# CELLULOSE BREAKDOWN IN SOIL AND IN MODEL SOIL ENVIRONMENTS

A thesis submitted in accordance with the requirements of

THE UNIVERSITY OF KENT AT CANTERBURY

for the degree of

Doctor of Philosophy

by

CAROL F. A. HOPE

Biological Laboratory

June 1983

*To my parents*

## ABSTRACT

Cellulose is the major cell wall polymer of plants and is deposited in enormous quantities in terrestrial environments. Thus an understanding of the processes involved in cellulose decomposition is of importance with reference to carbon cycling in the biosphere.

In order to degrade a polymeric insoluble substrate such as cellulose which is discontinuously distributed in soil, a microorganism must rely on extracellular enzymes which are likely to be rapidly inactivated. Furthermore, constitutive production and secretion of extracellular enzymes by soil microorganisms in the prolonged absence of substrate is an inefficient and ultimately fatal use of cell energy. It has been suggested that, in addition to enzymes associated with viable microorganisms, an immobilized and hence stabilized enzyme fraction may have a significant role in substrate decomposition.

In this work, we have developed assays and measured cellulase activities (exo- and endoglucanase, β-D-glucosidase) in air-dried soil using the substrates : Avicel, carboxymethyl cellulose, cellobiose and p-nitrophenyl-β-D-glucopyranoside. From an extensive characterization of these activities (in terms of pH-activity optima, thermostability and resistance to proteolysis), we have been able to speculate as to the origin and location of these enzymes in soil, and to evaluate the role of immobilized and other forms of enzymes in cellulolysis.

The persistence and efficacy (in terms of locating substrate) of 'free' (soluble), extracellular enzymes in soil was investigated using a novel technique developed for this purpose. This 'barrier ring plate' technique has allowed us to study extracellular enzyme diffusion and microbial growth through soil-like but carefully controlled environments.

Using the barrier ring diffusion plates we demonstrated that whole soil and bentonite (a high unit surface area smectite clay) impeded the diffusion of both β-D-glucosidase and endoglucanase and subsequent substrate hydrolysis. Whilst sand or kaolin (a low unit surface area clay) had no significant effect on enzyme diffusion or substrate hydrolysis.

We have further used barrier ring plates to demonstrate how soil components differentially affect the radial growth of three fungal soil isolates : *Trichoderma viride, T. koningii, Botryotrichum piluliferum*; and a *Streptomyces* sp. However, the interpretation of the data from these growth experiments is limited by our poor understanding of the genetic and environmental factors which influence the morphology of the developing colony. The main advantages of the barrier ring plates lie in their versatility and in the fact that they provide a vectorial system in which the influence of soil components on the comparatively complex spatial interactions between microorganisms, enzymes and substrates may be studied.

CONTENTS

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to:

Dr. R.G. Burns, my supervisor for his enthusiasm, guidance and encouragement throughout this research programme and for his constructive criticism of the manuscript.

J.M. Alexander and R.J. Geraghty for permission to quote the work on β-D-glucosidase. Fellow members of the fourth floor, especially P.J. Sallis, for helpful discussion and advice.

Dr. R.B. Freedman for advice on the enzymology, Dr. P. Jeffries and Dr. H. Wildman for help with identification of the fungal isolates. R. Newsam for help with the photography and all the support staff whose help made this work possible.

Dr. B. Morgan and E. Henness for advice on statistical treatment of my data and the Computing laboratory for their advice and the use of their facilities.

Mr. Amos for permission to collect soil from his land.

Miss Sylvia Reeve for her excellent typing of the manuscript.

Professor A.T. Bull and K.A. Stacey for allowing me to carry out this work in their department.

The ARC and the University of Kent for financial support.

# CHAPTER 1 : INTRODUCTION

Cellulose is the major cell wall polymer of plants and is thus regarded as an important renewable resource. The net worldwide production of cellulose is estimated (Spano, 1976) at $1 \times 10^{11}$ tonne annum$^{-1}$ and since a significant proportion of this (*ca.* 5 to 10%) occurs as municipal, industrial and agricultural waste, it is not surprising that great efforts have been made to understand the physical, chemical and microbiological factors which influence cellulose decay.

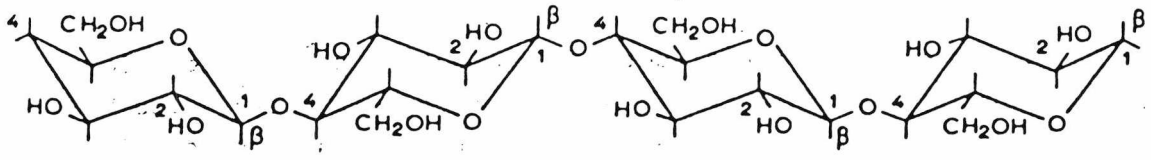
## 1.1 CELLULOSE STRUCTURE AND MORPHOLOGY

The natural fibres of cotton and wood are the most important commercial sources of cellulose. Cotton contains 98% dry weight cellulose while the level in wood is 40-50% depending on plant species. The celluloses of cotton and wood are linear polymers of D-anhydroglucopyranose units linked by β-1,4-glucosidic bonds (Fig. 1.1a). The number of glucose units per molecule ranges from as little as 15 to greater than 10 000, the latter indicating a molecular weight above $1.5 \times 10^6$ daltons and a chain length of about 5 μm. Numerous reviews (e.g. Ranby, 1969; Sihtola and Niemo, 1975; Virkola, 1975; Fan *et al.*, 1980a) on the composite structure of cellulose are available. This discussion therefore, will be limited to structural and morphological features of cellulose pertinent to understanding the mechanism of enzymic hydrolysis.

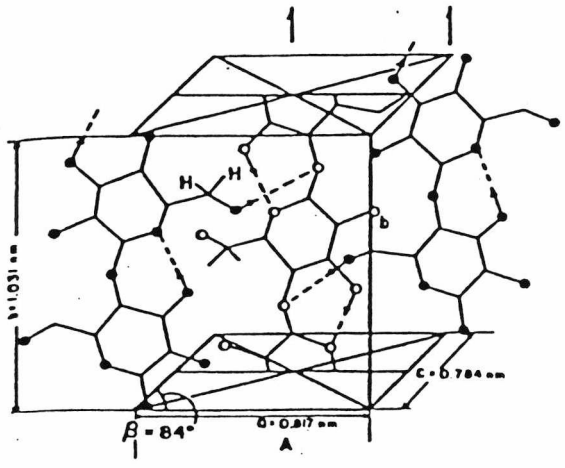
The repeat unit in the cellulose molecule is the glucose dimer, cellobiose. Individual cellulose molecules are linked together to form highly ordered structural aggregates called elementary fibrils or protofibrils. Elementary fibrils have approximate dimensions 3-4 nm x 10 nm and contain polymer chains firmly bound by hydrogen bonds (Fig. 1.1b). Elementary fibrils are the smallest structural units of cellulose and are grouped

FIGURE 1.1

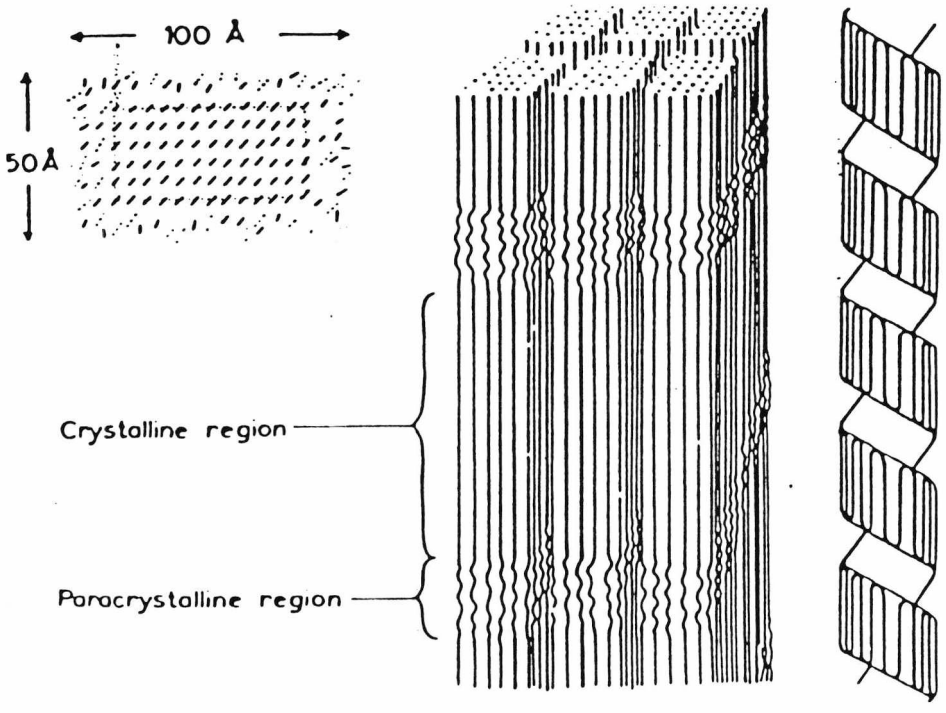
C E L L U L O S E  S T R U C T U R E



a) Conformational formula ($^4C_1$ chair form).



b) Arrangement of molecules and hydrogen bonds in a cellulose 1 unit cell.




c) Recent concepts in the structure of cellulose microfibrils. (Cowling and Kirk, 1976)

together to form microfibrils and fibres. Elementary fibrils and microfibrils are not visible in untreated cotton or wood and hence there is some controversy as to which are real and which are artificial structures. This controversy is illustrated by the three models proposed for the structure of the microfibril (Fig. 1.1c). It is generally accepted however, that cellulose contains both highly ordered, crystalline regions and less ordered amorphous or para-crystalline regions; the latter being more susceptible to enzymic hydrolysis.

Direct contact between the cellulase enzyme molecule and the insoluble cellulose surface is a prerequisite for hydrolysis. Fan *et al.* (1980b) investigated the relationship between the structural properties of cellulosic substrates, specifically, surface area and degree of crystallinity, and the rate of enzymic hydrolysis. They concluded that the rate of hydrolysis is determined by the degree of crystallinity of the substrate rather than by the surface area. They further showed that the total available surface area was not the major rate limiting factor in the later stages of hydrolysis as had been previously suggested (Stone *et al.*, 1969) and they proposed that the total surface area could be subdivided into regions which were susceptible to enzyme attack and regions which were resistant. Further evidence for the existence of greater than one type of surface in native cellulose comes from the work of Rowland (1975). Rowland (1975) used chemical reagents to measure the accessibility of the hydroxyl groups in the D-glucopyranosyl chains on the surfaces of the elementary fibrils and he proposed three types of surfaces (Fig. 1.2) based on the extent to which the O(3)H...O(5') and O(6)H...O(1'') hydrogen bonds were disrupted. The most inaccessible and hence highly ordered surfaces (A) were found adjacent to, and hydrogen bonded to, other highly ordered surfaces. The B surfaces were so called since they contained imperfections in the hydrogen bonding order while the C surfaces were disrupted as a result of twist and tilt in the

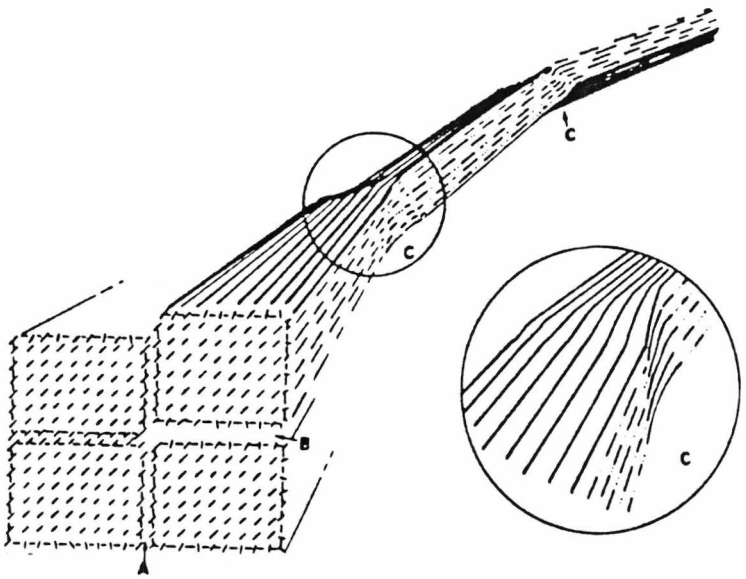



FIGURE 1.2 (from Rowland, 1975)

Scheematic representation of the cellulose elementary fibril to show: A, coalesced surfaces of high order; B, readily accessible, slightly disordered surfaces; and C, readily accessible surfaces of strain-distorted tilt and twist regions.

microfibril.

Apart from the degree of crystallinity and surface area, other structural features affecting the susceptibility of cellulosic substrates to enzymic degradation include the degree of water swelling, the presence of substituent groups and the content of associated materials such as lignin or pectin (Fan *et al.*, 1980a).

## 1.2 ENZYMIC CELLULOSE DECOMPOSITION

### 1.2.1 The Enzymes

Over the past thirty-five years much research has been directed at elucidating the mechanisms involved in the enzymic decomposition of cellulose. However, some controversy still exists and is reflected in the confused nomenclature of cellulases. A survey of the literature reveals a variety of names applied to cellulolytic enzymes and activities, many of which are only justified in relation to the substrate hydrolysed, e.g. CMCase and FPase (Sternberg, 1976), Avicelase (Tomita *et al.*, 1974), Hydrogen bondase (Eriksson, 1969) and Swelling factor (Nisizawa, 1973). Because of this confusion in terminology, the major types of hydrolytic enzymes involved in the decomposition of cellulose will be presented prior to a discussion on the postulated mechanisms.

Endo-1,4-β-glucanase (EC 3.2.1.4)

Systematic name: 1,4-(1,3;1,4)-β-D-Glucan-glucanohydrolase.

Reaction: Endo hydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans.

Alternative names: Cellulase, endoglucanase, CMCase.

Endoglucanase is in fact not one but a group of enzymes which are responsible for the hydrolysis of high molecular weight glucose chains. Acting on soluble cellulose derivatives (section 2.2.2) endoglucanases cause a rapid decrease in viscosity with a relatively slow increase in reducing end groups indicating a random cleavage of the β-1,4-linkages. Endoglucanases can also hydrolyse the soluble cellodextrins, the rate of hydrolysis increasing with the degree of polymerization within the limits of substrate solubility. The dimer cellobiose can be hydrolysed by some endoglucanases but not by all. Endoglucanases have been purified from several cellulolytic microorganisms: *Trichoderma viride* (Berghem *et al.*, 1976; Hakansson *et al.*, 1978; Shoemaker and Brown, 1978), *Trichoderma koningii* (Wood and McCrae, 1978), *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*) (Eriksson and Pettersson, 1975a), *Fusarium solani* (Wood, 1971), *Irpex lacteus* (Kanda *et al* ., 1979), *Pseudomonas* sp. (Ramasamy and Verachtert, 1980), *Cellulomonas flavigena* (Beguin and Eisen, 1978), *Cytophaga* sp. (Chang and Thayer, 1977). These enzymes have been found to be glycoproteins having molecular weights in the range 5 000 - 65 000 daltons. Wood and McCrae (1978) have purified four distinct endoglucanases from *T. koningii* and demonstrated differences in the degree of randomness of attack of these enzymes on carboxymethylcellulose (CMC) by plotting the decrease in viscosity against the change in reducing sugar concentration. A randomly-acting endoglucanase would be expected to produce a rapid decrease in viscosity in relation to the appearance of reducing sugars, while a less randomly-acting endoglucanase would produce more reducing sugars for an equivalent drop in viscosity.

Exo-1,4-β-glucanase

This group includes two classes of enzyme:

[1] Exo-1,4-β-D-glucosidase (EC 3.2.1.74)

Systematic name: 1,4-β-Glucan glucohydrolase.

Reaction: Hydrolysis of 1,4-linkages in 1,4-β-D-glucans resulting in the successive removal of glucose units.

Alternative name: Exoglucanase.

[2] Exo-cellobiohydrolase (EC 3.2.1.91)

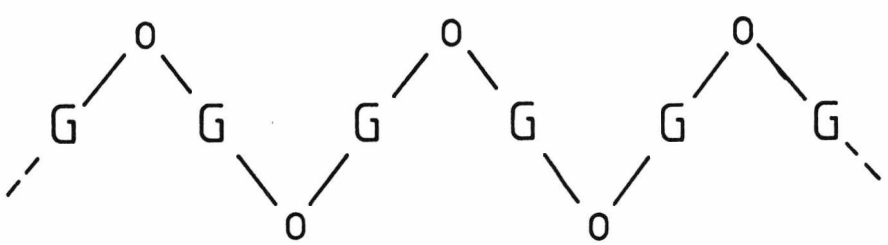
Systematic name: 1,4-β-D-Glucan cellobiohydrolase.

Reaction: Hydrolysis of 1,4-β-D-glucosidic linkages in cellulose, releasing cellobiose from the non-reducing ends of the chains.

Alternative names: CBH, exoglucanase.

No one has succeeded in purifying a specific exo-1,4-β-D-glucosidase and shown it to be distinct from endo-1,4-β-D-glucosidase and β-D-glucosidase. In the few instances where exo-1,4-β-D-glucosidase has been reported (Wood *et al.*, 1980), no synergism was observed with endoglucanases. The rest of this discussion will therefore refer to the exo-cellobiohydrolase enzymes, hereafter called exoglucanases. The term cellulase will be used to describe combined exoglucanase and endoglucanase activity. [It is more reasonable, in terms of active site-substrate interaction (Doonan *et al.*, 1970), for an enzyme moving along the glucose chain to cleave every alternative glucosidic linkage releasing cellobiose. This is because the repeat unit is cellobiose and not glucose (Fig. 1.3).]

FIGURE 1.3



Exoglucanases have been purified from *Trichoderma viride* (Berghem *et al.*, 1975), *T. koningii* (Wood and McCrae, 1972), *Penicillium funiculosum* (Wood *et al.*, 1980), *Sporotrichum pulverulentum* (Eriksson and Pettersson, 1975b) and other cellulolytic fungi.

In the absence of a specific assay for exoglucanase activity, the enzyme has generally been identified by its lack of activity against CMC and by its ability, when combined with endoglucanases, to bring about the extensive hydrolysis of crystalline cellulose. Activity against $H_3PO_4$-swollen cellulose has been reported for a number of exoglucanases (Wood and McCrae, 1972; Berghem and Pettersson, 1973; Wood *et al.*, 1980) and in all cases the principle product was cellobiose. The estimated molecular weights of the exoglucanases are in the range 40 000 - 62 000 daltons (Lee and Fan, 1980).

β-D-Glucosidase (EC 3.2.1.21)

Systematic name: β-D-Glucoside glucohydrolase.

Reaction: Hydrolysis of terminal non-reducing β-D-glucose residues with release of β-D-glucose.
Transfer of glucose units to other sugar alcohols.

Alternative names: Cellobiase, aryl-β-D-glucosidase.

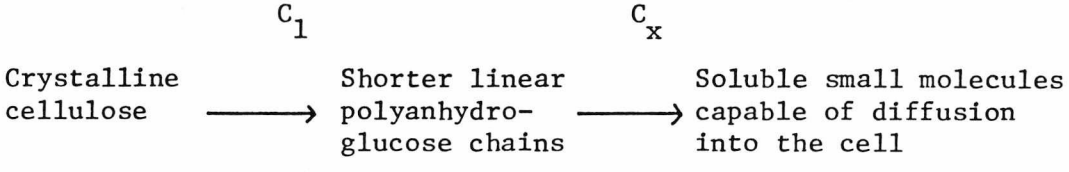
β-D-Glucosidases hydrolyse cellobiose and the lower cellooligosaccharides to produce glucose. β-D-Glucosidases have been isolated and purified from numerous fungi and bacteria (King and Vessal, 1969). The IUB Nomenclature Committee designation does not however distinguish between aryl-glucosidase and cellobiase and whilst β-D-glucosidases are mostly assayed using nitrophenyl-β-D-glucopyranoside as the substrate, it has been reported that some aryl-β-D-glucosidases are unable to hydrolyse cellobiose (Jermyn, 1955). Reported molecular weights of purified β-D-glucosidases are in the range 40 000 - 400 000 daltons.

Endoglucanases, exoglucanases and β-D-glucosidases constitute the three major classes of hydrolytic enzyme involved in cellulose decomposition (Table 1.1 summarizes the major properties of these enzymes). In addition, cellobiose quinone oxidoreductase (CBQ) and cellobiose oxidase have been shown to be produced by the white-rot fungus *Sporotrichum pulverulentum* (Eriksson, 1978) while some bacteria have been reported to cleave cellobiose by phosphorolysis (King and Vessal, 1969; Schimz *et al.*, 1979). Whilst the possible existence of other cellulolytic systems (containing novel enzymes) cannot be dismissed, it is likely that all of the enzyme activities so far reported, could be fitted into one of the three classes.

### 1.2.2 The Mechanism of Enzymic Cellulose Decomposition

The first multi-enzyme hypothesis to account for enzymic cellulose depolymerization was the $C_1$-$C_x$ system postulated by Reese *et al.* (1950) and invoked to explain the observation that some microorganisms were able to degrade amorphous cellulose but not crystalline cellulose (Fig. 1.4).

FIGURE 1.4: Cellulose depolymerization according to Reese *et al.* (1950).




