.4*	4.9*	3.6*	3.1*
8	1.3	1.3	1.6*	2.6*	3.9*	3.2*	3.1*
11	1.3	1.2	1.4	2.2*	3.9*	3.7*	4.0*
13	1.2	1.1	1.1	1.7*	4.9*	5.1*	4.1*
17	1.3	1.4	1.1	1.0*	2.7*	3.3*	3.4*
24	1.0	1.0	1.0	0.9	1.2*	3.6*	4.2*
36	0.9	0.9	0.8	0.7*	0.8	0.9	5.7*
51	0.9	0.8	0.7*	0.8	0.9	0.8	4.4*
70	1.0	0.9	1.0	0.9	0.9	1.2	5.2*
84	1.1	ND	ND	ND	1.1	1.1	1.0
100	1.0	ND	ND	ND	1.1	ND	1.1

ND = no determination.

* = significantly different (P = 0.05) from the control value.

comparing its evolution from soil by potassium ion displacement with that from unamended soil. The 2,4-D did not retard ammonia evolution (Table 67).

Another interesting point to emerge from this experiment was that not all the ammonia added to the soil was recovered in the boric acid - the method was 83 to 89% efficient. The amount of ammonia added was confirmed by pipetting a sample directly into the indicator followed by back-titration. This result suggests that the urease activities quoted are slight underestimates of the real values. However this finding may well be an artifact of the experimental procedure, since when ammonia was added to soil as ammonium nitrate (NPK), urea and pig slurry the recovery rates were 93 to 98%, 96 to 105% and 88 to 119% respectively.

Returning to the discussion of Table 66 and the ammonia levels of the soil control. From day 4 onwards the 2,4-D amended soil (at every concentration) evolved more ammonia than the control soil and the duration of this stimulation was concentration dependent. Thus at 250 ppm the increased ammonia levels of the soil had disappeared by day 8 but at 1000 ppm they persisted at least until day 70. That this increase did not occur under conditions known to prevent 2,4-D degradation (sodium azide treated and autoclaved soil) suggests that the ammonia levels of the soil were a result of the microbial utilisation of the herbicide. This hypothesis is supported by the fact that the

Table 67. Effect of 250 ppm 2,4-D on the evolution of added ammonia from soil.

Time (days)	Ammonia concentration (μ moles) Amount added	Evolved from unamended soil	Evolved from amended soil	Percent[a] evolved
0	29.5	26.2	25.2	89
1	29.1	25.1	25.8	86
2	29.6	26.2	27.0	89
7	28.1	24.4	24.3	87
13	29.4	24.3	24.4	83
26	29.0	25.7	25.1	89

a = derived from the unamended soil values.