The truly cellulolytic microorganisms (i.e. those capable of causing extensive hydrolysis of crystalline cellulose) were thought by Reese and coworkers to produce an extra component ($C_1$) believed to act on crystalline cellulose modifying it in such a way as to make it susceptible to hydrolysis by the $C_x$ enzymes (the subscript x emphasizes the multicomponent nature of this fraction). Many workers (e.g. Berghem and Pettersson, 1973; Halliwell and Griffin, 1973; Wood and McCrae, 1975) in attempting to relate their findings to this hypothesis have equated $C_x$ with the endoglucanases and $C_1$ (defined

TABLE 1.1 Enzymes Hydrolysing β-Glucosidic Linkages (after Barras *et al*, 1969)

| | ARYL-β-GLUCOSIDE HYDROLASES | β-D-GLUCOSIDE HYDROLASES | β-GLUCAN EXO-HYDROLASES | β-GLUCAN ENDO-HYDROLASES |
|---|---|---|---|---|
| Specificity for position of linkage, i.e. 1,3;1,4 etc. | Not applicable | Low | High or absolute | High or absolute |
| Specificity for glycosyl residue | High<br>Preference for β-glucopyranosyl | High<br>Preference for β-glucopyranosyl but probably others | High<br>Glucopyranosyl residues preferred but accepts 6-substituted and xylopyranosyl residues | Absolute |
| Specificity for aglycone | High<br>Aryl alchohols | Low<br>Alkyl, aryl and oligosaccharides | High<br>Glucans preferred but oligosaccharides cleaved | High (but variable)<br>Must involve β-glucanopyranosyl residue(s) |
| Rate of hydrolysis of oligomers | Not applicable | di>tri>tetra $\gg$ penta | tri<tetra<penta $\ll$ polymer | tri<tetra<penta $\ll$ polymer |
| Inhibition by 1,5 gluconolactone | unknown | $K_i$ low | $K_i$ high | Not inhibited |
| Configuration of anomeric OH released | unknown | β (i.e. configuration retained | α (i.e. configuration inverted | β (i.e. configuration retained |
| Transfer activity | + | + | $\pm$ | $\pm$ |
| Specificity for transfer acceptors | Low | Variable | unknown | High |

as the enzyme which in conjunction with the endoglucanases causes solubilization of highly ordered cellulose) with exoglucanase (cellobiohydrolase) activity. This practice has lead to considerable confusion for if a sequential process of saccharification is countenanced (i.e. one enzyme acting after another), it is more likely to be a $C_x$-$C_1$ sequence (i.e. exoglucanase acting on the free chain ends produced by the endoglucanase) than a $C_1$-$C_x$ sequence. In the light of these developments Reese (1977) modified his original $C_1$-$C_x$ hypothesis and suggested that $C_1$ was an endoglucanase but that it was different from the $C_x$ endoglucanases because it was:

1) active on crystalline cellulose,
2) able to disrupt hydrogen bonds,
3) inactive on CMC, and
4) unable to produce soluble products from crystalline cellulose.

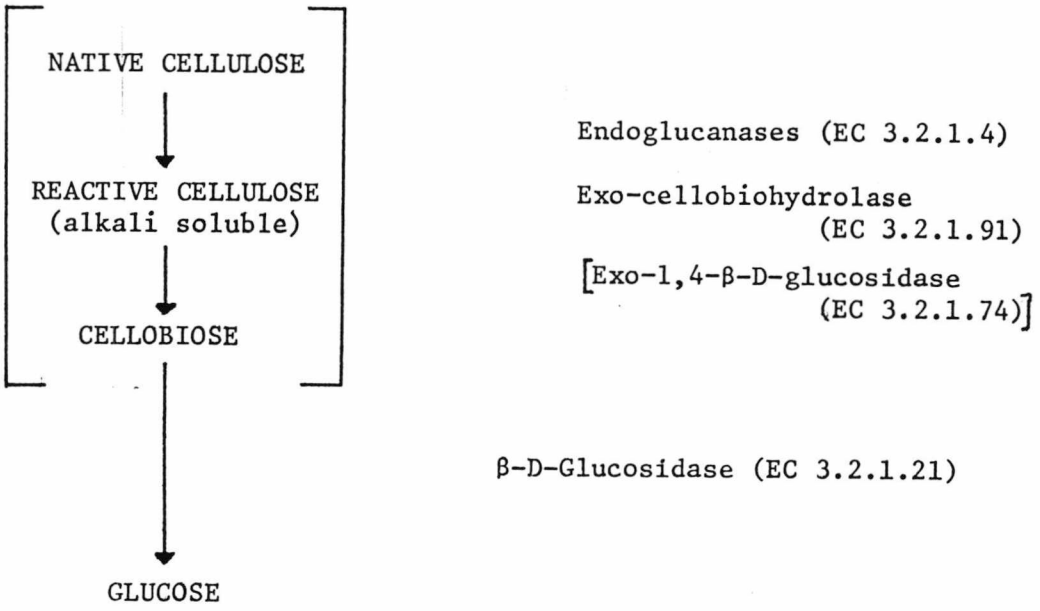
Because of the confusion with the $C_1$-$C_x$ terminology, and the likelihood that the hydrolytic events occur in parallel (i.e. the exo- and endoglucanase acting as a complex - see section 1.2.3) rather than sequentially, a more generally accepted and useful scheme is shown in Fig. 1.5. In addition to its hydrolytic role, the exoglucanase (cellobiohydrolase) may have a locating role in that it is known to be strongly adsorbed to cellulose (Peitersen *et al.*, 1977; Ghose and Bisaria, 1979; Reese, 1982) although it is the endoglucanase which makes the initial cleavage of the glucose chain. The exoglucanase then prevents the rejoining of the free ends produced by removing successive cellobiose units from the non-reducing end of the chain. β-D-Glucosidases are responsible for the hydrolysis of cellobiose to glucose.

### 1.2.3 Synergism

Evidence for the synergistic action of exo- and endoglucanases on crystalline cellulose substrates has come from studies by Wood and McCrae (1978). Working with *Trichoderma koningii* they observed synergistic action

FIGURE 1.5

ENZYMIC CELLULOSE HYDROLYSIS



between the single exoglucanase and each of the four distinct endoglucanases found in this species. Synergistic effects were also observed when exoglucanase ($C_1$) and endoglucanase ($C_x$) from various fungal sources were combined (Table 1.2; Wood and McCrae, 1979).

'Cross-synergism' (an expression used by Wood and McCrae, 1979) was most marked between the exoglucanase and endoglucanase components of fungi that freely release exoglucanase in the active form and the authors speculated that the two enzymes may interact in some way to form a complete cellulase complex. Protein-protein interactions have also been proposed by Leatherwood (1969) who, working with *Ruminococcus* spp., suggested that cellulose degradation by these organisms involved the interaction of an affinity factor and a hydrolytic factor which together formed an active cellulase complex. The affinity of the exoglucanase for the different endoglucanases of *Trichoderma koningii* could explain the different degrees of synergism observed (Wood and McCrae, 1978) and would support the notion of a non-hydrolytic (i.e. interaction with the endoglucanase) as well as a hydrolytic function of the exoglucanase.

Exo- and endoglucanases are inhibited by cellobiose (Mandels and Reese, 1963; Berghem *et al.*, 1975; Huang, 1975a; Hsu *et al.*, 1980) thus the activity of β-D-glucosidase is essential to maintain the momentum of hydrolysis. Increases in the rate of saccharification have been obtained by supplementing *Trichoderma viride* cellulase (containing only low levels of β-D-glucosidase) with β-D-glucosidase from *Aspergillus* spp. (Sternberg *et al.*, 1977).

## 1.3 CELLULOLYTIC MICROORGANISMS

### 1.3.1

Microorganisms producing enzymes capable of hydrolysing β-D-glucosidic linkages are widely distributed among the various taxa. The ability to utilize cellulose is found amongst bacteria (including actinomycetes) and fungi, although the rate and extent of cellulose decomposition differs

TABLE 1.2 (from Wood and McCrae, 1979)

a) Synergistic effect on cellulase activity shown by combination of $C_1$ and $C_x$ from *Trichoderma koningii*, *Fusarium solani* and *Penicillium funiculosum*.

| Source of $C_1$ component | Source of $C_x$ component | Solubilization of cotton (%) |
|---|---|---|
| *T. koningii* | *T. koningii* | 54 |
| *T. koningii* | *F. solani* | 79 |
| *F. solani* | *F. solani* | 59 |
| *F. solani* | *T. koningii* | 51 |
| *P. funiculosum* | *P. funiculosum* | 72 |
| *P. funiculosum* | *T. koningii* | 51 |

b) Synergism between the $C_1$ component of *Trichoderma koningii* and the $C_x$ culture filtrates of other fungi.

| | Solubilization of cotton (%) |
|---|---|
| *Myrothecium verrucaria* | 20 |
| *Stachybotrys atra* | 21 |
| *Gliocladium roseum* | 15 |
| *Memnoniella echinata* | 21 |

c) Synergism between the $C_1$ component of *Penicillium funiculosum* and the $C_x$ culture filtrates of other fungi.

| | Solubilization of cotton (%) |
|---|---|
| *Myrothecium verrucaria* | 20 |
| *Stachybotrys atra* | 11 |
| *Gliocladium roseum* | 14 |
| *Memnoniella echinata* | 18 |

between species. For instance, many fungi have been shown to produce exo- and endoglucanases as well as β-D-glucosidase and thus are able to grow using native cellulose as their sole carbon source and independent of other cellulolytic microorganisms (Enari and Markkanen, 1977). On the other hand, some fungi and many bacteria can only hydrolyse soluble cellulose derivatives (Reese *et al.*, 1950; Mandels, 1975) and presumably need to be part of a microbial community in order to grow on highly ordered insoluble cellulose.

Another major difference between the cellulase systems of different microbial species is in the number and molecular weights of the enzymes collectively termed exoglucanase, endoglucanase and β-D-glucosidase. Two exoglucanases (Gritzali and Brown, 1979) and two endoglucanases (Berghem *et al.*, 1976) have been purified from *Trichoderma viride*. As many as four endoglucanases have been reported for *T. koningii* (Wood and McCrae, 1978) and five for *Sporotrichum pulverulentum* (Eriksson and Pettersson, 1975a) which also produces two β-D-glucosidases (Deshpande *et al.*, 1978). Recent studies (Nakayama *et al.*, 1976; Gritzali and Brown, 1979) on the multiplicity of endoglucanases in *T. viride* enzyme preparations indicate that these may arise as a result of proteolytic degradation of a single parent endoglucanase. By contrast, Eriksson and Pettersson (1968) suggested that the two endoglucanases produced by *Sterum sanguinolentum* growing on powdered cellulose, differed only in respect to their carbohydrate moities. A third possibility is that the low molecular weight endoglucanases produced by some species Lee and Fan (1980), may be subunits or components of a high molecular weight enzyme. It is important, however, to remember that the different endoglucanases appear to differ in the nature of their attack on CMC (see section 1.2.1). Cellulolytic microorganisms also differ in the location and regulation of their cellulolytic enzymes.

1.3.2 Location of Cellulase Enzymes

Cellulose is insoluble in water, therefore to accomplish cellulolysis a microorganism must possess an extracellular enzyme system capable of producing soluble products that can pass into the cell.*

While considerable information is available on the nature of fungal cellulase systems there are relatively few studies on the physiological basis of extracellular cellulase secretion in fungi. The exo- and endo-glucanases are normally recovered in the culture filtrate from fungi grown on insoluble cellulose derivatives and it is generally assumed that these enzymes are secreted by living cells (Enari and Markkanen, 1977). However, the appearance of enzymes in the culture filtrate is not sufficient evidence to describe an enzyme as physiologically extracellular (Pollock, 1962). Berg and v. Hofsten (1976) using *Trichoderma viride* reported that cellulase enzymes only appeared in the culture medium after the stationary phase had been reached by which time transmission electron microscopy (TEM) studies revealed that extensive cell lysis had occurred. Analysis of the carbohydrates of the cell wall after hydrolysis revealed the presence of glucose, mannose and galactose - the same carbohydrates as reported to be present in purified endoglucanases from *T. viride* (Berg and Pettersson, 1977). This suggests that the enzymes may be bound to the cell wall of living cells and retain some of the associated carbohydrate on lysis. A similar view was taken by Kubicek (1981) who suggested that enzyme release occurred after cleavage of covalent bonds of certain wall polymers. Release of enzymes with different amounts of carbohydrate moiety could partially account for differences in molecular weights (Section 1.3.1).

---

*N.B. There is some confusion in the literature concerning the word extracellular and for the purpose of this discussion I would like to use the term to describe enzymes which are external to the cell wall and in contact with the surrounding medium. Thus extracellular enzymes may be cell wall bound as well as being located at a distance from their parent microorganisms.

Whether released by active secretion, leakage or following cell lysis, there can be no doubt that the majority of exo- and endoglucanases produced by fungi in axenic cultures retain activity when separated from their parent cell. Rosenberg (1980), using a diffusion chamber which allowed the continuous passage of enzymes and metabolites between a growing mold in one chamber and a moist lignocellulose substrate in another, reported that all twelve of the fungal species tested were able to bring about some degradation of carbohydrate in the sterile chamber.

Several cellulolytic bacteria are thought to retain their cellulases on their outer membrane. Ultrastructure studies of *Cellvibrio fulvus* and *Sporocytophaga myxococcoides* growing on different types of cellulose fibre (Berg *et al.*, 1972b) show vegetative bacteria in close contact with the cellulose. Supernatant liquids from centrifuged cultures of *S. myxococcoides* were completely inactive against Avicel and it was concluded that direct contact between the living bacterium and the cellulose was necessary for effective degradation to occur. Further investigations (Berg, 1975) showed that the location of cellulase in *Cellvibrio fulvus* depended on the carbon source and it was suggested that the presence of cellulose or a cellulose derivative in the medium may cause release of cellulase from cells.

v. Hofsten (1975) has proposed that cellulases are more effective when cell bound than when free in the medium. This is because the local enzyme concentration is increased and the formation of a series of enzyme-substrate complexes along the microfibril is thought to cause comformational changes in the molecule facilitating the hydrolysis of covalent bonds.

Investigations of the location of the cellulase complex are further complicated by the fact that many exo- and endoglucanases are readily bound to cellulose thus being removed from the culture medium (Béguin *et al.*, 1977; Peitersen *et al.*, 1977; Ghose and Bisaria, 1979; Lee *et al.*, 1980).

The adsorption of *Trichoderma viride* cellulase enzymes to cellulose produced by *Acetobacter xylinum* has been confirmed by White and Brown (1981). Using TEM they observed cellulose samples which had been incubated with cellulase solutions for 5 min. Particles were seen on the surface of the cellulose ribbons which had the same range of diameters as the particles seen in preparations of the enzyme system alone. These particles were not removed by washing with buffer. After 10 min exposure to cellulase the cellulose ribbons were splayed into bundles and after 30 min the ribbons were completely degraded.

In most cases β-D-glucosidases appear to be located intracellularly although an extracellular β-D-glucosidase has been reported for *Trichoderma viride* (Herr, 1979).

### 1.3.3 Regulation of Cellulase Enzymes

Cellulolytic microorganisms differ in the regulatory mechanisms used to control the production of their cellulolytic enzymes. The need to produce higher levels of cellulases for commercial use has stimulated interest into unravelling the complexities of these regulatory mechanisms. Sophorose has long been known to be a potent inducer of cellulases in *Trichoderma viride* (Mandels *et al.*, 1962) while glucose acts as a repressor (Nisizawa *et al.*, 1972). The regulation of cellulases in *Sporotrichum pulverulentum* has been discussed by Eriksson and Hamp (1978) who reported that, unlike the *T. viride* system, cellobiose was a better inducer of endoglucanases than sophorose. Catabolite repression of the cellulases of *Myrothecium verrucaria* (Hulme and Stanks, 1971) and of *Sporotrichum thermophile* (Canevascini *et al.*, 1979) has been demonstrated.

Unlike the inducible system of *Trichoderma viride*, cellulase has been shown to be produced constitutively in *Pseudomonas fluorescens*, its formation being subject to catabolite repression (Suzuki *et al.*, 1969). From studies on mutants of a *Cellulomonas* sp., Stewart and Leatherwood (1976) were able

to conclude that regulation of cellulase production in this organism is a complex mechanism including both an inducer and a repressor system. Berg (1975) suggested that the presence of cellulose or a cellulose derivative in the medium caused release of cellulase from the cell walls of *Cellvibrio fulvus* and could thus stimulate further enzyme synthesis. This is of interest since it would provide a control mechanism not requiring the presence of an intracellular inducer. A similar mechanism has been proposed by Ishmande (1970) for penicillinase in *Bacillus cereus*.

Gong and Tsao (1979) have produced a unified model for the regulation of cellulase biosynthesis which incorporates the observed diversity of induction and repression mechanisms (Fig. 1.6). The salient points of the model are as follows.

[1] The importance of β-D-glucosidase in the regulation of intracellular levels of glucose and cellobiose.

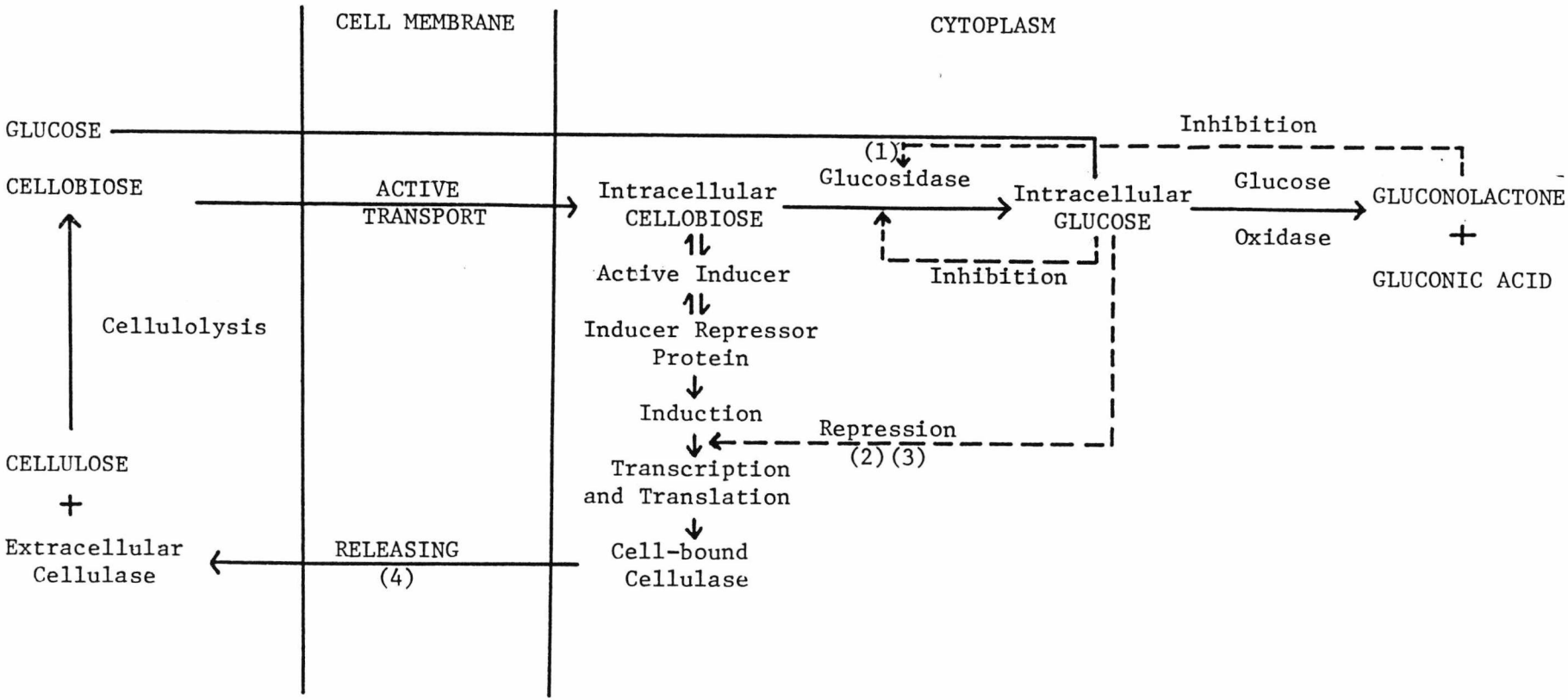
[2] The essential low-level constitutive synthesis of cellulase for providing initial inducer.

[3] Co-ordinate gene expression for both exo- and endoglucanases.

[4] Regulation of the actual amount of extracellular cellulase produced by active releasing mechanisms.

Exoglucanase is inhibited competitively by cellobiose and, to a much lesser extent, by glucose (Mandels, 1982). Hong *et al.* (1981) have produced an equation for the combined product and substrate inhibition of β-D-glucosidase from *Trichoderma viride* while inhibition of cellulases and β-D-glucosidases by a variety of physical and chemical factors has been reported by Mandels and Reese (1963).

FIGURE 1.6 Proposed Model for Regulation of Cellulase Biosynthesis (after Gong and Tsao, 1979)



## 1.4 CELLULOSE DECOMPOSITION IN SOIL

### 1.4.1

Enormous quantities of organic debris are deposited each year into terrestrial environments, plant litter alone accounting for 1.0-15.3 tonne hectare$^{-1}$ annum$^{-1}$ (Williams and Gray, 1974). Since cellulose is the major constituent of plant material (up to 98% dry weight), the decomposition of this carbohydrate in soil has a special significance in the biological cycling of carbon.

The rate at which cellulose is degraded in different soils varies with the soil's physical, chemical and biological properties. The major environmental factors (included amongst these) affecting transformations are; nitrogen availability, temperature, aeration, moisture, pH and the relative proportion of lignin and other recalcitrant polymeric molecules in the plant residue (Alexander, 1977). Cellulolytic microorganisms are common in field and forest soils, in manure and on decaying plant tissues and the large number of different species permits the transformations to occur in habitats with or without oxygen, at acid or alkaline pH, at low or high moisture levels and at temperatures from just above freezing to those tolerated by thermophilic microorganisms.

### 1.4.2 Biodeterioration Studies

Many different approaches have been adopted in order to investigate cellulose decomposition in soil (Burns, 1982a); one practice has been to incubate [$^{14}$C]-labelled plant material (or other cellulosic substances) with soil and to measure the rate of [$^{14}$C]-$CO_2$ evolution. Another rather different approach has been to observe the succession of microorganisms which colonize decaying wood or plant litter (Hancock and Benham, 1980).

The use of [$^{14}$C]-labelled fractions of plants has allowed investigations into the fate of plant cellulose in soil. Cheshire *et al.* (1973) showed that

over 20% of the cellulose still remained after soil mixed with 0.2% [$^{14}$C]-labelled rye had been incubated for 16 months. While Martin and Haider (1979) found that carbon losses from a Chino loam soil following addition of [$^{14}$C]-cellulose were independent of the initial concentration of added substrate in the range 1 - 50 000 µg (g soil)$^{-1}$.

Investigations regarding the organisms involved in soil cellulose decomposition, have revealed that faecal pellets of soil arthropods provide a more favourable habitat for litter microorganisms than whole leaf litter (Lodha, 1974). The suitability of dung material for microbial activity may be attributed to its high content of nitrogen and readily assimilable carbohydrate. Cellulose activity has been found in extracts prepared from soil molluscs and oligochaetes (Hartenstein, 1982). However, the origin of this activity (e.g. soil invertebrate or gut microflora) was not investigated. Many reports describe fungal succession occurring on wood and plant litter (Swift, 1977), while information concerning the microbial colonization of cellulose film buried in soil has been provided by the work of Tribe (1960). Tribe (1960) studied the colonization of cellulose film in various Canadian soils and observed a general pattern of succession in which the initial colonizers were cellulolytic fungi the predominant species being *Rhizoctonia solani*, *Humicola* spp. and *Botryotrichum* spp. Bacteria and nematodes did not appear until after the intial fungal colonization and the later stages of degradation and disintegration were caused by the larger soil fauna such as mites and collembola which devoured the partially decomposed cellulose film along with its microbial colonizers.

### 1.4.3 The Soil Environment

Measurements of the overall cellulose decomposing process in soil (i.e. $CO_2$ evolution) can be used for characterizing soils in terms of cellulose decomposing potential as well as for identifying the major factors which influence decomposition rates. However, these methods of investigation

do not allow a detailed study of the microbial ecology of soil cellulose decomposition. For this, it is necessary to study the individual organisms, or communities involved in cellulose decomposition and to measure the physical and chemical properties of the microenvironment in which they reside. It is also necessary to characterize the cellulolytic enzymes produced by these organisms or communities and to investigate their activity and persistence in a soil environment.

Microorganisms in soil, like plants and animals are components of integrated communities (Atlas and Batha, 1981). Ultimately, the success or failure of an individual species will be determined by the availability of suitable substrates within its immediate environment and the capacity of the microorganism to respond to those substrates.

The concept of the microbial microenvironment in soil has been discussed extensively in recent years (Stotzky, 1974; Burns, 1980; Stotzky and Burns, 1982). Implicit in these articles is the generally accepted premise that microbial activity predominates on, or in close proximity to the surface of soil particulates rather than in the bulk solution. As a result the most influential soil particles as far as microbial activity is concerned are the clays and humic material since they have:

[1] high surface to volume ratios;

[2] ionic properties such that, cations are usually attracted to their surfaces and anions repelled; and

[3] a high affinity for water.

The other principle soil particulates; sand and silt, have low unit surface areas, negligible ion exchange capacities, and do not accumulate organic matter to any great extent. They are thus relatively inert when compared to the colloid-size fraction.

The principle colloid-size particles in soil consist of the clay minerals; oxides and hydroxides of aluminium, silicon, iron, manganese and titanium; and the soil organic matter. Clay minerals are comprised of two basic arrangements of molecules exemplified by the two clay types; kaolinite and smectite (Table 1.3). Silicon oxide tetrahedra and aluminium oxide or hydroxide octahedra are assembled either in a 1:1 ratio (-Si.Al.Si.Al.Si-) or a 2:1 ratio (-Si.Al.Si.Si.Al.Si-). The layers of the 1:1 silicates are tightly held together by hydrogen bonds whilst those of the 2:1 silicates are loosely associated by van der Waal forces. Thus 2:1 smectite clays such as bentonite will expand on wetting and have a high surface area and cation exchange capacity (C.E.C.) compared to the 1:1 kaolinite clays.

Soil organic matter can be considered as being composed of three fractions (Burns, 1983).

[1] A macroscopic, physically recognizable fraction, containing plant, animal and microbial debris in the early stages of decay.

[2] A biochemically recognizable fraction derived from the breakdown of the material in fraction [1], e.g. carbohydrates, sugars, fatty acids, proteins and amino acids.

[3] A brown-coloured polymeric fraction the structure of which is traditionally differentiated into humic acids, fulvic acids and humins on the basis of their differential solubility in aqueous acids and bases.

This third organic matter fraction (the humic fraction) ranks with the expanding lattice clays in relation to its importance in influencing biological activities in soil. This is because humic polymers have a tertiary structure which permits them to expand on hydration exposing an extensive internal surface area (see Table 1.3). Furthermore, they usually carry a high proportion of negative charges arising from the pH-mediated dissociation of functional groups on their constituent molecules.

TABLE 1.3 (Burns, 1983)

Some Properties of Model Clays and Humic Materials

| Colloid type | Layering | Swelling | Surface area[1] ($m^2 g^{-1}$) | Cation Exchange capacity ($\mu eq\ g^{-1}$) | Basal spacing[2] (nm) |
|---|---|---|---|---|---|
| Kaolinites | 1.1 | non-expanding | 10-50 | 20-100 | 0.72 |
| Vermiculites | 2.1 | expanding | 500-750 | 1200-2500 | 0.93-1.57[3] |
| Smectites | 2.1 | expanding | 700-800 | 600-1300 | 0.95-2.2[3] |
| Humates | - | expanding | 500-800 | 200-7500 | - |
| | | | | (fulvic acids 5000-7500) | |
| | | | | (humic acids 3000-5000) | |
| | | | | (humins <3000 ) | |

[1]Determined by N-cetyl pyrimidinium bromide sorption
[2]Distance between repeating layers
[3]Variation due to level of hydration and species of interlayer cation

### 1.4.4 Production, Location and Persistence of Enzymes in Soil

It is appropriate at this point to consider the activity and location of enzymes in soil since extracellular enzymes are required for the decomposition of macromolecular or insoluble compounds which cannot be taken up by microbial cells. The persistence and activity of extracellular enzymes will therefore be important in determining the rate at which these compounds are degraded.

Burns (1982b) has divided the enzymes active in soil environments into ten categories according to the enzymes association with different soil constituents:

[1] Enzymes which function within the cytoplasm of proliferating microbial, animal and plant cells.

[2] Enzymes located within the periplasmic space of proliferating Gram-negative bacteria.

[3] Enzymes attached to the outer surface of the viable cell and whose active sites extend into the ambient medium.

[4] Enzymes secreted by living cells during normal cell growth and division and found in the aqueous phase of the soil.

[5] Enzymes within non-proliferating cells, e.g. spores and seeds.

[6] Enzymes attached to entire dead cells and cell debris.

[7] Enzymes leaked from extant cells or released by cell lysis and whose original function was within the cell but which may survive for a short period in the aqueous phase of the soil.

[8] Enzymes temporarily associated in enzyme-substrate complexes.

[9] Enzymes adsorbed to clay minerals either in the external surfaces or within the lattices of 2:1 silicates.

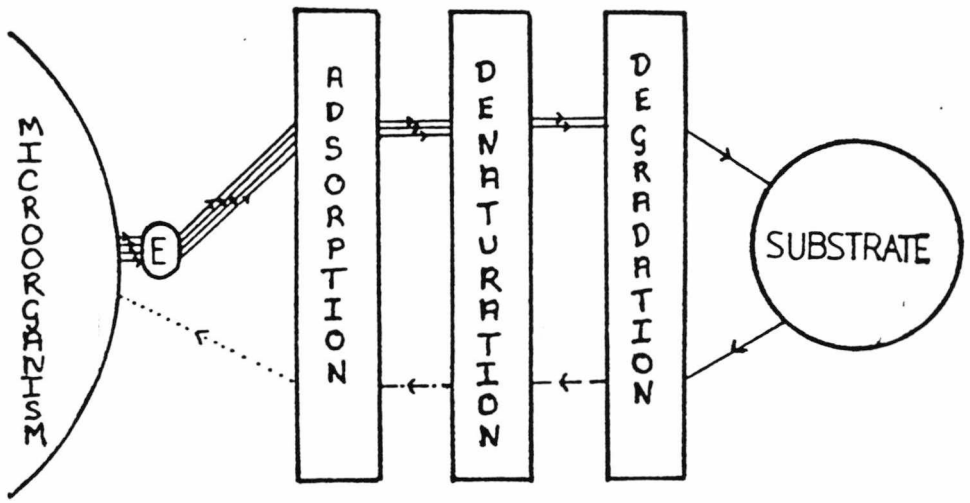
[10] Enzymes which become associated with the humic colloids due to adsorption, entrapment, or co-polymerization during humic matter genesis.

The word accumulated has been used to describe enzymes in categories 5-10 and the word immobilized for enzymes in categories 9 and 10. Immobilized enzymes by virtue of their chemical and physical bonding to the soil organic and inorganic colloids have long term protection from denaturation and degradation and half-lives considerably longer than the same enzymes in the soil aqueous phase. A number of important carbon, nitrogen, phosphorous and sulphur cycle enzymes are known to be immobilized in this way (Pettit, 1978; Lethbridge, 1978) and it has been proposed that immobilized enzymes may also be involved in cellulose degradation (Hope *et al.*, 1980).

Soil microorganisms relying on extracellular enzymes are faced with problems not normally encountered by their counterparts *in vitro* and in the absence of soil. In soil suitable substrates are unevenly distributed (both temporally and spatially) and if an enzyme is to be successful in locating its exogenous substrate it must resist inactivation due to a combination of adsorption, denaturation and degradation. Subsequently, at least a portion of the product from an extracellular catalysis must survive long enough to be utilized by the microorganism or community which produced the enzyme in the first place (Fig. 1.7). Experiments in which enzymes have either been added to non-sterile soil, or those in which production has been stimulated reveal that new enzyme is rapidly destroyed or inactivated (Roberge, 1970; Drozdowicz, 1971; Zantua and Bremner, 1976).

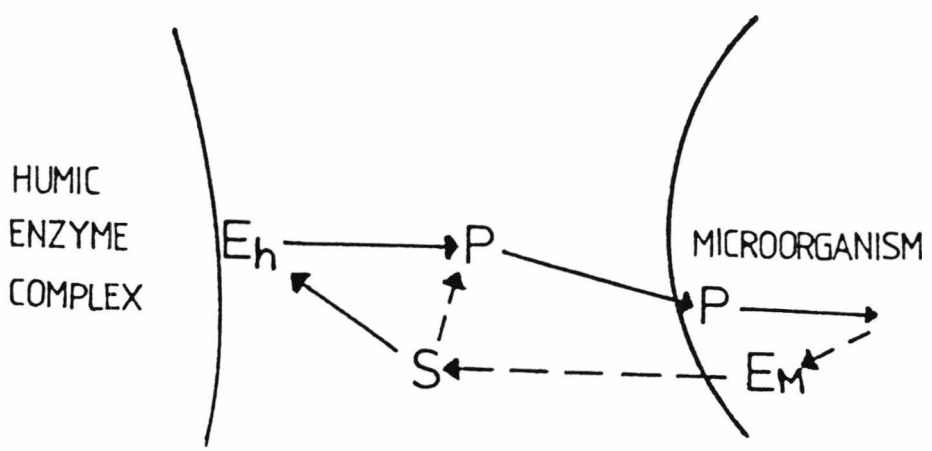
Quite apart from the obvious hazards faced by extracellular enzymes in soil it seems improbable (and energetically impossible) that soil microorganisms secrete enzymes continuously and at random in the hope that a suitable substrate exists in their immediate environment. The concept of soil containing sub-optimal concentrations of available substrate, unevenly distributed in both space and time is supported by investigations

FIGURE 1.7




Obstacles encountered by extracellular enzymes and the products of their activity in soil (Burns, 1980)

FIGURE 1.8




Possible function of the immobilized enzyme complex in relation to adjacent microorganisms (Burns, 1980). $E_h$, immobilized enzyme; $E_m$, microbial enzyme; S, exogenous substrate; P, product.

into the growth rate of bacteria in soil. Gray (1976) has estimated a mean generation time for bacteria in a deciduous woodland soil of about 20 days while Shields *et al.* (1973) suggested that the mean generation time for microorganisms in prairie soils is also low and they calculated that the specific maintenance coefficient for soil bacteria growing *in situ* might be about $0.002h^{-1}$. More recently Jenkinson and Powlson (1976) have developed a method of estimating soil microbial biomass which suggests that arable soils contain about 2% of their organic carbon in the biomass (Biomass = 200µg C $(g\ soil)^{-1}$). From measurements of the annual input of organic carbon into an unmanured plot they estimated that the mean generation time for soil microorganisms would be about two years! The implication from these calculations is that either the growth of soil organisms is continuous and extremely slow or that it occurs in short but rapid bursts interspersed with long periods of inactivity. The latter is more likely and the most generally accepted view. Surprisingly, despite its overall low metabolic activity the adenylate energy charge (AEC) of the soil biomass is comparable to that of actively growing cells *in vitro* (Oades and Jenkinson, 1979) while the level in spores is significantly lower. Quite how the biomass obtains sufficient nutrients to maintain its high ATP content is not fully understood however it does appear that the microbial cells in soil can be likened to resting units which are 'charged and ready-to-go' on receipt of a suitable trigger. Clearly an induction mechanism whereby a small amount of enzyme is produced constitutively (Gong and Tsao, 1979) is not feasible for soil microorganisms because of the scarcity of substrate. It has been proposed (Burns, 1980) that immobilized enzymes may play an ecological role in the induction of extracellular enzymes (Fig. 1.8). In this model substrate is hydrolysed by enzyme (Eh) associated with the humic matter. Product P, is then taken up by the cell. If the concentration of P (and therefore S) is high enough the organism can start degrading S directly by secreting the

appropriate enzyme (Em). In addition the products of the Eh-S interaction may diffuse some distance and initiate a positive chemotactic response. At present, there is little evidence to support this model.

## 1.5 AIMS OF PROJECT

The overall aim of the project was to investigate the dynamics of cellulose breakdown in soil at the level of the soil microenvironment. To this end we attempted to:

[1] Develop and refine assays for the enzymes of the cellulase complex that could be used in heterogenous soil systems as well as *in vitro*.

[2] Measure and characterize soil cellulase activities.

[3] Develop a technique with which we could model the soil microenvironment and which could be used to investigate the behaviour and efficacy of extracellular enzymes in soil.

**ABBREVIATIONS**

| | | |
|---|---|---|
| CETMAB | = | Cetyltrimethyl ammonium bromide |
| CMC | = | Carboxymethyl cellulose |
| CSF | = | Colloidal-size fraction |
| DP | = | Degree of polymerization |
| DS | = | Degree of substitution |
| FP | = | Filter paper |
| rgr | = | Radial growth rate |

# CHAPTER 2 : DEVELOPMENT AND ADAPTATION OF ASSAYS

## 2.1 INTRODUCTION

A large number of different and contradictory assays for cellulolytic enzymes can be found in the literature. Although there is a tendency in all enzymology for workers to develop their own assay procedures, most of the confusion is inherent in the multiplicity of substrates and in the range of enzymes collectively described as cellulases. Table 2.1 lists just some of the substrates which have been used in the study of cellulases.

Cellulose has a simple chemical structure which suggests a comparatively rapid rate of hydrolysis. However, when the length and physical arrangement of the glucose chains within the polymer are considered, a variety of complex forms are revealed which show various degrees of susceptibility to enzymic hydrolysis (section 1.1.2). Emert *et al*. (1973) have classed cellulosic substrates into six groups depending on their degree of hydration and solubility (Table 2.2). The first three types (i.e. crystalline cellulose, native cellulose, and purified wood cellulose) are insoluble in water and are relatively resistant to enzymic degradation. Amorphous (swollen) cellulose, even though insoluble, is much more susceptible to hydrolysis due to its reduced degree of crystallinity, and represents an intermediate state between insoluble and soluble (derivatized cellulose and cellooligosaccharides) forms. Within each group, variant forms exist having different degrees of crystallinity or substitution.

The range of enzymes collectively described as cellulases has been previously discussed (section 1.2.1). The use of ill-defined units of activity such as loss in weight, reduction in tensile strength, formation of short fibres and release of dye, make it difficult to know exactly which enzyme is being measured. For this reason we have confined our attention to the substrates which best allow the identification of the three major types

TABLE 2.1. SUBSTRATES USED FOR THE ASSAY OF CELLULOLYTIC ENZYMES

| ENZYME | SUBSTRATE | PRODUCTS |
|---|---|---|
| β-D-Glucosidase (Cellobiase) | Cellobiose | Glucose |
| | Cellodextrins | |
| | Salicin | Saliginin |
| | p-Nitrophenyl-β-D-glucopyranoside | p-Nitrophenol |
| | o-Nitrophenyl-β-D-glucopyranoside | o-Nitrophenol |
| Endoglucanase | Carboxymethyl cellulose | Decrease in viscosity |
| | Hydroxyethyl cellulose | Reducing sugars |
| | Amorphous cellulose (e.g. Walseth) | |
| | Cellodextrins | |
| | 3,4-dinitrophenyl glycosides | 3,4-dinitrophenol |
| Exoglucanase | Amorphous cellulose | |
| | Microcrystalline cellulose (e.g. Avicel) | Cellobiose |
| | Cellodextrins | |
| Cellulase (Exo- and Endoglucanase) | Microcrystalline cellulose | Clearing zones |
| | Filter paper | Loss in weight |
| | Cotton | Reducing sugars |
| | Cellodextrins | Reduction in turbidity |
| | | Soluble carbohydrate |
| | | Glucose |
| 'Filter paper'ase' | Filter paper | Uptake of alkali |
| 'Swelling factor' | Cotton | Reduction in tensile strength |
| 'Cellulase' | Cotton thread | Release of dye |
| | Dyed cellulose | |

TABLE 2.2. CLASSIFICATION OF CELLULOSIC SUBSTRATES (Emert *et al.*, 1973)

| GROUPS | EXAMPLES | DP |
|---|---|---|
| 1. Crystalline cellulose | Avicel | 100 - 200 |
| 2. Native cellulose | Cotton<br>Dewaxed cotton | 8 000 - 100 000 |
| 3. Purified wood cellulose | Filter paper<br>Cellulose powder | 200 - 700 |
| 4. Amorphous (swollen) cellulose | Mercerized cellulose (25% NaOH)<br>Walseth cellulose (85% $H_3PO_4$) | 1 200 - 1 400 |
| 5. Derivatized (soluble) cellulose | CMC<br>Hydroxyethyl cellulose<br>Methyl cellulose | 370 - 2 140 |
| 6. Soluble cellooligosaccharides | Cellobiose-cellohexaose | 2 - 6 |

of enzyme activity; exoglucanase, endoglucanase, and β-D-glucosidase.

Even within these limits, there is controversy as to the best method for measuring enzyme activities. This is most noticeable in the case of exoglucanase where there is no specific assay unless a purified enzyme preparation is obtained. Insoluble cellulose derivatives are suitable for determining conjoint exo- and endoglucanase activity which, in some cases has been claimed to bear a direct and quantifiable relationship to the activity of the exoglucanase alone. However, in view of the various synergistic effects observed (Wood and McCrae, 1979), there is unlikely to be a predictable relationship between exo- and endoglucanse activity.

Another related area in which controversy exists is the kinetics of enzymic cellulose hydrolysis.

2.1.2 Kinetics

The cellulase - insoluble cellulose system is very different from conventional enzyme - soluble substrate systems to which standard kinetic treatments are most applicable.

Lee *et al.* (1980) have defined the major difficulties involved in the kinetic characterisation of the cellulase - insoluble cellulose system. These are:

[1] mass transfer limitations on the diffusion of enzymes and products;

[2] enzyme adsorption to, and desorption from cellulose;

[3] fragmentation of fibrils giving rise to changes in the surface area of the insoluble substrate during hydrolysis and therefore heterogeneity; and

[4] product inhibition.

Several kinetic models of varying complexity have been proposed. Amemura and Terui (1965) derived an amended Michaelis-Menten equation to predict the hydrolysis of native cellulose to glucose by enzymes from *Penicillium variabile*. In this model the total substrate concentration $[S]_t$ is replaced by the effective substrate concentration $[S]_a$ and a factor is introduced to account for product inhibition. Several other similar models have been proposed for different cellulase systems all of which are based on the Michaelis-Menten equation but which are modified to take into account either competitive or non-competitive product inhibition. These models are discussed by Lee *et al* (1980), but are only applicable to the early stages of the reaction since they do not take into account substrate heterogeneity [3]. Furthermore, the rates of enzyme adsorption and desorption [2] and of mass transfer of enzymes and products [1] are not considered limiting. In contrast, McLaren (1963) believed that the initial rate of hydrolysis for an insoluble substrate-enzyme system is proportional to the surface area of the substrate in contact with the enzyme rather than being a linear function of the total enzyme concentration and he used the Langmuir isotherm to relate the

concentration of the adsorbed enzyme to the initial free enzyme concentration. McLaren's description of this structurally-restricted cellulose-cellulase system has been further developed by Huang (1975b) who combined an enzyme adsorption equation and an equation of the Michaelis-Menten type, and by Kim (1974) whose model takes into account cellulase adsorption and desorption [2], negligible mass transfer limitations [1] and product inhibition [4] as well as cellulase deactivation. Yet another approach (Suga *et al.*, 1975) to cellulase kinetics has been to consider the mode of action of different cellulase components on substrate molecules with different degrees of polymerization.

From this discussion it can be seen that a unified model has yet to emerge which would serve to describe the kinetics of the cellulose-cellulase system. In order to minimize the changes in the hydrolysis rate we have chosen substrates which are chemically well-defined and, more importantly and as far as is possible, physically homogeneous. Furthermore, we have attempted to measure initial reaction rates over the range in which the rate of product formation is approximately linear but we have not attempted further analysis in terms of conventional steady state kinetic parameters. As a result, the rates thus expressed may not be valid for the later stages of hydrolysis or for different initial Avicel concentrations - even though we are able to express activities in standard units (i.e. reducing sugars produced $h^{-1}$).

## 2.2 SUBSTRATES

### 2.2.1 Avicel PH

Avicel PH microcrystalline cellulose (FMC Corporation) is a purified, depolymerized alpha cellulose derived from fibrous plants. It is insoluble in water, organic solvents and dilute acids and only partially soluble in dilute alkali. The two grades used in this work were PH101 and PH105 which

have an average particle size of 50 and 20 μm, respectively. Plate 2.1 is a scanning electron micrograph of Avicel PH105 and illustrates the range of particle size and shapes which occur with this substrate. To ensure the removal of any residual glucose the Avicel was first washed three times in distilled water (50 g Avicel dispersed in 450 ml distilled water and stirred for 30 min before centifuging (10 000 x g, 15 min) dried and stored in a sealed jar at room temperature until required.

Avicel was chosen in preference to filter paper since it is reported to be a better substrate for measuring activities which include exoglucanase (v. Hofsten, 1975). Avicel contains many short microfibrils whereas the glucosidic chains in filter paper fibres have a much higher degree of polymerization and correspondingly fewer bonds available for hydrolysis by exoglucanase. Endoglucanase alone is not able to attack Avicel and hence Avicel was used to measure the combined activity of exo- and endoglucanase.

### 2.2.2 Carboxymethyl Cellulose (CMC)

Carboxymethyl cellulose is a soluble cellulose derivative widely used to measure endoglucanase activity. The structure of CMC is given in Fig. 2.1, the degree of substitution (DS) being the average number of hydroxyl groups substituted per anhydrous glucose unit. Substituents on glucose units are thought to prevent cleavage of adjacent linkages (Lee and Fan, 1980), cellulases having a high specificity for the glycosyl residue. This means that endwise action due to exoglucanase activity is restricted. Endoglucanase activity against CMC is normally measured either by determining the decrease in viscosity of a CMC solution or by measuring the increase in reducing sugars.