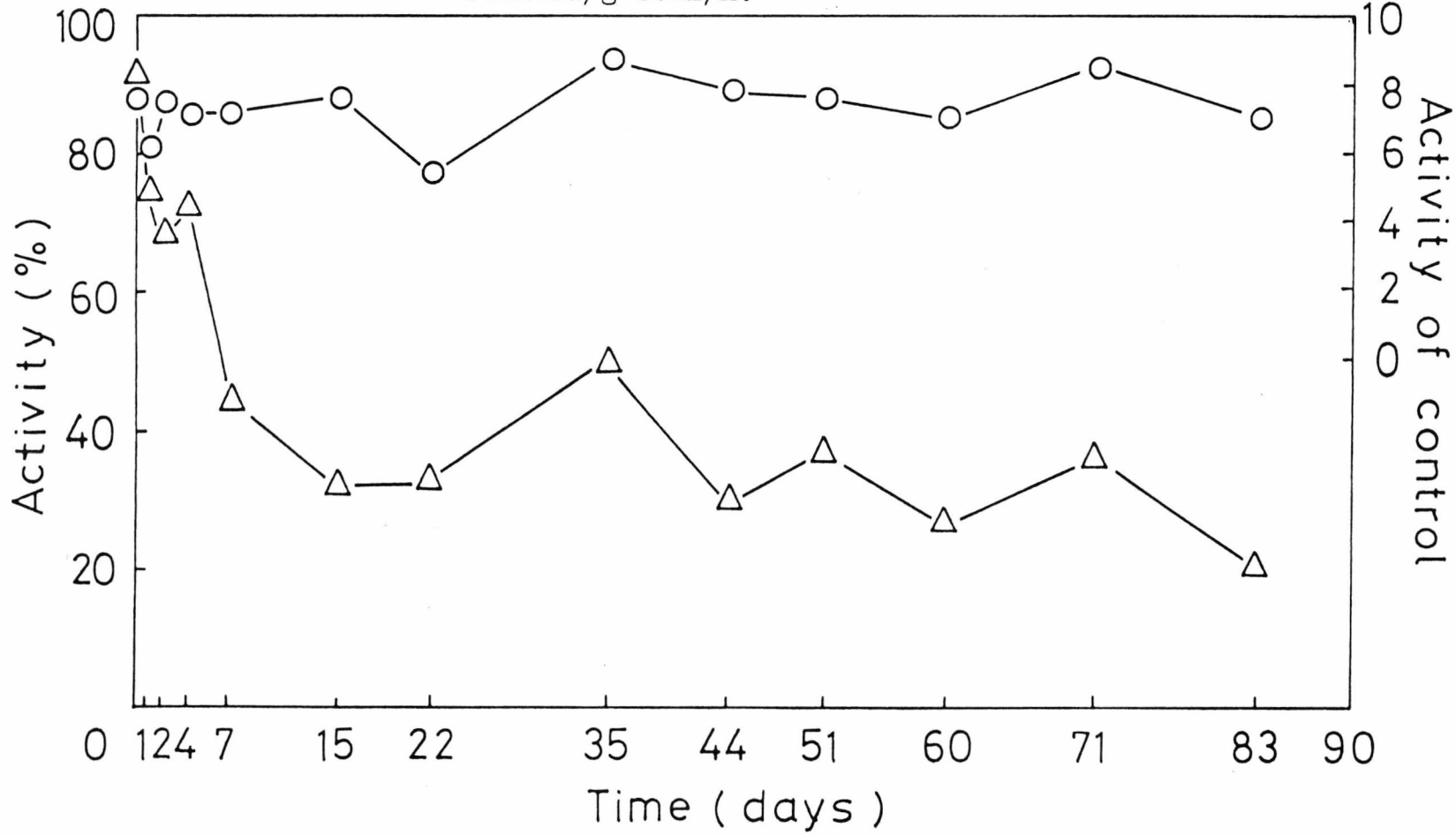
duration of the stimulation was proportional to 2,4-D concentration. It seems likely that during the microbial degradation of 2,4-D, mineralisation of soil protein occurred producing ammonia. The ammonia thus produced eventually disappeared as it was immobilised in microbial biomass and converted to nitrite and nitrate in the nitrification process. Lenhard (1959) has also observed this phenomenon, but not to the same extent. Soil amended with 100 ppm 2,4-D consistently produced more ammonia than the unamended control over the 14 day incubation period.

In April soil, urease, unlike glucanase, responded to 1000 ppm 2,4-D in a very similar manner to that in the November soil, so that when the inhibition levelled off about 70% of the original activity had been lost (compared to 65% in November soil) (Figure 24). This suggests that although the two soils have very different urease activities the various accumulated enzyme fractions which together make up the total activity are present in similar ratios in both samples.

No inherent effect on jack bean and Bacillus pasteurii urease activities was detected when the assay was performed with an amount of purified enzyme which gave similar activity to 1 g of soil (20 to 30 μ moles ammonia evolved/h) in the presence of 250 μg 2,4-D.

The 2,4-D sample used in the experiment was the acid and the 0.5 mg/ml standard solution from which all the soluble concentrations were prepared (i.e. 0.5 ml/g =

FIGURE 24 Effect of 1000 ppm 2,4-D on urease activity in April soil; O = absolute activity of unamended control; Δ = activity of 2,4-D amended soil expressed as a percentage of the unamended control soil activity. Activity is expressed in μ moles ammonia evolved/g soil/h.