The influence of DP and DS on the viscometric method of determining enzyme activity (for a number of cellulases) was investigated by Almin and Eriksson (1968). They found that when using different substrate CMCs the

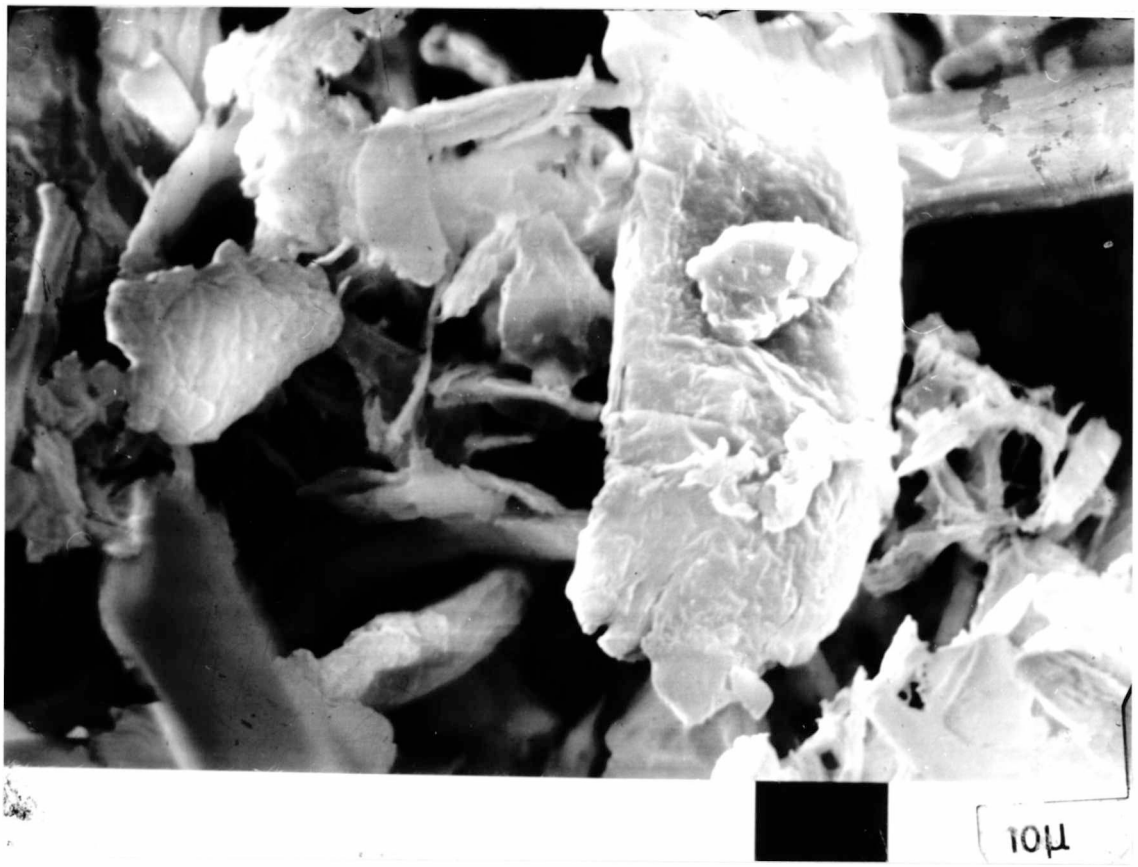
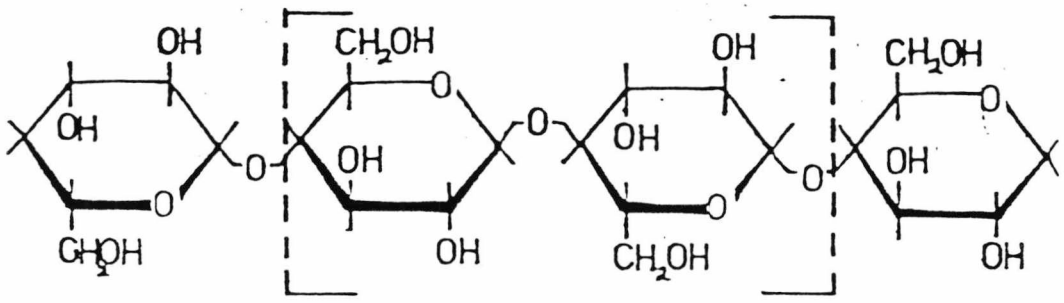



PLATE 2.1

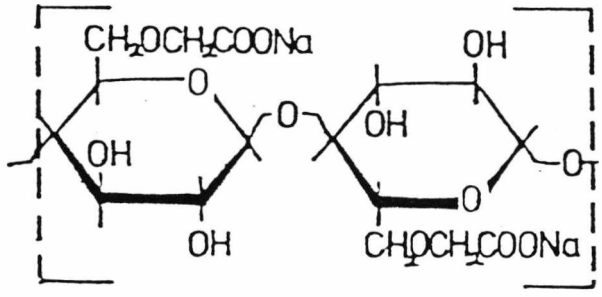
Scanning electron micrograph of Avicel PH105 (x 1 700).

FIGURE 2.1

STRUCTURE OF CELLULOSE



IDEALIZED UNIT STRUCTURE OF CELLULOSE GUM, WITH A DS OF 1.0



CMC 9M8F

9 = Degree of substitution (DS) = 0.9

M = Medium viscosity, molecular weight - 250 000
degree of polymerization (DP) - 1100

8 = Maximum viscosity = 800centipoises at 25°C

F = Food grade

number of units of activity (A) recorded for a particular enzyme varied with both the DS and DP of the CMC, even though the enzymes could still be ranked in the same order with respect to their activities. The possibility of finding a consistent relationship between the A value and the DS or DP was prevented according to the authors, by an unknown structural characteristic of the substrate. The viscosity of CMC solutions is affected by the pH and the ionic strength of the solvent (Iwasaki *et al.*, 1963) and the use of neutral hydroxyethyl cellulose (HEC) has been advocated by some (Child *et al.*, 1973). The disadvantage of HEC is that the substituent groups may be different sizes as a result of polymerization and may thus affect the susceptibility of regions of the substrate to hydrolysis. This disadvantage, together with the fact that CMCs with defined DS and DP are readily-available has lead to the widespread adoption of CMC as a substrate for measuring endoglucanase activity. It is important, however, to remember the influence of DP and DS on activity and these properties should always be reported when the assay procedure is described; a detail which is overlooked by many (e.g. Kanamoto *et al.*, 1979; Batistic *et al.*, 1980; Galas *et al.*, 1981).

CMC substrates of varying DP and DS were supplied by Hercules Inc. Table 2.3 outlines the major properties of the types used in this study.

TABLE 2.3. PROPERTIES OF THE CMC TYPES USED IN THIS STUDY

| CMC Type | Substitution range | Viscosity range in centipoise at 25°C for 2% (w/v) solution | Degree of polymerization |
|---|---|---|---|
| 12M8F | 1.20-1.40 | 400-800 | ~1 100 |
| 9M8F | 0.80-0.95 | 400-800 | ~1 100 |
| 7L | 0.65-0.85 | 25-50 | ~400 |

### 2.2.3 Cellobiose and P-nitrophenyl-β-D-glucopyranoside

Cellobiose and p-nitrophenyl-β-D-glucopyranoside (Sigma) were both used to assay β-D-glucosidase activity. An attempt was made to prepare soluble cellooligosaccharides by acid hydrolysis and separation on a charcoal column (72x300 mm) according to the method of Miller *et al.* (1960a). This attempt was unsuccessful and a mixture of cellooligosaccharides for use as TLC standards was kindly supplied by Dr. P. Linnet (Shell Research, Sittingbourne, Kent).

## 2.3 VISCOSITY MEASUREMENTS

The measurement of viscosity changes provides a sensitive and specific assay for endoglucanase activity. Almin *et al.* (1967) have developed a method by which viscosity changes can be expressed in absolute units, i.e. the number of β-1,4-glucosidic bonds broken per unit time.

In this work we measured viscosity changes in CMC/cellulase mixtures initially using an Ubbleholde viscometer and subsequently a Ferranti spindle viscometer. Using the spindle viscometer a linear relationship between $(\eta_{sp})^{-1}$ and time of incubation was obtained (Table 2.4). However, because of the size of the spindle viscometer the minimum quantities on which measurements could be made were 100 ml CMC solution requiring 10 mg of crude *Trichoderma viride* cellulase preparation (BDH) to produce a sufficient change in viscosity from which rate measurements could be made.

Measurements made using the Ubbleholde viscometer were very time consuming and since this procedure could not be used with soil (due to its particulate nature), further attempts at increasing the sensitivity of the assay were abandoned in favour of the DNS reducing sugar assay (section 2.4.2).

TABLE 2.4. The change in viscosity of 2% (w/v) CMC 7L at 30°C (in 0.1M-acetate buffer pH 5.0) following addition of 10 mg *Trichoderma viride* cellulase preparation (BDH) ($\eta$ = viscosity of acetate buffer = 0.8 cp)

| Time (min) | Viscosity (cp) | $S_p$ Viscosity $(\eta/\eta_o - 1)$ | $1/\eta_{sp}$ |
|---|---|---|---|
| 4 | 20.81 | 25.01 | 0.040 |
| 7 | 18.80 | 22.50 | 0.044 |
| 12 | 16.03 | 19.04 | 0.053 |
| 17 | 13.88 | 16.35 | 0.061 |
| 21 | 12.68 | 14.85 | 0.067 |
| 38 | 10.14 | 11.56 | 0.086 |

Regression equation: $Y = 0.036 + 0.00136X$

$r = 0.992$

## 2.4 MEASUREMENT OF REDUCING SUGARS

### 2.4.1 Nelson-Somogyi Method

Reducing sugars in the Avicel hydrolysates were assayed using the Nelson-Somogyi copper reduction method (Spiro, 1966).

Reagents

Alkaline copper reagent (Somogyi, 1952).

Solution 1

15 g potassium sodium tartrate and 30 g anhydrous $Na_2CO_3$ were dissolved in 300 ml water. 20 g $NaHCO_3$ were added (Solution A). A solution of 180 g anhydrous $Na_2SO_4$ in 500 ml water was heated to expel air and after cooling combined with Solution A and the mixture made up to 1 ℓ with water.

Solution 2

5 g $CuSO_4 5H_2O$ and 45 g anhydrous $Na_2SO_4$ were dissolved in 250 ml water.

Just prior to use 4 vol of Solution 1 were combined with 1 vol of Solution 2.

Arsenomolybdate reagent

21 ml concentrated $H_2SO_4$ were added to a stirred solution of 25 g ammonium molybdate in 450 ml water; 3 g sodium arsenate were dissolved in 25 ml water and added to the molybdate solution with mixing. After incubation for 24-48 h at 37°C the solution was stored in a glass-stoppered brown bottle and just prior to use was diluted with 2 vol 0.75M-$H_2SO_4$.

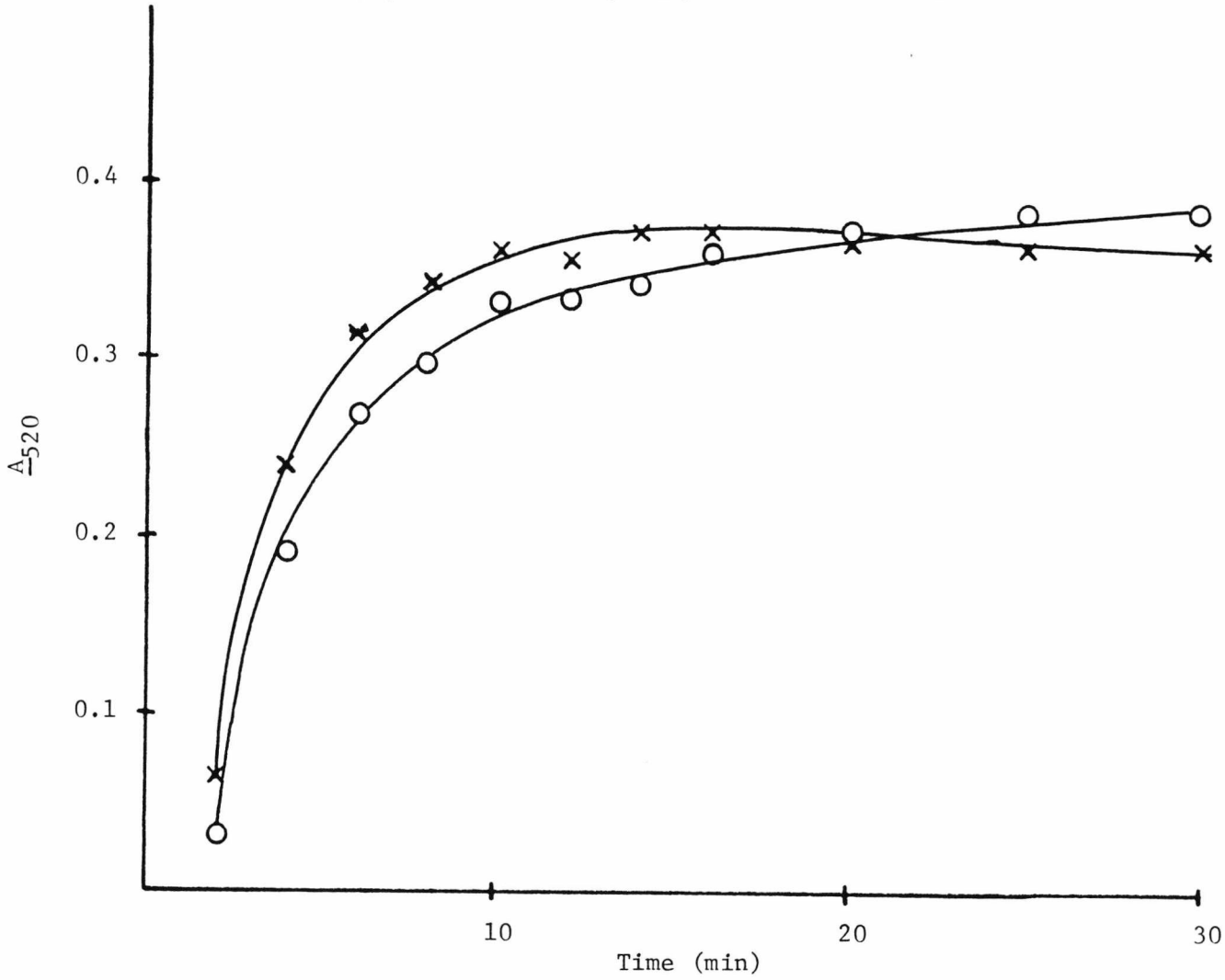
Assay Procedure

To 1 ml of suitably diluted supernatant was added 1 ml of alkaline copper reagent. After mixing, the tubes were stoppered with glass marbles and placed in 100°C water bath for 20 min (Fig. 2.2). The tubes were then removed and cooled. 1 ml of arsenomolybdate reagent was added to each tube followed by 3 ml of distilled water and the contents mixed by inversion. The absorbance of the resulting blue colour was measured within 1 h against a reagent blank, at 520 nm using 1 cm, 3 ml cuvettes in a Pye Unicam SP500 spectrophotometer. Reducing sugar concentration as glucose equivalents was calculated from a glucose standard curve (0.05-0.4mM) freshly prepared each day. The correlation coefficient of the straight line was always greater than 0.995.

Despite the reported use of the Nelson-Somogyi method for measuring the production of reducing sugars from CMC (Batistic *et al.*, 1980), it was found that the arsenomolybdate reagent caused precipitation of any unhydrolysed CMC present in the sample. Removal of the precipitate by centrifuging also removed some of the colour so that the concentration of reducing sugars present in the sample was underestimated. The DNS method of Miller *et al.*, (1960b), as recommended by Pettersson and Porath (1966), was therefore adopted

FIGURE 2.2

Nelson- Somogyi Reducing Sugar Assay
Development of chromatogen with time in 100°C water bath.
×, 2.5mM Glucose; ○, 2.5mM cellobiose.



for measuring reducing sugars produced from CMC.

2.4.2 DNS Method

Reagents

CMC Reagent

A 2% (w/v) CMC 7L solution was prepared in 0.1M-sodium acetate buffer pH 5.0 and 10 mg $\ell^{-1}$ merthiolate (sodium ethylmercurythiosalicylate) added as a preservative. The solution was stored at 4°C until required.

DNS Reagent

10 g dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulphite and 200 g potassium sodium tartrate were dissolved in 500 ml 2% (w/v) NaOH and diluted to 1 $\ell$.

Assay Procedure

Tubes containing 0.2 ml of enzyme solution and 2 ml of CMC were incubated in a stationary water bath at 40°C. After 30 min, 3 ml DNS reagent was added and the tubes heated in a 100°C water bath for 15 min, cooled to room temperature and the absorbance at 640 nm recorded against a reagent blank. [Zero-time controls in which the CMC was added at the end of the 30 min incubation at 40°C were also prepared]. Reducing sugars were calculated with reference to a standard glucose curve prepared using 0.2 ml glucose (5-40 mM) + 2 ml CMC.

The DNS assay was initially investigated using a *Trichoderma viride* commercial cellulase preparation (BDH). However, it was found that following the preparation of an enzyme solution, reducing sugars were produced in the absence of added substrate (Table 2.5), suggesting that unhydrolysed substrate was present in the commercial cellulase.

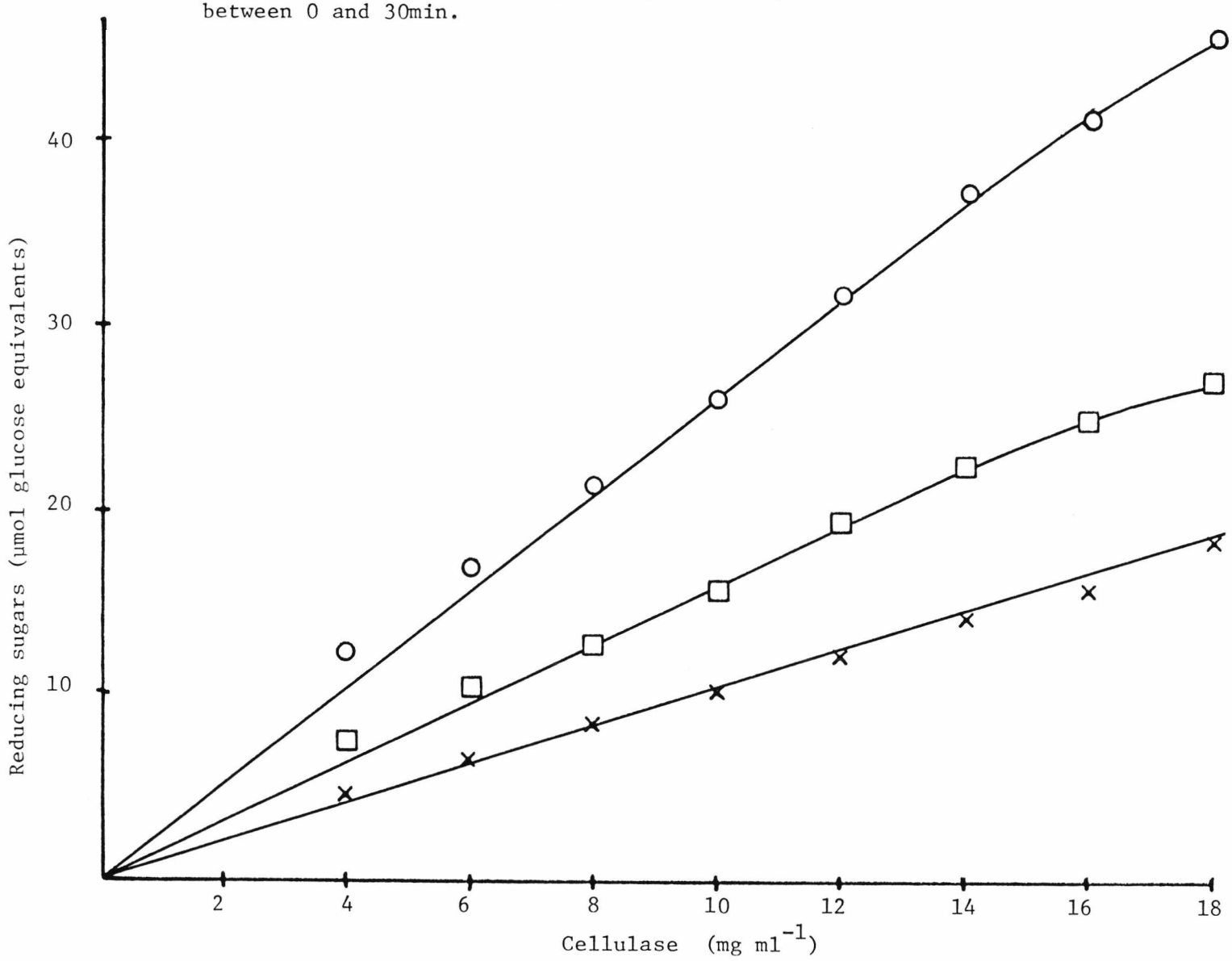
TABLE 2.5. Measurement of reducing sugars from BDH cellulase (5 mg $m\ell^{-1}$) in 0.1M-acetate buffer pH 5.0 incubated at room temperature ($22\pm3^{o}C$).

| Time of incubation (min) | Reducing sugars as glucose equivalents (mM) |
|---|---|
| 0 | 4.4 |
| 30 | 8.6 |
| 60 | 9.8 |
| 90 | 10.4 |
| 120 | 11.2 |
| Maintained at $0^{o}C$ | |
| 120 | 6.6 |

*Trichoderma viride* commercial cellulase preparation (Sigma) contained some reducing sugars [*ca.* 1.0 µmol reducing sugars as glucose equivalents (mg enzyme preparation)$^{-1}$], but appeared to be free from unhydrolysed substrate since the reducing sugar concentration did not increase on incubation of an enzyme solution (10 mg $m\ell^{-1}$) for 4 h, either at room temperature ($22\pm3^{o}C$) or at $40^{o}C$. Fig. 2.3 is a standard curve for *T. viride* cellulase preparation (Sigma) showing reducing sugars produced after 30 min incubation at $40^{o}C$.