250 ppm) had a pH of 3. Although the 2,4-D caused an initial drop in soil pH (Table 68), this was counteracted by the buffering capacity of the 0.5 M tris-maleate in the urease assay such that assay pH was not affected.

TABLE 68 Effect of 2,4-D on soil pH

Time (days)	Soil pH		
	2,4-D concentration (ppm w/w)		
	0	250	1000
0	5.4	5.3	5.1
2	5.7	5.5	5.2
8	5.9	5.8	5.3
13	6.2	6.2	5.7
22	5.7	5.8	5.8
35	5.3	5.3	5.4

It is unlikely that the temporary drop in soil pH was the cause of the decreased urease levels, since at 250 ppm the pH had returned to that of the control by the time enzyme activity started to decline and even at 1000 ppm, where pH recovery took a little longer, it was back to that of the control only a few days after the onset of inhibition.

There are two reports of soil urease - 2,4-D interactions, one involving inhibition (Zinchenko and

Osinkaya, 1969) and the other stimulation (Zinchenko et al., 1969) of activity and they have both been discussed previously (see Results and Discussion, B.1.a.2). 2,4-D has also been shown to decrease dehydrogenase levels when applied to soil at 100 to 700 ppm (Lenhard, 1959).

PART FOUR - CONCLUSIONS

The work described in this thesis was performed in order to evaluate the usefulness of soil enzymes as indicators of agrochemical effects in the soil. The observations clearly show that formulations of the pesticides 2,4-D, benzoylprop ethyl, diallate, glyphosate and malathion, when applied to air-dried or field-wetness soil at 5 times the recommended field rate had no effect on the steady state levels of glucanase and urease. Temporarily enhanced levels of these enzymes (induced by additions of glucose, cellulose and pig slurry) were also unaffected by the same five pesticides. However, the experimental conditions were vastly different to those encountered in the field, i.e. higher and constant temperature and water holding capacity, reduced microbial numbers (due to air-drying), pesticide incorporated uniformly, small volumes of sieved soil and no plant effects. As a result of the non-stressed nature of the experiment, extreme caution should be applied when attempting to extrapolate from the *in vitro* to the *in situ* environment. Laboratory experiments are a useful pointer towards what might be happening in the field but do not allow accurate predictions to be made. Indeed Wingfield, Davies and Greaves (1977) have shown that bacterial counts and dehydrogenase activity can be modified by soil preparations and mode of pesticide application. The

same authors reported that the effects of the herbicide dalapon were less marked in surface sprayed soil cores than in disturbed samples (sieved and air-dried or just sieved) in which the herbicide was incorporated uniformly. Nevertheless, with this proviso in mind, the results in this thesis strongly suggest that the pesticide formulations described will have no effects on soil glucanase and urease in the field situation, at least when used in the recommended manner.

Despite the obvious and frequently aired criticisms concerning laboratory experiments and microbial activity they will undoubtedly continue to provide the majority of our information on agrochemical soil environment interactions. This is not surprising, for only in the laboratory can environmental conditions be precisely monitored such that any experimental variables are defined. This type of rigid control is impossible in the field and as a direct result experimental variation is increased so that not only is it difficult to elucidate the exact cause of an observed effect but the extent of replication (which has to be large even in the laboratory) has to be increased to be certain that the effect is of significance. It is important to realise that since the field situation is such a complex environment (due particularly to climatic and macro-organic factors not encountered in the laboratory), then effects are more likely to be caused by a number of interacting factors rather than a single one. An understanding of these complex interactions by stepwise

progression at the bench may eventually facilitate extrapolation from the laboratory to the field.

It is apparent that the preparation of soil for these laboratory investigations requires careful consideration since herbicide degradation rates may be reduced in air-dried soils and those stored at field-wetness when compared to fresh soil (Lay and Ilnicki, 1975; Wingfield et al., 1977). Under these circumstances the effect of the chemical might be changed in nature or extent. This project has shown that air-drying of soil not only reduces viable counts of microorganisms but accumulated enzyme levels as well. Glucanase levels were enhanced in glucose-amended air-dried soil but not in glucose-amended field-wetness soil indicating that on occasions results obtained with the former are artifacts. Wingfield et al. (1977) have proposed that agrochemical effects should be monitored in surface sprayed, freshly collected soil cores for two reasons. Firstly, the mode of application is closely related to the way in which these chemicals are applied in practise. Secondly, they will provide information on the vertical distribution of the agrochemical which, when compared to the depth of cultivation and crop root penetration, will have a considerable bearing in evaluating the importance of any effects. However this approach is not ideal for all pesticides since the volatile ones, such as diallate, have to be incorporated into the soil by cultivation.

The pesticide concentrations used in these investigations should be chosen carefully so as to reflect the aims of the research. Many workers use concentrations vastly in excess of those needed for efficient pest control in order to emphasise potential effects. This is only reasonable if the object is to obtain fundamental information about pesticide soil microenvironment interactions by disrupting various processes but results from such studies should not be extrapolated to the field situation. The theory that high concentrations of pesticides should be used in screening programmes so that they can be rapidly eliminated if they have no effect at these levels (Grossbard, 1976) is in this author's opinion invalid. It is wrong to assume that if a chemical has no effect at excessive concentrations it will have no effect at field rates, since the former levels might be toxic to a proportion of the soil microflora, whereas the latter levels merely available as a growth substrate. If the avowed object of the exercise is to monitor pesticides for effects in the field situation then concentrations in the region of those recommended by the manufacturers should be used, although it is important to incorporate a small safety margin to account for human error in preparation, spray overlap, uneven distribution in soil and concentration effects due to adsorption and partitioning.

Having decided upon the type of soil and amendment concentration it is then necessary to devise a routine

testing programme which should be as diverse as the available manpower and resources will allow (Johnen and Drew, 1977). At the moment specific soil enzyme activity estimations play a minor role in these monitoring procedures but the results of this project suggest that they have certain advantages over some of their counterparts:-

1) Soil enzymes are assayed within their natural environment and hence the results are likely to be more meaningful than those obtained from pure culture studies of microorganisms isolated from soil.

2) Soil enzymes are far more reproducible than microbial counts lending increased confidence that the observations are real. The coefficients of variation for the glucanase and urease assays varied between 2 to 8% and 2 to 6% respectively, whereas those for the microbial counts usually fell in the range 30 to 70% although values of over 100% were not uncommon. As a direct consequence of their reproducibility (and simplicity), the enzyme assays were rapidly and easily performed compared to microbial counts. Each glucanase and urease assay was replicated 4 and 3 times respectively, compared to 15 times for the microbial counts. This permits the screening of more agrochemicals in any given experiment.

3) Enzyme activity estimations do not pose the long-term, constant temperature storage problems associated with microbial enumeration. Depending on the activity of the enzyme, answers can be arrived at within a 24 h period, whereas some soil microorganisms are slow growers and the plates must be incubated for days.

All these advantages make soil enzyme activities seem like an obvious choice for monitoring agrochemical effects in soil. However, it is important to bear in mind that by their very stable nature soil enzymes are unlikely to be the most sensitive indicators of change and no testing programme should be based entirely upon measurements of their activity.

Some research groups, notably those at ICI (Johnen and Drew, 1977) and WRO (Greaves et al., 1977), have incorporated soil enzyme assays (dehydrogenase, phosphatase and nitrogenase) into their testing programmes but unfortunately in every case artificial substrates are being used. These should be avoided wherever possible since their rates of turnover may bear no relationship to those of the natural substrates.