Substrate limitation is a major problem when using CMC for assaying cellulase enzymes. The tendency for substituent groups to be concentrated at certain points within the molecules (Nisizawa, 1973) makes it impossible to predict accurately the number of bonds available for hydrolysis. On the other hand, attempts to compensate for substrate limitation by increasing the concentration of CMC in the reaction mixture are frustrated by the fact that solutions containing greater than 2% (w/v) CMC are unmanageable due to their high viscosity. However, if it is assumed that two consecutive unsubstituted residues are required for hydrolysis to occur, and substituent groups are randomly distributed throughout the molecule, then for CMC type 7L (MW 90 000), it is estimated that 9% of the bonds should be available for

FIGURE 2.3 DNS Cellulase Assay
Standard curve for *Trichoderma viride* commercial cellulase preparation (Sigma). Reducing sugars as glucose equivalents produced $ml^{-1}$ at. ×, 0 time; and ○, 30min. □, Difference between 0 and 30min.



hydrolysis yielding a maximum of 16 μmol glucose equivalents from 2 mℓ of 2% CMC solution. Therefore, substrate limitation is the most likely explanation for the levelling off of the curve at cellulase concentrations greater than 14 mg $m\ell^{-1}$.

## 2.5 MEASUREMENT OF GLUCOSE

In some of the assays the production of glucose from Avicel, rather than reducing sugars, was measured since this reflects the combined activities of exoglucanase(s), endoglucanase(s) and β-D-glucosidase(s).

### 2.5.1 Glucose Oxidase/Peroxidase Method (adapted from Benefield, 1971)

Reagents

o-Tolidine dihydrochloride

The dihydrochloride was precipitated from a solution of o-tolidine in ethanol (8 g in 200 mℓ) using concentrated HCl. The precipitate was filtered, washed with ethanol and ether and after drying stored in a dark bottle at 4°C. Prior to use 200 mg were dissolved in 25 mℓ distilled water.

Glucose Oxidase/Peroxidase

The glucose oxidase was of fungal origin (Sigma Type V) repurified to remove carbohydrate hydrolases and containing *ca*. 1000 units $m\ell^{-1}$. [1 unit oxidises 1.0 μmol of β-D-glucose to D-gluconic acid and $H_2O_2$ $min^{-1}$ at pH 5.1 35°C]. Horseradish peroxidase (Sigma Type I) had an activity of *ca*. 120 units $mg^{-1}$ [1 unit will form *ca*. 1 mg purpagallin from pyrogallol in 20s at pH 6.0 20°C].

300 units of glucose oxidase were mixed with 5 mg peroxidase in 125 mℓ of 0.1M-sodium acetate buffer pH 4.2.

The reagents were stable for up to 3 days when stored at 4°C.

Assay Procedure

0.5 mℓ sample was mixed with 2.5 mℓ of glucose/peroxidase reagent and 0.5 mℓ o-tolidine reagent and incubated at room temperature in the dark for 35 min (Fig. 2.4). The absorbance at 365 nm was recorded against a reagent blank and the glucose concentration calculated from a standard curve (0.05 - 0.3 mM) prepared on each occasion.

Hydrogen peroxide formed during the dehydrogenation of glucose by glucose oxidase is catalysed by peroxidase to oxidise o-tolidine.

$$\text{Glucose} + O_2 + DH_2 \xrightarrow[\text{peroxidase}]{\text{glucose oxidase}} \text{Gluconic acid} + H_2O + D$$

Unfortunately the glucose oxidase (Sigma type V) was completely inhibited by 0.2% $NaN_3$ as used routinely in all the soil cellulase assays (section 3.2.4). For this reason the Boehringer GOD-Perid method was investigated since the glucose oxidase in this kit preparation was insensitive to $NaN_3$ inhibition.

2.5.2 Glucose Test-Combination Boehringer GOD-Perid Method

Reagent

The reagent when dissolved in water, contained 0.1M-phosphate buffer pH 7.0; peroxidase > 0.8 U $m\ell^{-1}$; glucose oxidase >10 U $m\ell^{-1}$ and diammonium 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTMS) 1 mg $m\ell^{-1}$.

Assay Procedure

0.5 mℓ of sample was mixed with 5 mℓ reagent and incubated for 20 min at room temperature in the dark. The absorbance at 610 nm was recorded and the glucose concentration calculated by reference to a standard curve (0.1 - 0.6 mM).

Despite the reported use of GOD-Perid for glucose measurement in cellulase assays (Pycraft and Howarth, 1980), an early problem was caused by the presence of β-D-glucosidase in the reagent kits which resulted in a slow increase in absorbance when cellobiose was present in the sample. The presence of

FIGURE 2.4

Glucose Oxidase Assay (Benefield, 1971).
Development of chromatogen with time.
×, 0.3mM Glucose; ○, 15mM cellobiose.

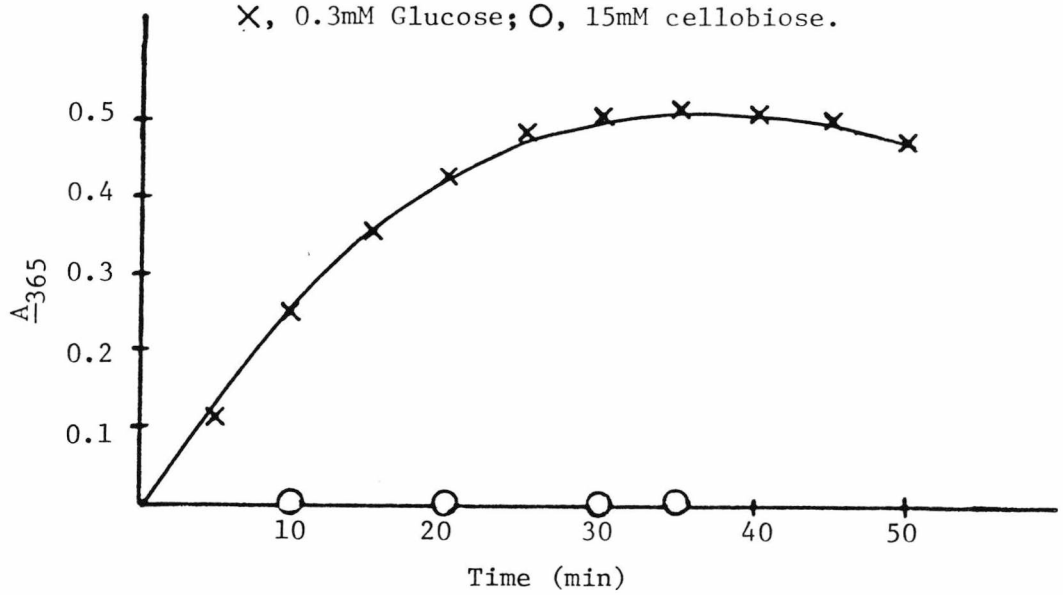
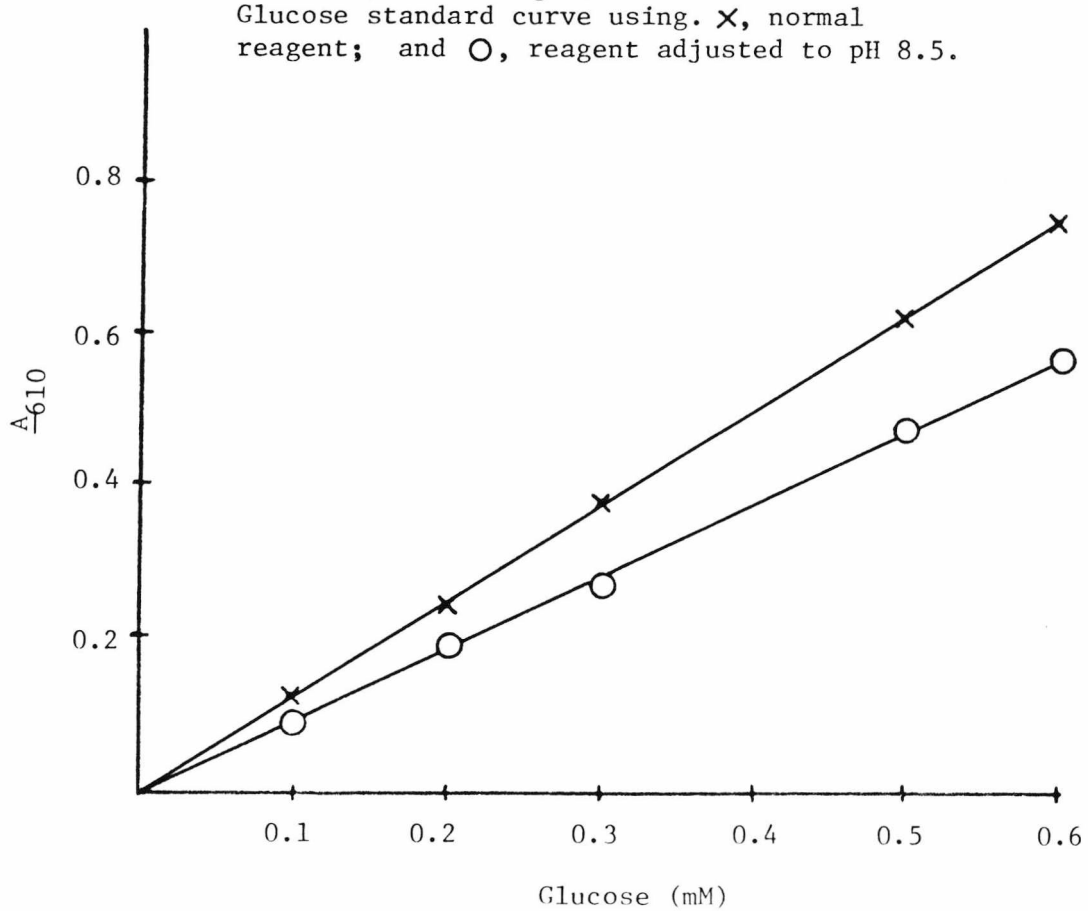



FIGURE 2.5

GOD Assay (Boehringer)
Glucose standard curve using. ×, normal reagent; and ○, reagent adjusted to pH 8.5.



β-D-glucosidase was confirmed by the determination of rate constants for the hydrolysis of cellobiose to glucose at various initial cellobiose concentrations. Canesvascini and Meyer (1979) reported that the contaminating β-D-glucosidase was totally inhibited by adjusting the pH of the enzyme-buffer reagent to 8.5 with 1M-Tris (Tris(hydroxymethyl)aminomethane) solution immediately prior to use. Our results were in agreement with this observation, although the $\underline{A}_{610}$ appeared to be lower when using the reagent at an elevated pH (Fig. 2.5) and the stability of the chromatogen was somewhat reduced. Nonetheless, by reading the absorbance of the samples between 20 and 30 min after addition of the reagents and by reference to a simultaneously prepared standard curve, the glucose concentration in the sample could be accurately determined. In some of the assays where glucose was present in low concentrations the quantity of reagent added to 0.5 mℓ sample was reduced to 3 mℓ.

## 2.6 CHROMATOGRAPHY

To further investigate the nature of the products following hydrolysis of Avicel, various chromatographic techniques were considered. The use of paper chromatography (both ascending and descending) for separating cellooligosaccharides has been widely reported in the literature (Stutzenberger, 1972; Kaufmann *et al.*, 1976; Lamot and Voets, 1976; Kanda *et al.*, 1979; Shewale and Sadana, 1979) as has the use of TLC (Stutzenberger, 1972; Streamer *et al.*, 1975; Wood *et al.*, 1980), GLC (Molton *et al.*, 1979; Storer *et al.*, 1979), HPLC (Sternberg *et al.*, 1977; Wells and Lester, 1979; Ferchak *et al.*, 1980) and LPLC (Ladisch *et al.*, 1979). The various chromatographic methods for separating oligosaccharides are described in greater detail in the review by Bailey and Pridham (1962) and by Kumar (1980).

Paper chromatography according to Shewale and Sadana (1979) and TLC on silica gel using a variety of solvents and detection agents were investigated. The most satisfactory results were obtained using either Merck $F_{254}$ Fertigplatten or plates spread to 0.25 mm thickness with 2% (w/v) silica gel type G (Sigma).

A double ascension in ethyl acetate/propan-2-ol/water (18:13:9, by vol) was performed, the plates dried and the separated oligosaccharides detected using anisaldehyde/sulphuric acid (Stahl and Kaltenbach, 1961). Anisaldehyde/sulphuric acid, although not as sensitive to the presence of reducing sugars as silver nitrate, is not affected by the small amount of salts which are present in the normal reaction mixtures.

Using AR glucose and cellobiose and the following sugar mixture from the wood hydrolysis (section 2.2.3), the following Rf values were obtained:

| | Rf |
|---|---|
| Xylose | 0.831 |
| Glucose | 0.705 |
| Cellobiose | 0.550 |
| Cellotriose | 0.350 |
| Cellotetraose | 0.179 |

Due to the double ascension some variation (*ca.* 10%) was found in the Rf values between plates and for this reason a sample containing the five standards was run each time.

# CHAPTER 3: CELLULASE ACTIVITY IN SOIL

## 3.1 INTRODUCTION

In the past, cellulase activity in soil has often been measured using ill-defined and heterogeneous substrates, such as pieces of buried cellulose film (Tribe, 1961) and cotton (Latter and Howson, 1977), the results being expressed semiquantitatively, in terms of loss in weight or reduction in tensile strength. However, carbon dioxide evolution from labelled (Ibister *et al.*, 1980; Zunino *et al.*, 1982) and unlabelled (Sato, 1981) cellulose has also been used as a measure of cellulolysis, allowing a more specific and accurate determination of the cellulose decomposing potential of a particular soil (Zunino *et al.*, 1982). These methods, while being useful in predicting the cellulose decomposing potential of a soil are of limited value in detailed investigations of cellulolytic enzymes and microorganisms.

Cellulase activity in soil has been assessed by many workers who have incubated CMC with soil under laboratory conditions and measured the production of reducing sugars (Ambroz, 1973; Batistic *et al.*, 1980; Pancholy and Rice, 1973). Incidentally, the use of the term 'cellulase' to describe activity against CMC is somewhat misleading since the choice of this substrate determines that it is only endoglucanase activity which is being measured.

Benefield (1971) measured cellulase activity by incubating cellulose powder with soil under laboratory conditions and measuring the glucose produced after 48 h at 50$^{o}$C using a glucose oxidase/peroxidase system. There are two main criticisms of this work. 1) Cellulose powder is a mixture of Avicel and CMC and no attempt was made to investigate to what extent each component was degraded. 2) A plot of glucose produced vs time

was non-linear after 24 h. This meant that, by choosing a 48 h assay period the results could not be expressed in standard units (i.e. glucose produced $g^{-1}$ $h^{-1}$).

Ross (1974) measured cellulase activity in a New Zealand soil using an adaptation of the Benefield assay in which cellulose powder was incubated with soil at either 30°C or 50°C and reducing sugars measured using the Nelson-Somogyi method. While the substrate again contained a mixture of microcrystalline cellulose and CMC they found that the production of sugars was linear for the first 48 h.

In the work reported here we have attempted to measure and characterize (in terms of pH-activity profile, thermostability etc.) the combined exo- and endoglucanase activity in soil by using Avicel as a substrate and measuring the production of reducing sugars. Similarly, β-D-glucosidase and endoglucanase activities have been measured using pNPG and CMC respectively. The extensive characterization of these activities has a dual purpose. Not only does it provide a detailed description of the individual enzyme activities in soil, from which deductions may be made concerning the origins of the enzymes and the nature of their association with the humic polymers (Burns, 1978), but it also convinces us that we are actually working with a biological and not a chemical catalyst since it has not proven possible to purify cellulases or indeed other soil immobilized enzymes.

## 3.2 MATERIALS AND METHODS

### 3.2.1 The Soil

#### (i) Soil Characterization

A silt loam soil (Hamble Series - Soil Survey Record No. 14) from Spring Grove Farm, Wye, Nr. Ashford, Kent (N.G.R. TR037466) was used throughout

this work. Samples were collected from the surface 25 cm (A horizon), hand crumbled, air-dried and sieved. The fraction which passed through a 2.0 mm mesh was stored in dark glass bottles at room temperature ($22\pm3^{o}C$) until required. Table 3.1 describes the major properties of the soil.

TABLE 3.1: MAJOR PROPERTIES OF HAMBLE SOIL

| | | |
|---|---|---|
| Sand (2 mm - 50 μm) | 9% | (i) |
| Silt (50 μm - 2 μm) | 72% | (i) |
| Clay (< 2 μm) | 19% | (i) |
| Organic matter | 6.0% | (ii) |
| pH | 6.4 | (iii) |
| Water holding capacity | 0.57 ml $g^{-1}$ | (iv) |
| %C | 2.0 | (v) |
| %N | < 0.5 | (v) |
| Carbohydrate as glucose | 7.8 mg $g^{-1}$ | (vi) |

(i) Soil Survey analysis (Green and Fordham, 1973)

(ii) Determined by high temperature ignition of oven-dried soil

(iii) pH of 1:2.5, soil:water slurry after 30 min mixing

(iv) Hilgard cup method (Gibson, 1977)

(v) Hewlett Packard automatic analyser

(vi) 5 g air-dried soil was hydrolysed with 10 ml 1.5M-$H_2SO_4$ at $85^{o}C$ for 24 h (Brink *et al.*, 1960). The soluble carbohydrates in the hydrolysates were then measured using anthrone (Roe, 1955) and converted to glucose equivalents by means of a standard curve.

| Soil collection | pH | %Water content |
|---|---|---|
| 21/11/1979 | 6.7 | 23.2±0.5 |
| 20/5/1980 | 6.4 | 14.7±0.8 |
| 23/3/1982 | 6.4 | 26.3±0.3 |
| 19/10/1982 | 6.3 | 24.6±0.2 |

Percent water content was determined by drying 1 g field-moist soil in a pre-weighed foil boat at 105°C overnight. After cooling in a dessicator the samples were weighed and returned to the oven. The procedure was repeated until no further weight loss occurred (Bascomb, 1974).

$$\% \text{ Water content} = \frac{\text{Mass of sample taken - Mass of oven-dried sample}}{\text{Mass of sample taken}}$$

Unless otherwise stated soil collected 20/5/80 was used for the experiments.

(ii) Separation of the colloidal-size fraction from soil (Gibson, 1977)

Soil (50 g), suspended in 250 ml of distilled water, was homogenized in a Waring Blender for 5 min. This method was used in preference to the conventional method of dispersion which uses sodium hexametaphosphate, in order to minimize any biological changes caused by excess inorganic phosphate. Water was then added to bring the liquid level up to the 1103 ml mark in a sedimentation cylinder (a figure which compensates for the volume of soil when using distilled water). After 14 h 52 min the colloidal-size fraction (CSF) was syphoned off and collected by centifugation (10 000 x g, 30 min).

(iii) Removal of organic matter from the colloidal-size fraction (Avery and Bascomb, 1974)

i) Carbonate removal

10 g of CSF was placed in a 250 ml polypropylene centrifuge bottle and 120 ml 0.6M-sodium acetate buffer, pH 5.0 added. After effervescence had ceased the mixture was agitated on a

reciprocating shaker for 1 h, centrifuged (10 000 x g, 30 min) and the clear supernatant discarded.

ii) Organic matter removal

50 ml of 40 vol $H_2O_2$ was added to the decarbonated sample, the bottle covered with a watch glass and left overnight. The sample was then heated on a steam bath for 4 h (care being taken not to allow the sample to dry) and after cooling, centrifuged (10 000 x g, 30 min). The $H_2O_2$ treatment was repeated and the final pellet washed twice with distilled water, air-dried and ground prior to use.

Results from the carbon and nitrogen analysis of the fractions are given in Table 3.2.

### 3.2.2 Viable Counts of Soil Microorganisms

Triplicate samples (1 g) of the fresh soil (collected 20/5/1980 and sieved to < 2 mm) were suspended in 100 ml sterile phage buffer.* The flasks were shaken for 1 h at 30°C to dislodge the microorganisms from the soil particles and a ten-fold dilution series in phage buffer prepared. Three 0.1 ml aliquots of three consecutive dilutions were spread onto agar plates. Plates were incubated at 14°C and 25°C.

TABLE 3.2: CARBON AND NITROGEN ANALYSIS OF CSF

| | % Carbon | % Nitrogen | Carbon remaining as % of that in original sample |
|---|---|---|---|
| CSF | 4.32 | 0.64 | 100 |
| CSF (after 1st $H_2O_2$ treatment) | 0.91 | 0.17 | 21.1 |
| CSF (after 2nd $H_2O_2$ treatment) | 0.40 | 0 | 9.3 |

*see media (appendix I)

Media

Bacteria 1) Nutrient agar; 2) BC: Berg MM* + 0.5% (w/v) CMC (BDH low viscosity) + 1% (w/v) purified agar (Oxoid); 3) B/BA: Berg MM + 1% (w/v) purified agar overlayed with 10 ml Berg MM + 1% (w/v) Avicel PH105 + 1% (w/v) purified agar.

Fungi 1) Malt Extract agar (MEA); 2) Czapek Dox (CZD); 3) SC: Sternberg MM* + 0.5% (w/v) CMC (BDH low viscosity) + 1% (w/v) purified agar; 4) S/SA:Sternberg MM + 1% (w/v) purified agar overlayed with 10 ml Sternberg MM + 1% (w/v) Avicel PH105 + 1% (w/v) purified agar.

In order to prevent the growth of bacterial colonies on the fungal plates, filter sterilized streptomycin sulphate was added to the cooled autoclaved media at a concentration of 400 $\mu g\ ml^{-1}$ prior to pouring.

3.2.3 β-D-Glucosidase

Assay Procedure

0.2 g air-dried soil
+ 1.5 ml 0.1M-buffer
+ 1.0 ml 25 mM-pNPG
↓ 4 h, 30°C
0.5 ml 0.5M-$CaCl_2$
+ 1 ml pNPG to controls
Reaction stopped by addition
of 2 ml 0.5M-Tris, pH 10.5
↓ Shaken, centrifuged (2 500 x g, 10 min)
Supernatant diluted and
absorbance read at 400 nm

*see media (appendix I)

Five replica treatments and three controls were used for each determination. Soil-immobilized β-D-glucosidase activity was characterized by J.M. Alexander and R.J. Geraghty (M.Sc thesis, U.K.C., 1983).

### 3.2.4 Total Cellulase Activity

#### (i) The Assay

Six samples (1 g) of air-dried soil were placed in 25 ml Erlenmeyer flasks and 5 ml 0.1M-acetate buffer, pH 5.5, containing 0.2% (w/v) $NaN_3$, added to each. Washed Avicel PH105 (0.5 g) was added to three of the flasks to give a substrate concentration of 100 mg $ml^{-1}$. All the flasks were then sealed with Nescofilm and incubated at 40°C in a shaking water bath (Mickle Co.) for 16 h. Avicel (0.5 g) was added to the control flasks at the end of the incubation period and the reaction stopped by centrifugation (2 500 x g, 10 min). Each supernatant was assayed in duplicate for reducing sugars and/or glucose using the Nelson-Somogyi copper reduction method or the Boehringer GOD kits adjusted to pH 8.5 (see section 2.4.1/2.5.2).

#### (ii) Development of the assay

a) Air-drying of soil

Loss of activity on air-drying was determined for Hamble soil (collected 19/10/1982) (Table 3.3).

TABLE 3.3: LOSS IN ACTIVITY ON AIR-DRYING

| | Activity [nmol reducing sugars expressed as glucose equivalents (g dry wt. soil)$^{-1}$ $h^{-1}$] |
|---|---|
| Field-moist soil | 111.3 |
| Air-dried soil | 39.8 |

The decline in activity (*ca.* 60%) is similar to that observed by Lethbridge (1978) for β-1,3-glucanase activity in Hamble soil. He proposed that the physical stress of air-drying denatured much of the extracellular unbound (and hence unprotected) enzyme free in the soil solution. Reduction of enzyme activities (up to 66%) on air-drying has also been reported for urease, protease and phosphatase (Speir and Ross, 1975).

The effects of air-drying and storage, on the microbial populations in soil were investigated by Sparling and Cheshire (1979) using a dilution plate technique. Sparling and Cheshire (1979) observed a 50% drop in the number of bacteria and fungi on air-drying to *ca.* 1.5% moisture. During the subsequent 6-month storage period the number of viable bacteria and to a lesser extent fungi, continued to decline. This pattern of loss (initial drop on air-drying followed by gradual loss on storage) has also been observed with soil urease and phosphatase (Pettit, 1978). Therefore, it may be that in addition to free enzyme, some of the labile enzyme activity measured in field moist soil is associated with viable microorganisms.

Air-dried soil was used for the majority of the work because after a decline during air-drying and the initial storage period, enzyme activities are stable (Lethbridge, 1978; Pettit, 1978) and believed to represent the soil-bound or immobilized fraction (Burns, 1982a). In addition, air-drying renders the soil in a state convenient for storage and subsequent use.

The extractable reducing sugar concentration (estimated in the soil control) of field-moist soil was low ($< 0.1$ μmol $g^{-1}$) and did not increase during the incubation period. After air-drying and storage the reducing sugar concentration in the controls was below 0.1 μmol $g^{-1}$ at zero time but increased to 0.2-0.4 μmol $g^{-1}$ after 52 h at 40°C. This suggested that a fraction susceptible to hydrolysis was released or exposed during air-drying.

This fraction could be removed by an inital incubation procedure in which the soil was incubated with buffer and bacteriostat for 24 h and then centrifuged. The supernatant was discarded, the soil resuspended in buffer, the mixture again centrifuged and the resulting pellet used for the assay. This procedure, however, was not routinely used since it reduced the levels of reducing sugars in the reaction mixtures to near the lower limits of sensitivity of the Nelson-Somogyi reducing sugar assay.

b) Buffer Selection

Arguments concerning the use of buffers in soil enzymology are well documented (Burns, 1978) and the main advantages of using buffered assays have been listed by Lethbridge (1978). Sodium acetate buffer was chosen for the standard assay since the pH-activity optimum of both the β-D-glucosidase and the total cellulase were within its pH range. Furthermore, from the series of buffers evaluated by Lethbridge (1978) sodium acetate and sodium succinate were the only two which did not extract large amounts of humic matter from the soil. In assays where it was necessary to use other buffers, e.g. determination of pH-activity profiles, extracted humic matter was precipitated by the sequential additions of 100 µl 1.2M-$(CH_3COO)_2Pb3H_2O$ and 100 µl 1.2M-$(COOK)_2H_2O$ (J.M. Sarkar, U.K.C. personal communication).

c) Temperature

Assays using fresh soil were performed at 25°C as well as at 40°C; a temperature used by many workers to measure in vitro activity against filter paper (Berghem *et al.*, 1976) and CMC (Pettersson and Porath, 1966). When assays using air-dried soil were carried out at 25°C the amount of reducing sugars produced was at the lower limit of detection when using the Nelson-Somogyi method; therefore 40°C was used.

d) Length of incubation and the need for an inhibitor of microbial growth

Skujins (1976) and Lethbridge (1978) have discussed the advantages and disadvantages of the various microbial inhibitors used in soil enzymology. In an early experiment the production of reducing sugars from Avicel was monitored for 48 h (Fig. 3.1) in the presence and absence of 10% (v/v) toluene. In the absence of toluene the glucose concentration increased for 4 h and then declined indicating that microbial utilization of cellulose breakdown products was occurring. A similar observation was made by Lethbridge (1978) on incubating soil with laminarin in the presence and absence of a microbial inhibitor. Toluene is not ideal for use in soil enzyme assays since it is immiscible with water, is decomposed by some soil microorganisms (Kaplan and Hartenstein, 1979) and it alters cell membrane permeability (Jackson and DeMoss, 1965). Lethbridge (1978) demonstrated the usefulness of sodium azide as a microbial inhibitor in soil enzyme assays, by showing that the incorporation of as little as 0.1% (w/v) sodium azide into nutrient agar plates prevented growth of microorganisms from a soil slurry inoculum for up to three weeks. However, in our experiments there was no significant difference in the cellulase activities measured in the presence of 10% (v/v) toluene and 0.2% (w/v) sodium azide. Sodium azide was used as the microbial inhibitor in all routine experiments for, even though it is also far from ideal (Rozycki and Bartha, 1981) it has some advantage over toluene in that it can be applied in aqueous solution and it does not affect membrane permeability.

A plot of reducing sugars released versus time shows a change in the rate of hydrolysis after 24 h (Fig. 3.2). This experiment was not continued for long enough to determine whether we were observing a levelling off in the rate of hydrolysis or whether a genuine two phase hydrolysis was occurring. However, there are many reports in the literature (e.g. Mandels *et al.*, 1976; Fan *et al.*, 1980b) of a biphasic enzymic attack on Avicel and several different kinetic models have been proposed to account for this (section 2.1.2).

FIGURE 3.1

Production of reducing sugars [µmol glucose equivalents (g dry wt soil)$^{-1}$] in the presence (×) and absence (●) of a microbial inhibitor (10% v/v toluene).

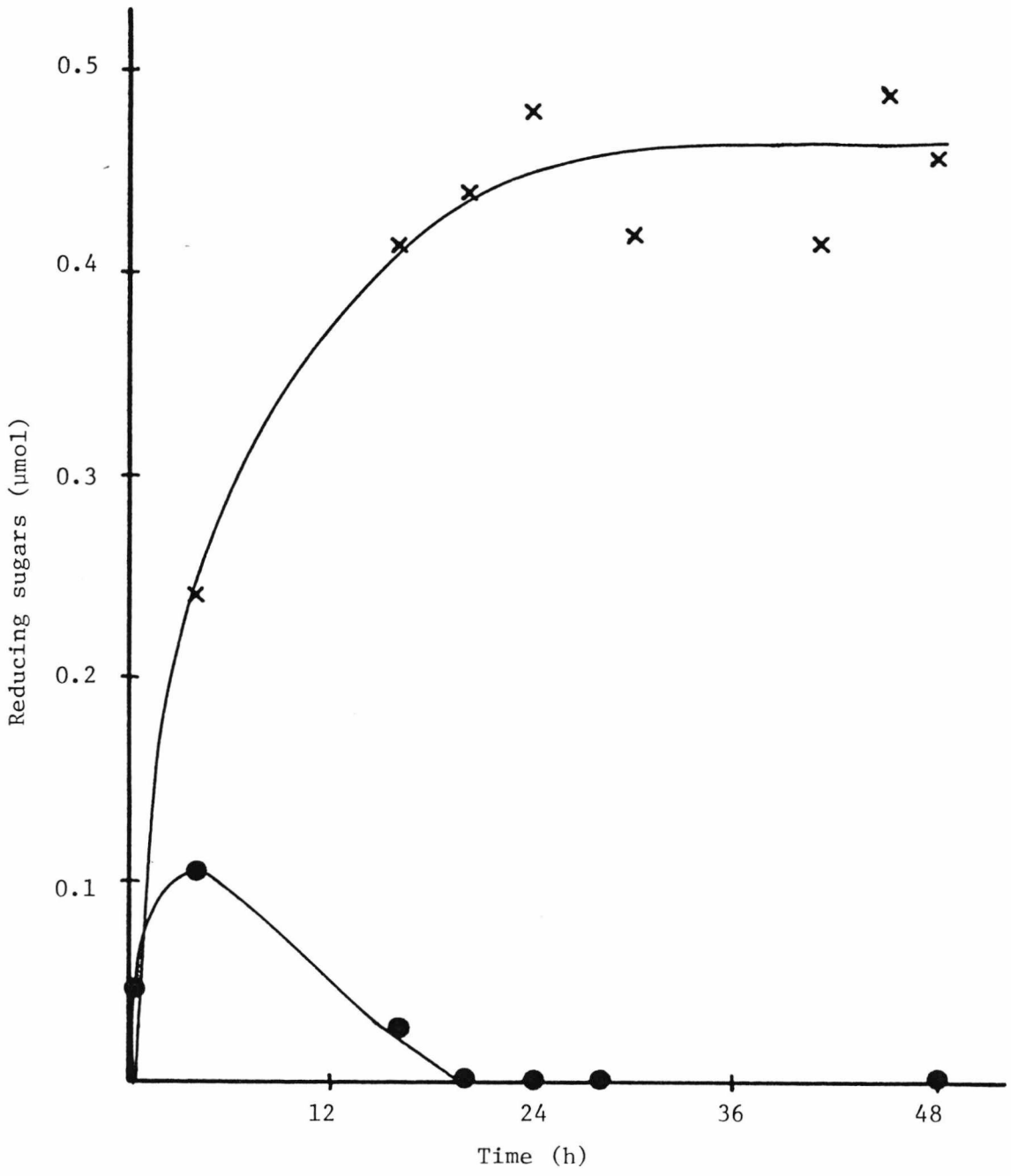
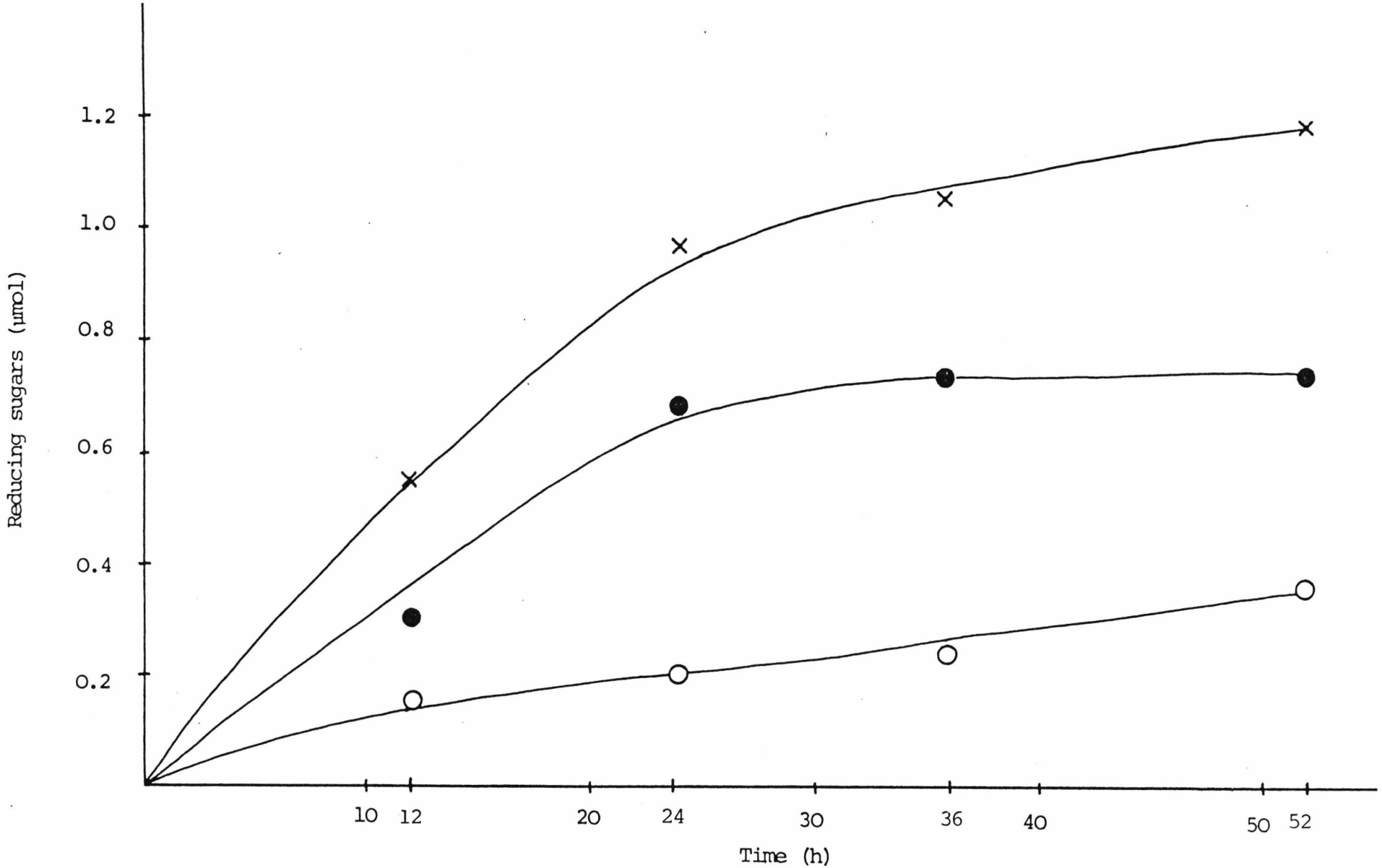


FIGURE 3.2 Production of reducing sugars {µmol glucose equivalents (g dry wt soil)$^{-1}$} with time. ×, Avicel + soil; ○, soil control; ●, treatment less control.



An incubation period of 16 h was chosen because the rate of product formation is constant over this period (Fig. 3.3) and hence the results could be expressed in standard units (section 2.1.2).

e) Substrate concentration

The effect of substrate concentration on the rate of reaction was investigated using 0.002 - 0.2 g $ml^{-1}$ Avicel. The soil:buffer ratios were 1:5 for air-dried soil (40°C) and both 1:5and 1:2 for field-moist soil (25°C). Substrate saturation kinetics were not observed (Fig. 3.4). There are a number of possible explanations for this phenomenon.

[1] We are investigating a system in which the enzymes are thought to be soil-bound or immobilized and the substrate is insoluble. Therefore, it may be impossible to saturate all the active sites because of steric hinderance and restricted diffusion of interactants. Analysis of the system is further complicated by the fact that we are not measuring a single enzyme activity but the activity of a group of enzymes (section 1.2.2 and section 2.2.1).

[2] The substrate, while being chemically homogeneous, is not physically homogeneous and some regions will be more resistant to hydrolysis than others. By increasing the substrate concentration the number of sites susceptible to rapid enzyme hydrolysis will be increased (see section 1.1).

At the lower Avicel concentrations ($< 0.05$ g $ml^{-1}$) the variation between replicas was relatively large and in order to minimize this the comparatively high substrate concentration of 0.1 g $ml^{-1}$ was chosen for the routine assay.

The activity measured in shaken assays was 2.95 x greater than that measured in stationary assays.

(iii) Fate of glucose produced during cellulose degradation

Ross (1974) suggested that soil-immobilized glucose oxidase may interfere

FIGURE 3.3

Production of reducing sugars { µmol glucose equivalents (g dry wt soil)$^{-1}$} with time. ×, Avicel+soil; ○, soil control; ●, assay minus control.

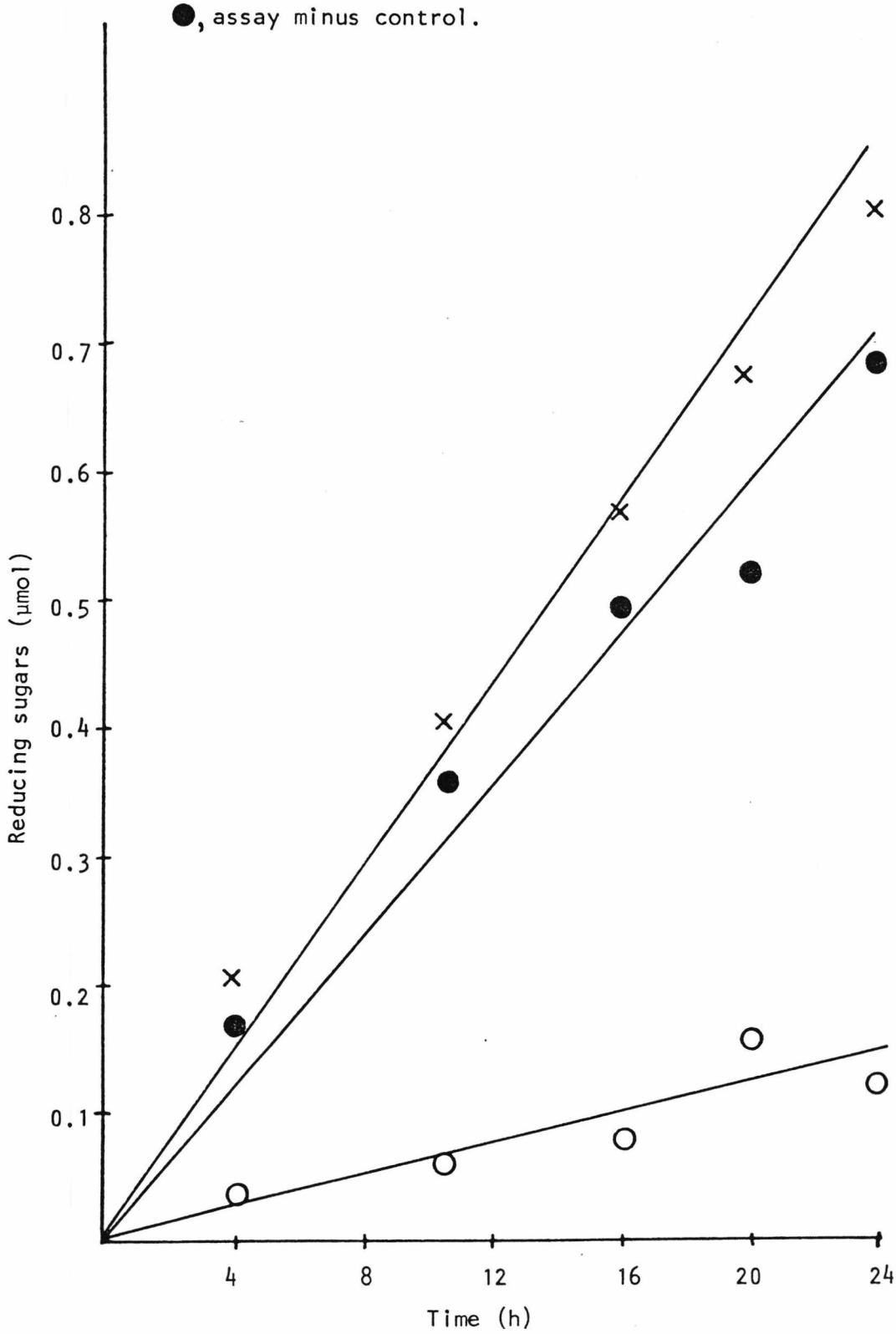
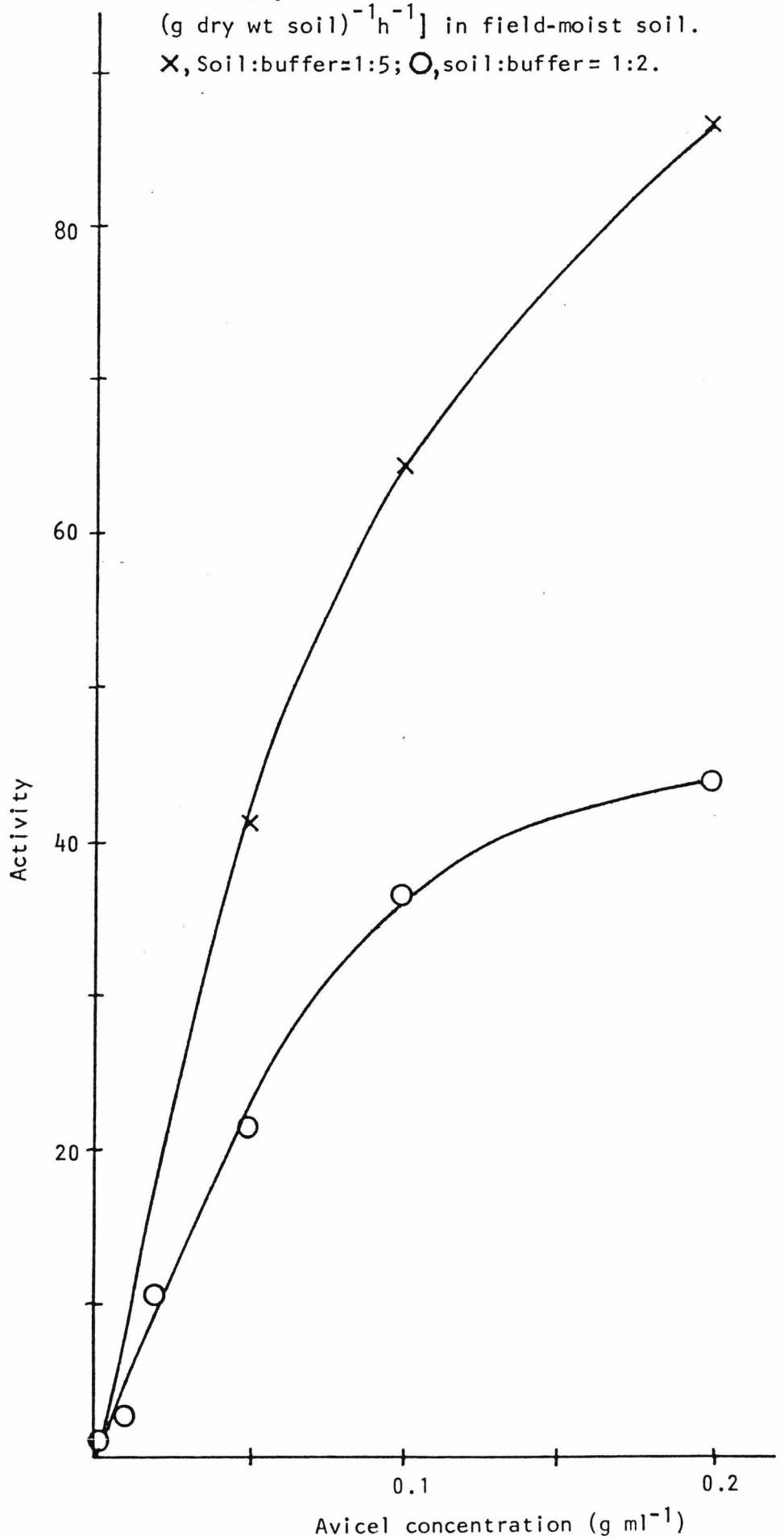


FIGURE 3.4

Effect of substrate concentration on cellulase activity [nmol reducing sugars as glucose equivalents $(g \text{ dry wt soil})^{-1} h^{-1}$] in field-moist soil.
×, Soil:buffer=1:5; O, soil:buffer= 1:2.



with those soil cellulase assays in which the production of glucose is measured. Microbial growth and utilization of glucose over the 16 h assay period was prevented by the addition of a microbial inhibitor; however, it was also possible that some of the glucose produced in the assays was being adsorbed by the soil colloids (Greenland, 1956a; 1956b; Novakova, 1972a; 1972b). In order to examine the fate of glucose under the conditions of the cellulase assay a routine assay was set up except that Avicel was replaced by glucose. Reducing sugars and glucose were measured immediately after addition (Fig. 3.5:2), after 16 h incubation at 40°C (Fig. 3.5:3) and following PbAc/KOx treatment (Fig. 3.5:4). The results were compared with a standard glucose curve (Fig. 3.5:1). There was no significant difference in the regression analysis for lines 1-3, indicating that glucose was not being adsorbed by the soil colloids or being oxidised by a soil immobilized glucose oxidase. Following the precipitation of extracted humic acids only 80% of the added glucose could be detected. This was probably due to coflocculation of glucose with the humic acids and a compensation factor (x 5/4) was introduced for assays in which this treatment was used.