Despite the plethora of research communications concerning the effects of pesticides on soil microorganisms little progress has been made towards an understanding of the factors involved in these interactions (Domsch, 1972). Indeed, Johnen (1977 personal communication) even

went so far as to say that, in his opinion, the contribution of such studies to our understanding of soil microbial ecology has been negligible. Whether he is correct or not is open to debate but one thing is certain; authors should refrain from simply publishing data without attempting to explain their observations. After nearly 30 years of soil-organic pesticide interaction research it is a pity that we still lack the basic knowledge to interpret these results in terms of soil fertility. Thus, although we know that soil microorganisms and enzymes play a vital role in this context it is difficult to assess the impact of changes in these activities upon soil fertility.

It is not surprising that there have been very few investigations of accumulated enzyme activity in soil containing growing plants (Skujins, 1978), since rhizosphere effects only serve to complicate further what is an already complex situation. However it seems likely that the future will see a change of emphasis in the monitoring of agrochemicals for soil microenvironment effects from root-free soil to soil containing growing plants (Greaves, 1978). If soil enzyme activity estimations are to be included in these routine testing programmes then the soil enzymologist should turn his attention in this direction.

PART FIVE - REFERENCES

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APPENDIX ONE

Enzyme Nomenclature

E.C. Number	Recommended Name	Other names	Systematic Name
Oxidoreductases			
1.1.3.4	glucose oxidase		β-D-glucose: oxygen 1-oxidoreductase
1.7.3.3.	urate oxidase	uricase	urate: oxygen oxidoreductase
1.11.1.6	catalase		hydrogen-peroxide: hydrogen-peroxide oxidoreductase
1.11.1.7	peroxidase		donor: hydrogen-peroxide oxidoreductase
1.13.11.11	tryptophan 2,3-dioxygenase	tryptophanase	L-tryptophan: oxygen 2,3-oxidoreductase (decyclizing)
1.14.18.1	monophenol monooxygenase	polyphenol oxidase[a] catechol oxidase[a] o-diphenol oxidase[a] p-diphenol oxidase[b]	monophenol, dihydroxyphenylalanine: oxygen oxidoreductase
Transferases			
2.4.1.5	dextransucrase		sucrose: 1,6-α-D-glucan 6-α- glucosyltransferase
2.4.1.10	levansucrase		sucrose: 2,6-β-D-fructan 6-β-fructosyltransferase

2.6.1 -	amino-transferases	transaminases	
2.8.1.1	thiosulphate sulphur transferase	rhodanese	thiosulphate: cyanide sulphurtransferase
Hydrolases			
3.1.1.1	carboxylesterase	malathion esterase	carboxylic-ester hydrolase
3.1.1.3	triacylglycerol lipase	lipase	triacylglycerol acyl-hydrolase
3.1.1.6	acetylesterase		acetic-ester hydrolase
3.1.3.1.	alkaline phosphatase	phosphomono-esterase	orthophosphoric-monoester phosphohydrolase (alkaline optimum)
3.1.3.2	acid phosphatase	phosphomono-esterase	orthophosphoric-monoester phosphohydrolase (acid optimum)
3.1.3.26	6-phytase	phytase	myo-inositol hexakisphosphate 6-phosphohydrolase
3.1.6.1	arylsulphatase	sulphatase	aryl-sulphate sulphohydrolase
3.2.1.1	α-amylase		1,4-α-D-glucan glucanohydrolase
3.2.1.2	B-amylase		1,4-β-D-glucan maltohydrolase
3.2.1.4	cellulase	endo-1,4-β-glucanase	1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase
3.2.1.6	endo-1,3(4)-B-D-glucanase	1,3-β-glucanase laminarinase	1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase
3.2.1.7	inulinase	inulase	2,1-β-D-fructan fructanohydrolase

APPENDIX ONE (Contd)