(iv) pH-Activity profile and thermal stability of soil cellulase activity

a) pH-Activity profile

The reaction mixture was buffered in the following ranges:

| | | |
|---|---|---|
| field-moist soil: | 4.75 - 6.0; | 0.1M-sodium acetate |
| | 5.0 - 8.0; | 0.1M-tris/HCl |
| air-dried soil: | 4.75 - 6.0; | 0.1M-sodium acetate |
| | 5.5 - 6.75; | 0.1M-sodium acid maleate |
| | 5.5 - 8.0; | 0.1M-tris maleate/NaOH |

FIGURE 3.5

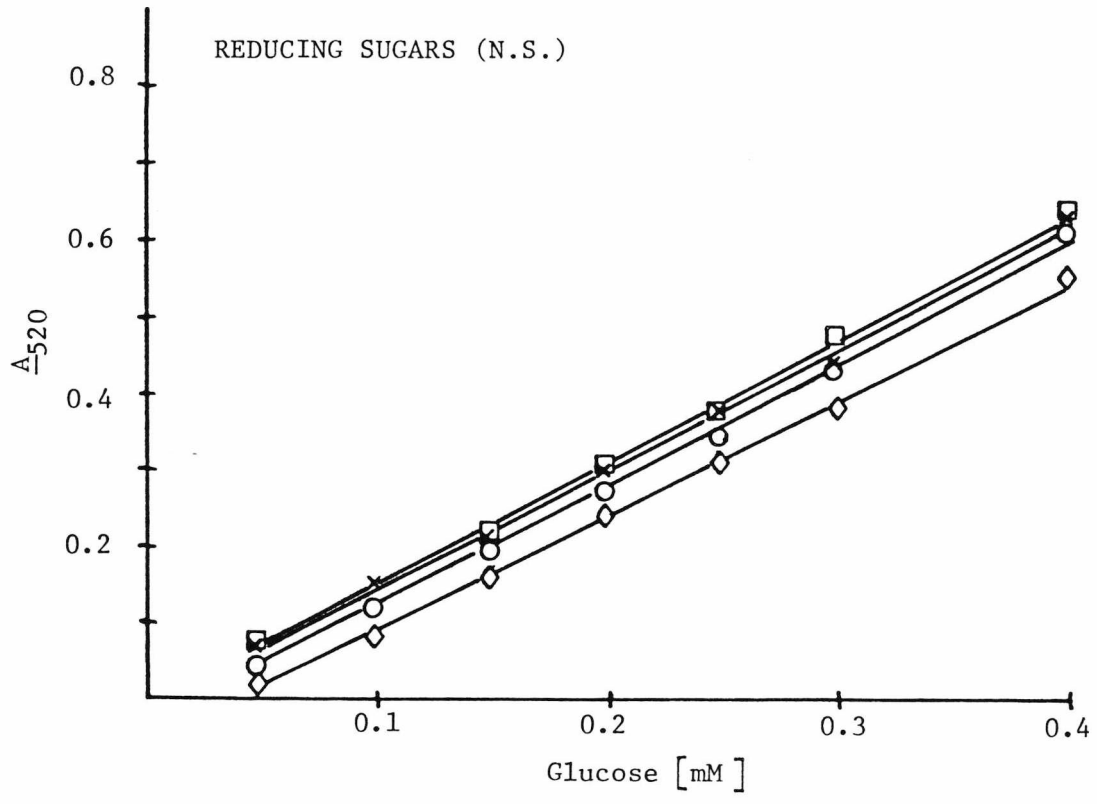
Recovery of Glucose added to Soil.

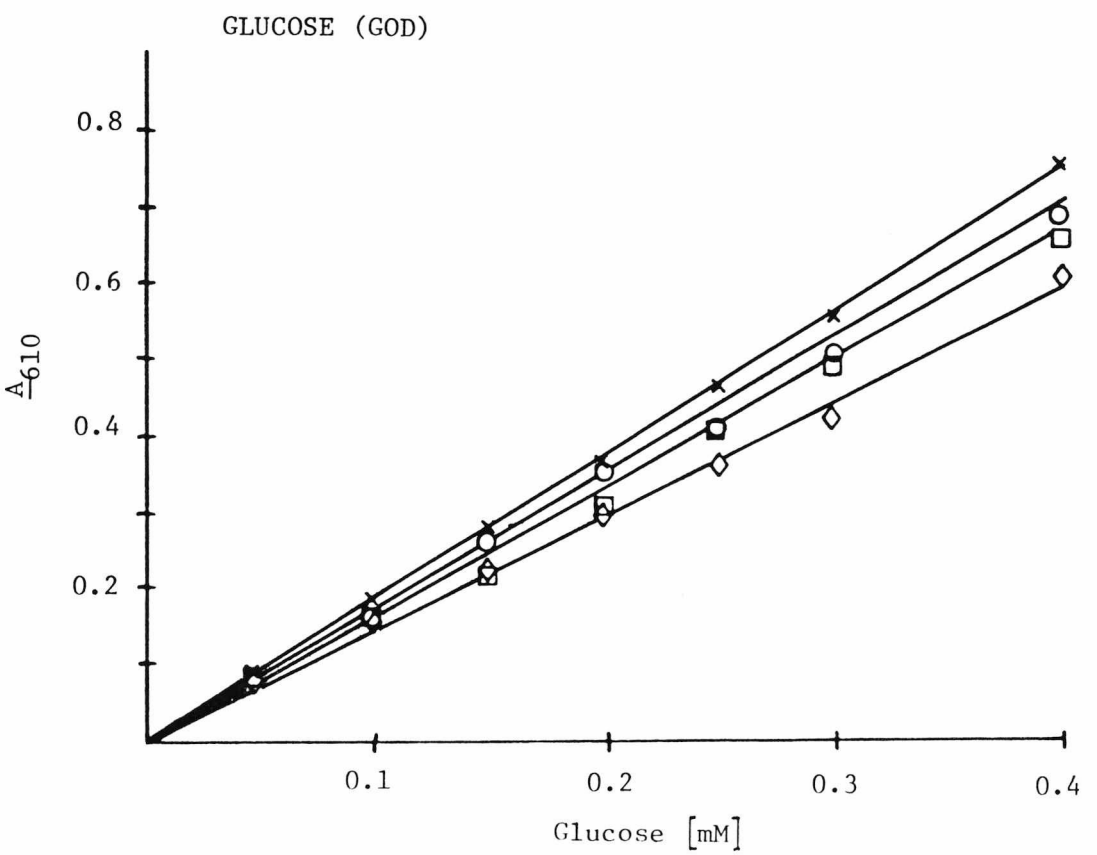
(1) ×, Standard glucose curve,

(2) ○, glucose + soil assayed immediatly following addition,

(3) □, glucose + soil assayed 16h after addition,

(4) ◇, glucose + soil + PbAc/KOx treatment.



b) Thermal stability

Air-dried soil brought to *ca*. 85% w.h.c. (0.5 ml $H_2O$ containing 0.2% (w/v) $NaN_3$ as a bacteriostat (g soil)$^{-1}$) was stored in 25 ml Erlenmeyer flasks at 25°C or 40°C for up to 14 days.

(v) Resistance to proteolysis

The resistance of soil cellulase and *Trichoderma viride* commercial cellulase (BDH) to proteolytic attack by a non-specific protease (Sigma type VI) was investigated. Because immobilized proteases are already present in soil which degrade added protein, three protease additions were made in each experiment in order to maintain a 'free' protease activity above that to which the commercial cellulase was subjected. Soil samples (1 g) at 85% w.h.c. (0.5 ml 0.1M-tris/HCl buffer, pH 7.0 containing 0.2% $NaN_3$ and 3 mg protease ml$^{-1}$) were kept at 25°C. Additional protease (1.5 mg) was added after 24 h and 67 h. Total protease activity was measured by the method of Burns *et al.*, 1972 and the level of 'free' protease calculated by subtracting from this the level of immobilized protease as measured in the control containing unamended soil.

Protease assay (from Burns *et al.*, 1972)

Protease treated soil (1 g dry weight) was placed in the outer well of a Conway Microdiffusion Dish (Gallenkamp). 1.5 ml 0.1M-Tris/HCl buffer, pH 7.0 and 1.0 ml 50mM-α-N-benzoyl-L-arginine amide (BAA) (Sigma) were added to the soil and after mixing the dishes incubated at room temperature (22°C±3°C). After 2 h, BAA substrate was added to the controls and the reaction terminated by addition of 0.5 ml 100mM-$HgCl_2$. 2.5 ml Boric acid indicator was added to the central wells of the Conway Microdiffusion Dishes and $NH_3$ released from the soil-buffer mixture by addition of 1 ml 3M-$K_2CO_3$. After 18 h the $NH_3$ in the boric acid indicator was estimated titrimetrically using 10mM-HCl.

Fig. 3.6 shows the level of immobilized protease activity present in Hamble soil and the levels of 'free' protease activity in the amended soil. *Trichoderma viride* commercial cellulase (1 mg $ml^{-1}$) was subjected to protease (1 mg $ml^{-1}$ and 0.1 mg $ml^{-1}$) which is equivalent to protease activities of 1.37 µmol $NH_3$ $h^{-1}$ and 0.14 µmol $NH_3$ $h^{-1}$ respectively, the latter being well below the level of 'free' activity in the protease treated soil. Cellulase activities in treated and untreated soil were assayed in the routine manner except that 4.5 ml 0.1M-acetate buffer was used. The treated and untreated commercial cellulase preparations were assayed as in section 4.2.1(i).

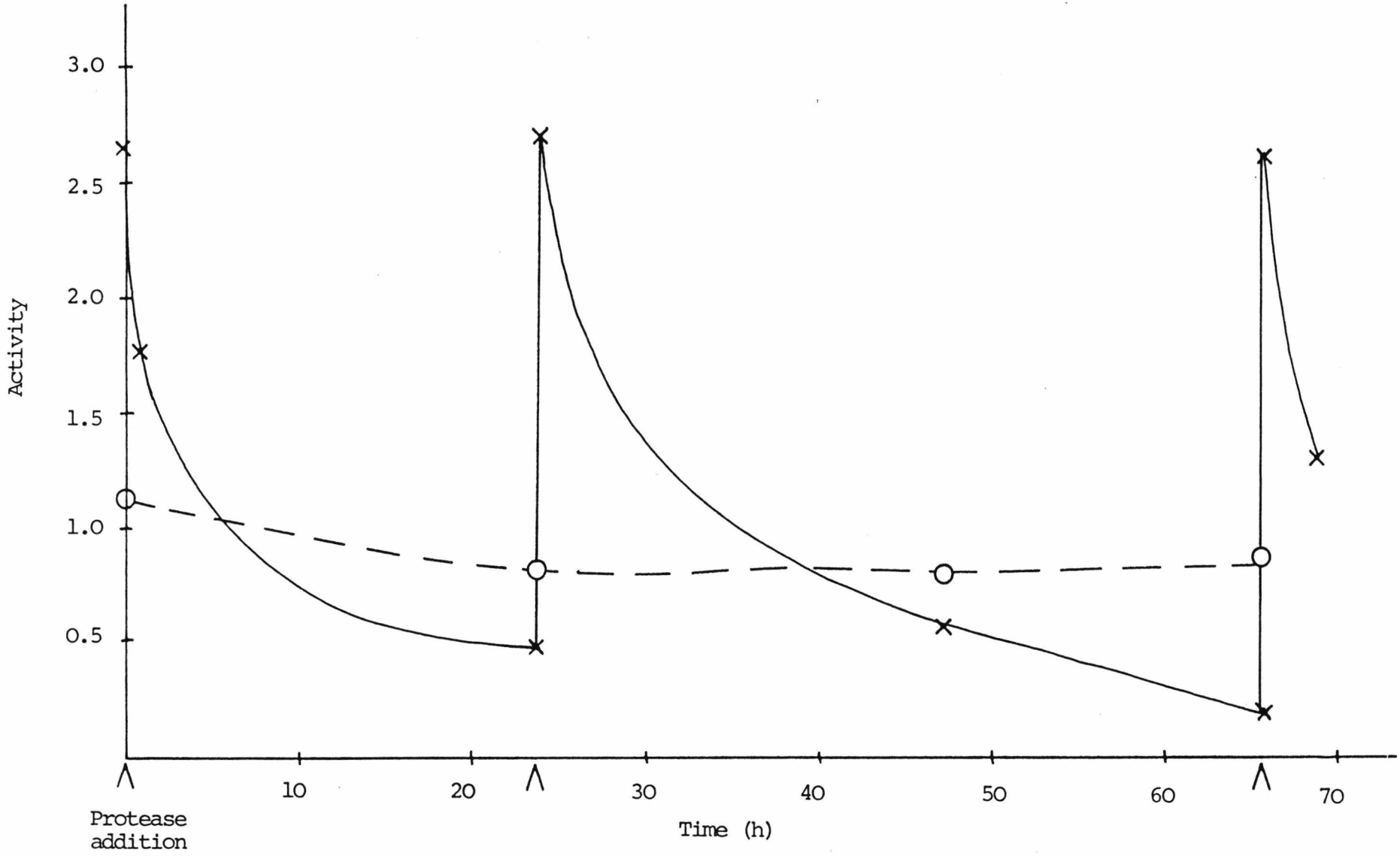
### 3.2.5 Endoglucanase Activity

A brief study was made on the soil-immobilized endoglucanase activity. The problems inherent in using CMC as a substrate have been discussed previously (section 2.2.2) and include such factors as the pH-dependent ionization of substituent groups and the high viscosity of solutions. These may well present even greater difficulties when CMC is used as a substrate to measure endoglucanase activity in soil.

#### The Assay

Flasks containing 10 g soil and 25 ml 2%(w/v) CMC (type 7L prepared in 0.1M-acetate buffer) + 25 ml 0.1M-acetate buffer (containing 0.2% (w/v) $NaN_3$) were agitated on a rotary shaker (150 rpm) at 40°C for 16 h. Controls contained soil + buffer only or 1% (w/v) CMC in buffer only. Aliquots (5 ml) were withdrawn and centrifuged (38 000 x g, 10 min) in a MSE HS18 centrifuge to remove the soil. Samples (2.2 ml) of the supernatants were then analysed for reduing sugars using the DNS method (section 2.4.2).

FIGURE 3.6 Persistence of added protease activity {µmol $NH_3$ released from BAA (g dry wt soil)$^{-1}h^{-1}$}. O, Native protease activity; ×, added 'free' protease activity.



## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Viable Counts of Soil Microorganisms

Viable counts of microorganisms in Hamble soil (20/5/80) are given in Table 3.4-6 (a detailed description of the media is given in section 3.2.2).

The dilution plate technique when used to estimate the number of microorganisms in soil has considerable limitations (Gray and Williams, 1971). The method depends on the assumption that a single colony arises from a single vegetative cell or a spore although, in practice, cells are not well dispersed and are often aggregated or remain attached to soil particles. In addition, cells may adsorb to the pipette walls or spores may fail to germinate. Finally any medium chosen is necessarily selective for certain nutritional groups of microorganisms as are the incubation conditions (e.g. temperature, presence or absence of oxygen etc.). It is generally agreed that the above factors will conspire to give an under-estimate of the number of microorganisms in the soil sample. For example, Skinner *et al.*, (1952) showed that the number of soil microorganisms estimated using the dilution plate technique was less than 10% of that obtained from a direct count. Notwithstanding, the microbial counts on Hamble soil described here were performed with the aim of estimating cellulose-degrading organisms as a percentage of the total population rather than absolute numbers and of isolating a representative selection of cellulolytic organisms for further study. Therefore the dilution plate technique, despite its disadvantages, was thought adequate for our purposes.

Incubation at either 14°C or 25°C did not affect the counts on nutrient agar (NA) and Avicel (B/BA) plates although bacterial growth (as judged by the rate of appearance of macroscopic colonies) at 14°C was much slower than that at 25°C. In contrast, CMC (BC) plates

TABLE 3.4

Viable counts of bacteria (including actiomycetes) in Hamble soil.

[Numbers (g dry wt soil)$^{-1}$ and standard deviation ($\sigma_{n-1}$) are given with the incubation time.]

| MEDIUM | 14°C | 25°C |
|---|---|---|
| NA | $2.56 \pm 0.58 \times 10^7$ (27d) | $3.78 \pm 0.51 \times 10^7$ (7d) |
| BC (CMC) | $0.13 \pm 0.10 \times 10^7$ (27d) | $1.00 \pm 0.08 \times 10^7$ (16d) |
| B/BA (AVICEL) | $1.05 \pm 0.28 \times 10^7$ (27d) | $0.70 \pm 0.16 \times 10^7$ (7d) [$1.15 \pm 0.39 \times 10^4$ cleared zones] |

TABLE 3.5

Viable counts of actinomycetes in Hamble soil.

[Numbers (g dry wt soil)$^{-1}$ and standard deviation ($\sigma_{n-1}$) are given with the incubation time.]

| MEDIUM | 14°C | 25°C |
|---|---|---|
| NA | N.D | $2.6 \pm 1.2 \times 10^6$ (7d) |
| BC (CMC) | N.D | $3.3 \pm 0.2 \times 10^6$ (16d) |
| B/BA (AVICEL) | N.D | $5.6 \pm 1.4 \times 10^6$ (7d) |

TABLE 3.6

Viable counts of fungi in Hamble soil.

[Numbers (g dry wt soil)$^{-1}$ and standard deviation ($\sigma_{n-1}$) are given with incubation time.]

| MEDIUM | 14°C | 25°C |
|---|---|---|
| MEA | 8.2 ± 2.5x10$^5$ (20d) | 8.1 ± 4.1 x10$^5$ (7d) |
| | | 10.0 ± 4.4 x10$^5$ (10d) |
| CZD | 5.9 ± 4.1x10$^5$ (20d) | 6.6 ± 0.9 x10$^5$ (7d) |
| | | 6.0 ± 2.0 x10$^5$ (10d) |
| SC (CMC) | 5.5 ± 2.8x10$^5$ (20d) | 3.8 ± 1.7 x10$^5$ (7d) |
| S/SA (AVICEL) | 3.5 ± 2.2x10$^5$ (20d) | 7.2 ± 3.9 x10$^5$ (7d) |

supported significantly fewer colonies at 14°C than at 25°C. Even at 25°C bacterial growth was much slower on BC than on B/BA.

From the total bacterial counts it appeared that *ca.* 25% of the soil bacteria were able to grow using Avicel as their sole carbon source although only a small proportion of this fraction produced clearing zones on the Avicel plates (indicative of extensive hydrolysis). A major proportion (*ca.* 80%) of this cellulolytic population was actinomycetes. [Reports in the literature on the cellulase systems of actinomycete species (Stutzenberger, 1972; Hagerdal *et al.*, 1978; Crawford and Sutherland, 1979; Hagerdal *et al.*, 1980; Ishaque and Kuepfel, 1980) suggest that these organisms possess a cellulase system more akin to that of the fungi than the bacteria. Therefore, for the rest of this discussion actinomycetes will be regarded as a group distinct from both the bacteria and the fungi]. One factor limiting growth on Avicel plates is that many cellulolytic bacteria need to align themselves along the cellulose fibril in order to be efficient at substrate hydrolysis. This is because their cellulases are not secreted into the surrounding medium but are retained at the outer membrane (section 1.3.2). Therefore, these organisms may have difficulty in growing on Avicel plates because the cellulose microcrystalline particles are incorporated in the agar and many are not directly accessible to the microorganisms growing on the surface of the plate.

Ten actinomycete and five bacterial isolates were taken from the Avicel plates and purified by sub-culturing on NA and B/BA plates. They were maintained on NA slopes at 4°C.

There was no significant difference in the number of fungal colonies growing on MEA and CZD plates. These media may have supported different components of the soil fungal population but this could not be proven without lengthy identification procedures especially as the colony

morphology of a single species may well be different on different media. Fungal counts on S/SA plates were not significantly different from those on MEA plates while the counts at 25°C on SC plates were *ca.* 45% of those on MEA plates. The proteose peptone and the Tween 80, in the medium may have supported the growth of non-cellulolytic fungi on SC and S/SA plates. Nonetheless, *ca.* 50% of the colonies on SC plates were surrounded by clearing zones following flooding of the plates with 10 ml 1% (w/v) cetyltrimethylammonium bromide, indicating that hydrolysis of CMC had occurred. Unlike the bacterial B/BA plates clearing zones were not obvious on the S/SA plates. With many of the fungal colonies, the dense sporulating mycelium masked any possible clearing and on the more densely populated plates diffusible products from cellulose breakdown may have supported the growth of species unable to use microcrystalline cellulose as their sole carbon source.