E.C. Number	Recommended Name	Other names	Systematic Name
Hydrolases (Contd)			
3.2.1.8	endo-1,4-β-xylanase	xylanase	1,4-β-D xylan xylanohydrolase
3.2.1.11	dextranase		1,6-α-D-glucan 6-glucanohydrolase
3.2.1.15	polygalacturonase	pectinase	poly (1,4-α-D-galacturonide) glycanohydrolase
3.2.1.20	α-glucosidase	maltase	α-D-glucoside glucohydrolase
3.2.1.21	β-glucosidase	gentiobiase cellobiase, emulsin	β-D-glucoside glucohydrolase
3.2.1.22	α-galactosidase	melibiase	α-D-galactoside galactohydrolase
3.2.1.23	β-galactosidase	lactase	β-D-galactoside galactohydrolase
3.2.1.26	β-fructofuranosidase	sucrase invertase saccharase β-fructosidase	β-D-fructofuranoside fructohydrolase
3.2.1.65	levanase		2,6-β-D-fructan fructanohydrolase
3.5.1.1	asparaginase		L-asparagine amidohydrolase
3.5.1.2	glutaminase		L-glutamine amidohydrolase
3.5.1.5	urease		urea amidohydrolase
3.6.1.10	endopolyphosphatase	meta phosphatase polyphosphatase	polyphosphate polyphosphohydrolase

Lyases			
4.1.1.12	aspartate 4-decarboxylase		L-aspartate 4-carboxy-lyase
4.1.1.15	glutamate decarboxylase	aspartate 1-decarboxylase[c]	L-glutamate 1-carboxy-lyase
4.1.1.25	tyrosine decarboxylase		L-tyrosine carboxy-lyase
4.1.1.28	aromatic L-amino-acid decarboxylase	DOPA decarboxylase tryptophan decarboxylase	aromatic-L-amino-acid carboxy-lyase

a previously 1.10.3.1 -diphenol: oxygen oxidoreductase

b previously 1.10.3.2 p-diphenol: oxygen oxidoreductase

c previously 4.1.1.11 aspartate 1-decarboxylase

APPENDIX TWO Pesticide Nomenclature

Common Name	Systematic Name
Herbicides	
Atrazine	2-chloro-6-ethylamino-4-isopropylamino-1,3,5-triazine
Chloropropham	isopropyl N-(3-chlorophenyl)-carbamate
Dalapon	2,2-dichloropropionic acid
Dinoseb	4,6-dinitro-2-S-butylphenol
Diuron	N-(3,4-dichlorophenyl)-NN-dimethyl urea
Fenuron	NN-dimethyl-N-N-phenyl urea
Linuron	N-(3,4-dichlorophenyl)-N-methoxy-N-methyl urea
Monuron	N-(4-chlorophenyl)-NN-dimethyl urea
Neburon	N-butyl-N-(3,4-dichlorophenyl)-N-methyl urea
Paraquat	1,1-dimethyl-4,4-bipyridylium-2A
Phenmedipham	methyl m-hydroxycarbanilate m-methyl-carbanilate
Prometryne	4,6-bis(isopropylamino)-2-methyl-thio-1,3,5-triazine
Pyrazone	5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone
Siduron	1-(2-methylcyclohexyl)-3-phenyl urea
Simazine	2-chloro-4,6-bis(ethylamino)-1,3,5-triazine
TCMB	2-(thiocyanomethylthio)benzothiazole
Tillam	n-propyl N-ethyl-N-n-butylthiolcarbamate
2,3,6-TBA	2,3,6-trichlorobenzoic acid
Trifluralin	2,6-dinitro-NN-di-n-propyl-ααα-trifluoro-p-toluidine
Tordon	4-amino-3,5,6-trichloropicolinic acid
Insecticides	
Accothion	O,O-dimethyl-O-3-methyl-4-nitrophenyl phosphorodithioate
Thimet	O,O-diethyl S-2-(ethylthio) methyl phosphorodithioate
Fungicide	
Perenox	Cuprous oxide

APPENDIX THREE - Metric - Imperial Conversion Table

1 cm	=	0.39 in
1 kg/ha	=	0.89 lb/ac
1 ha	=	2.47 ac
1 litre	=	0.22 gallons
1 kg	=	2.2 lb
1 tonne/ha	=	8 cwt/ac
1 litre/ha	=	0.71 pints/ac