Several fungi including those tentatively identified following microscopic examination as belonging to the genera; *Trichoderma*, *Botryotrichum*, *Cladosporium*, *Paecilomyces*, *Acremonium*, *Verticillium*, *Mucor* and *Gliomastix*, were isolated from the SC and S/SA plates and stored at 4°C.

### 3.3.2 β-D-Glucosidase

Table 3.7 summarizes the characteristics of the immobilized β-D-glucosidase activity in Hamble soil. For a more detailed discussion the reader is referred to R.G. Geraghty (M.Sc Thesis, U.K.C., 1983).

### 3.3.3 Characterization of Total Cellulase Activity

#### (i) pH-Activity profile

The relationship of total cellulase activity to pH is shown for air-dried (Figs 3.7-8) and field-moist (Fig. 3.9) soil. All points are

TABLE 3.7 Properties of β-D-glucosidase in soil (R.J. Geraghty, 1983)

1. Activity of air-dried soil (non-specific : pNPG → pNP) = 0.88μmol pNP (g soil)$^{-1}$ h$^{-1}$
   r = 0.9909
   (specific : cellobiose → glucose) = 0.44μmol glucose (g soil)$^{-1}$ h$^{-1}$

2. Activity of humic-enzyme extract (non-specific) = 0.09μmol pNP (g soil)$^{-1}$ h$^{-1}$

3. pH-optimum of soil and extract = pH 5.2 - 5.5

4. Temperature-optimum of soil and extract = 60°C

5. Kinetics of air-dried soil β-D-glucosidase

| | Lineweaver-Burk | Eadie Hofstee | Direct |
|---|---|---|---|
| $K_m$ (mM) | 0.5176 | 0.5535 | 0.6450 |
| $V_{max}$ (μmol pNP (g soil)$^{-1}$ h$^{-1}$) | 0.8414 | 0.8672 | 0.9053 |

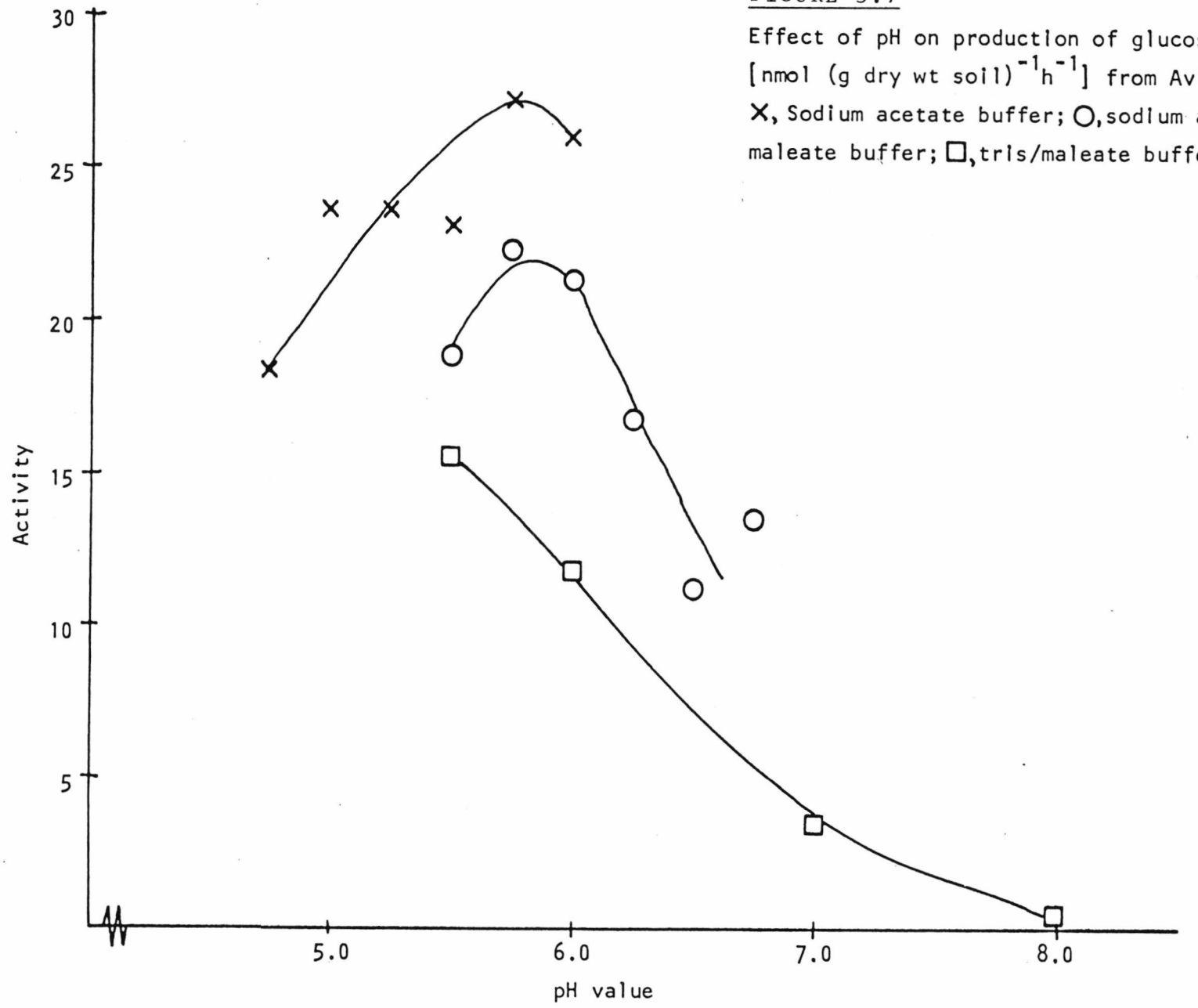



FIGURE 3.7

Effect of pH on production of glucose [nmol (g dry wt soil)$^{-1}$h$^{-1}$] from Avicel. ×, Sodium acetate buffer; ○, sodium acid maleate buffer; □, tris/maleate buffer.

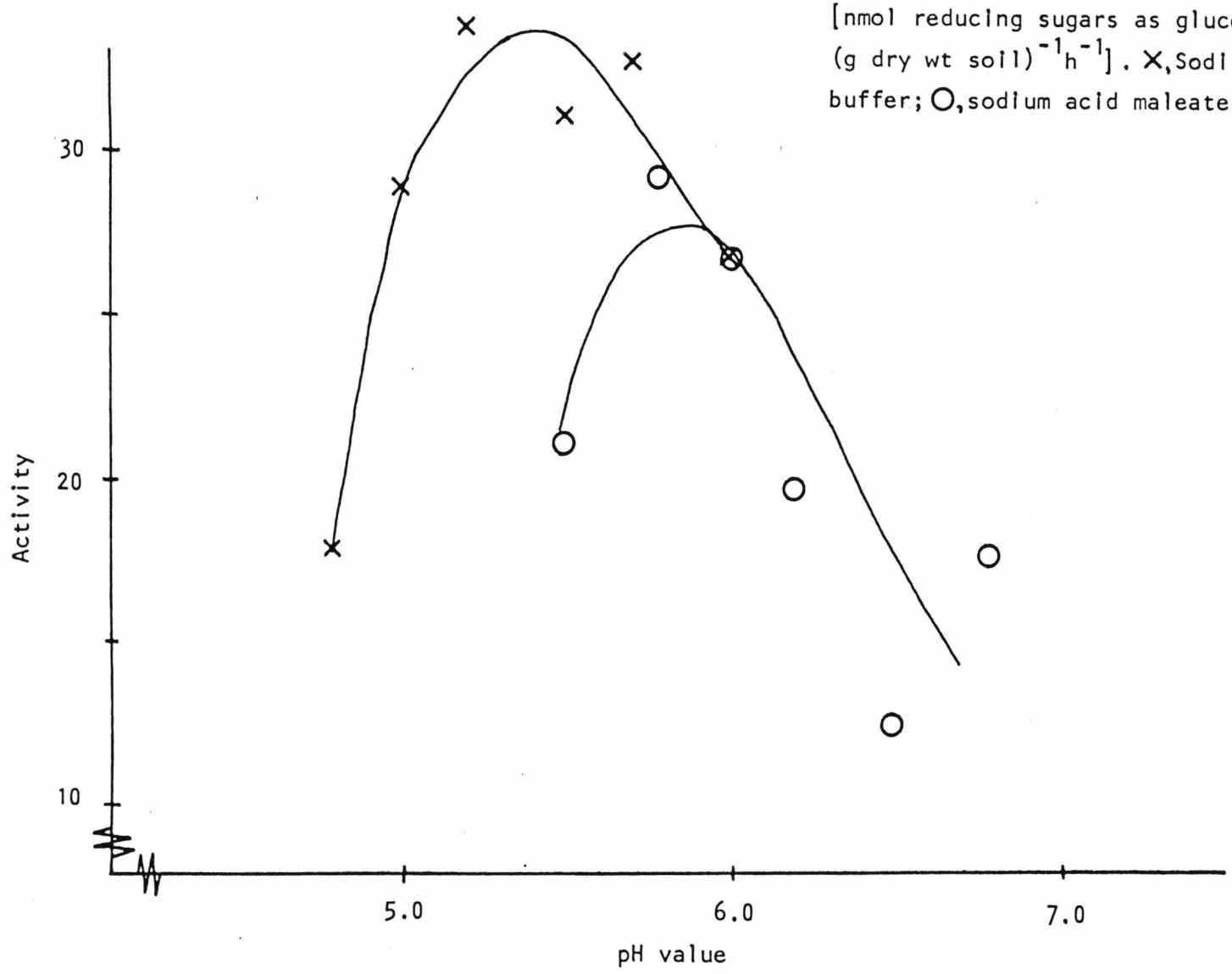



FIGURE 3.8

Effect of pH on soil cellulase activity [nmol reducing sugars as glucose equivalents $(g \text{ dry wt soil})^{-1} h^{-1}$]. ×, Sodium acetate buffer; ○, sodium acid maleate buffer.

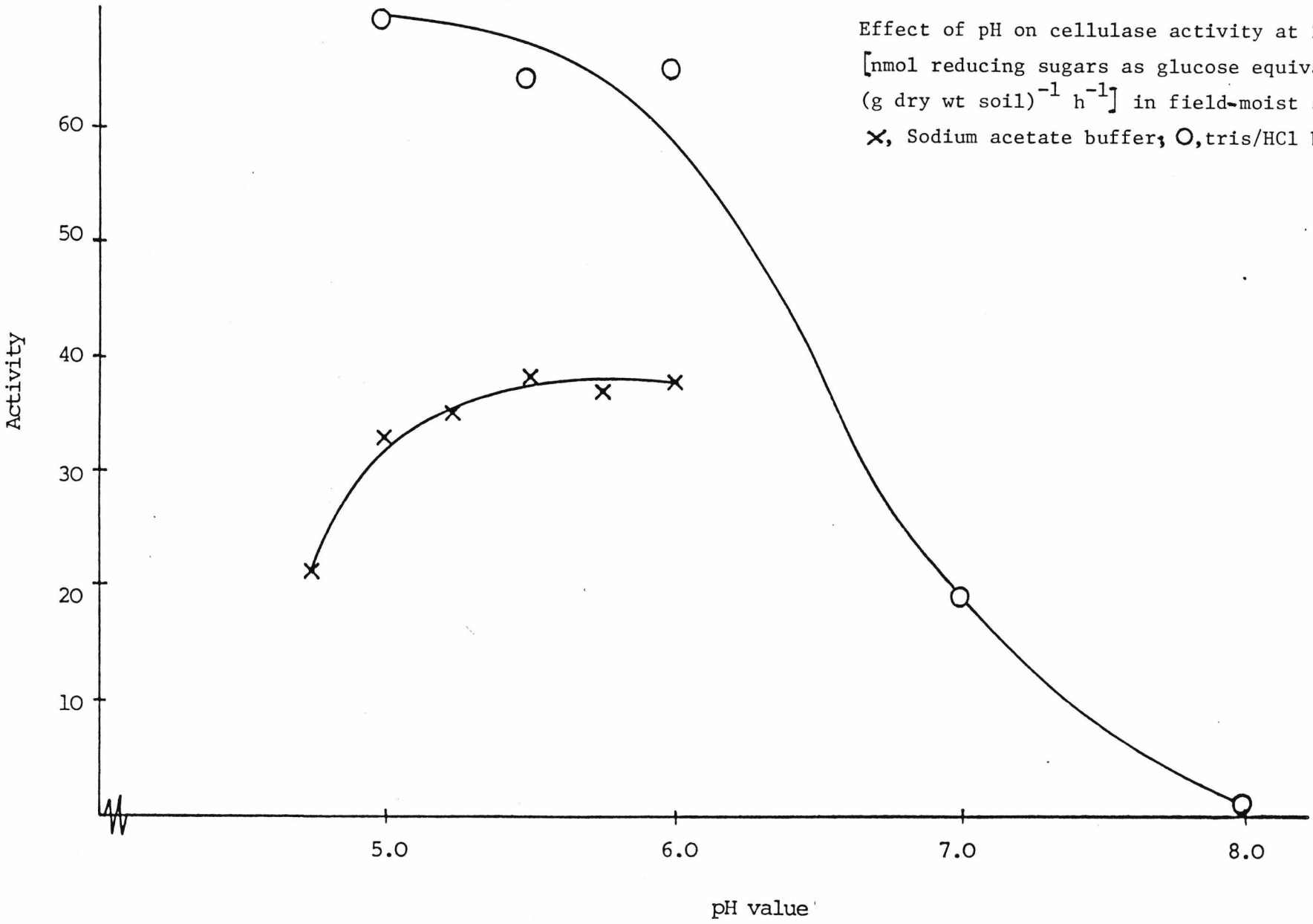



FIGURE 3.9

Effect of pH on cellulase activity at 25°C [nmol reducing sugars as glucose equivalents $(g \text{ dry wt soil})^{-1} h^{-1}$] in field-moist soil. ×, Sodium acetate buffer; O, tris/HCl buffer.

the mean of triplicate determinations and a glucose standard curve was constructed at each pH since the redox potential of the sugars is pH dependent. [The low activities measured in field-moist soil using acetate buffer (Fig. 3.9) were due to inadequate shaking]. The activity is highest between pH 5.25 and 5.75. Other workers (Benefield, 1971; Ross, 1974) have assayed soil cellulase activity against Avicel at pH 5.0 but have not quoted a pH optimum. The pH optimum of fungal cellulases is in the range 4.0-5.0 (5.5) while bacterial and actinomycete cellulases have pH optima in the range 5.6-7.0. Comparisons of the properties of soil enzymes (whose origins are uncertain) with those from known sources (e.g. microorganisms) must be interpreted with caution for a number of reasons.

[1] The pH of the soil-buffer slurries in which these assays are performed may not be the same as at the soil-solution interface where the immobilized enzyme/substrate interaction is occurring. In fact the pH at the soil colloid surface may be considerably more acid than that of the bulk solution due to the accumulation of $H^+$ ions within the diffuse double layer (DDL) (McLaren and Skujins, 1968; Stotzky, 1974).

[2] The activity and thus the pH-activity profile of a particular soil enzyme will often reflect the action of a group of enzymes of different origins (i.e. microbial, plant, animal) rather than just one enzyme (Pettit, 1978).

Nevertheless, fungi are known to be the principal producers of extracellular cellulases (Enari and Markkanen, 1977) and since the true pH optimum of the soil cellulase activity (taking into account the DDL effect) is likely to be below 5.5, the cellulase activity being measured is probably

of fungal origin. Measurement of glucose (GOD method) as compared with reducing sugars (Nelson-Somogyi method) suggested that glucose accounted for 65-80% of the reducing sugars produced during the 16 h assay.

(ii) Thermal stability

Table 3.8 shows the effect of storage at 25°C and 40°C on the total cellulase activity. After storage at 40°C for 7 - 14 days the variation between replica samples was large, possibly due to azide decomposition at the elevated temperature and the resulting microbial proliferation.

(iii) Resistance to proteolysis

The effect of added protease on cellulase activity in soil compared with a *Trichoderma viride* commercial cellulase preparation (BDH) is shown in Fig. 3.10. Virtually all *T. viride* commercial cellulase activity was lost by 24 h after treatment with 1.0 mg $ml^{-1}$ protease while only 42% of the activity remained at 72 h after treatment with 0.1 mg $ml^{-1}$ protease. The soil activity, on the other hand, was resistant to attack by protease at the concentrations measured in section 3.2.4 (v). The resistance of soil cellulase to proteolytic attack is not surprising considering the high level of immobilized protease activity found in Hamble soil. Only resistant enzymes could survive in soil.

(iv) Cellulase activity in soils collected at different times of the year

Table 3.9 shows the cellulase activities in air-dried samples collected June 1980, March 1982 and October 1982 together with the standard deviations ($\sigma_{n-1}$).

TABLE 3.8

Effect of incubation at 25°C and 40°C on cellulase activity in air-dried soil brought to ca. 85% w.h.c.

Activity against Avicel measured as:

(1) nmol reducing sugars as glucose equivalents (g dry wt soil)$^{-1}$ h$^{-1}$

(2) nmol glucose (g dry wt soil)$^{-1}$ h$^{-1}$

Results given with standard deviation ($\sigma_{n-1}$).

| Time (d) | Reducing Sugars (1) | Glucose (2) |
|---|---|---|
| 25°C | | |
| 0 | 34.4 ± 4.1 | 22.3 ± 1.3 |
| 3 | 40.8 ± 3.0 | 25.7 ± 3.1 |
| 7 | 32.8 ± 2.9 | 18.4 ± 2.0 |
| 14 | 32.8 ± 2.3 | 19.3 ± 1.3 |
| 40°C | | |
| 0 | 34.4 ± 4.1 | 22.4 ± 1.3 |
| 3 | 35.9 ± 2.3 | 24.7 ± 3.2 |
| 7 | 35.8 ± 3.2 | N.D |
| 14 | 30.2 ± 4.2 | 19.6 ± 5.1 |

FIGURE 3.10 Effect of pronase on free and soil-associated cellulase activity (expressed as a percentage of the untreated control(X)).

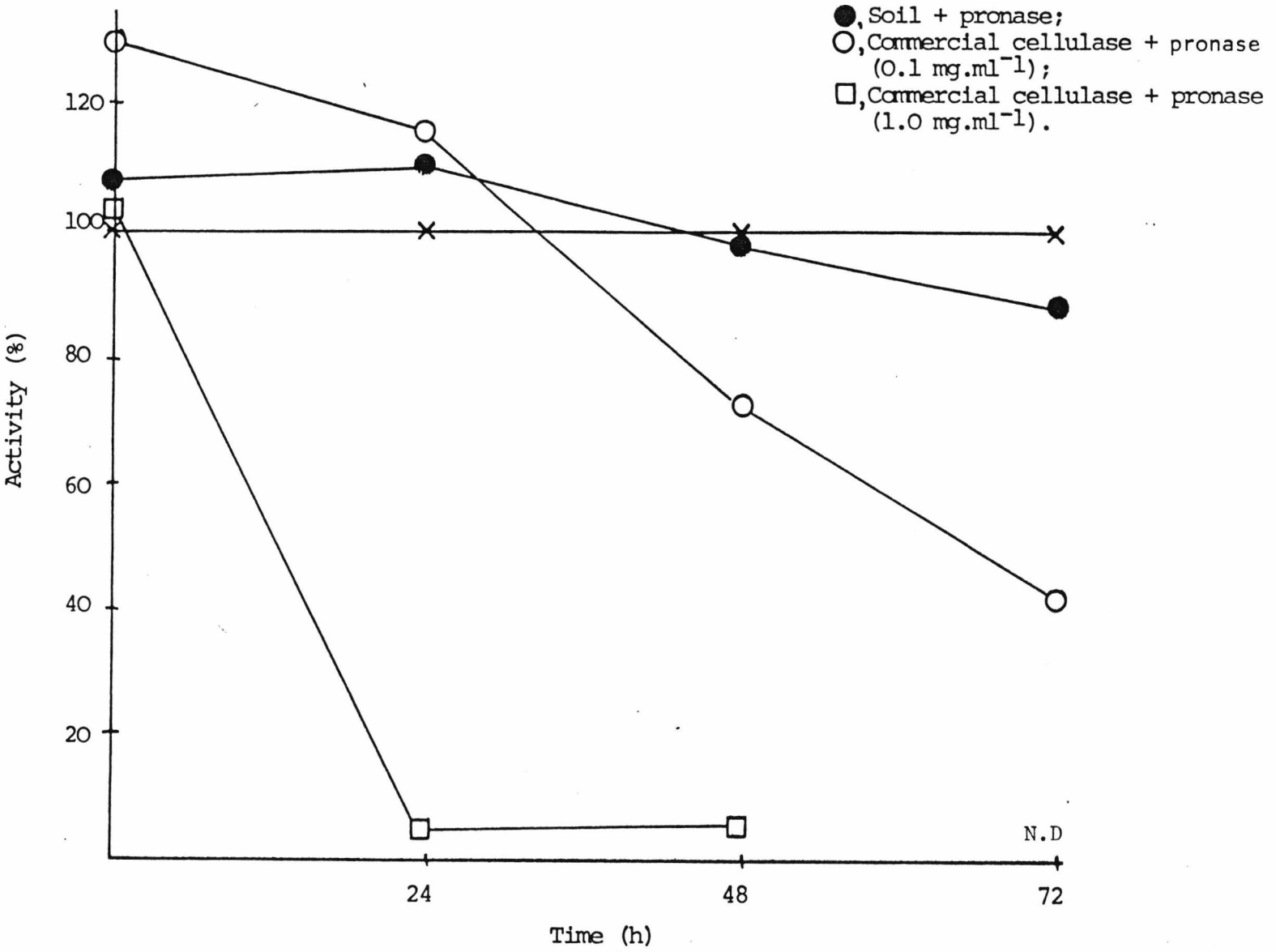


TABLE 3.9

| Collection Date | Activity [nmol reducing sugars as glucose equivalents (g dry wt soil)$^{-1}$ h$^{-1}$] |
|---|---|
| June 1980 | 38.2±4.5 |
| March 1982 | 34.3±2.3 |
| October 1982 | 39.8±4.9 |

(v) Comparison of cellulase activity in different soils (field-moist)

Table 3.10 shows cellulase activities measured (at 40°C) in field-moist grassland and woodland soil as compared to Hamble soil (19/10/1982).

TABLE 3.10

| Soil | pH | Activity [nmol reducing sugars as glucose equivalents (g dry wt soil)$^{-1}$ h$^{-1}$] |
|---|---|---|
| Hamble (19/10/1982) | 6.30 | 111.3 |
| Grassland | 6.15 | 191.6 |
| Woodland | 3.70 | 175.5 |

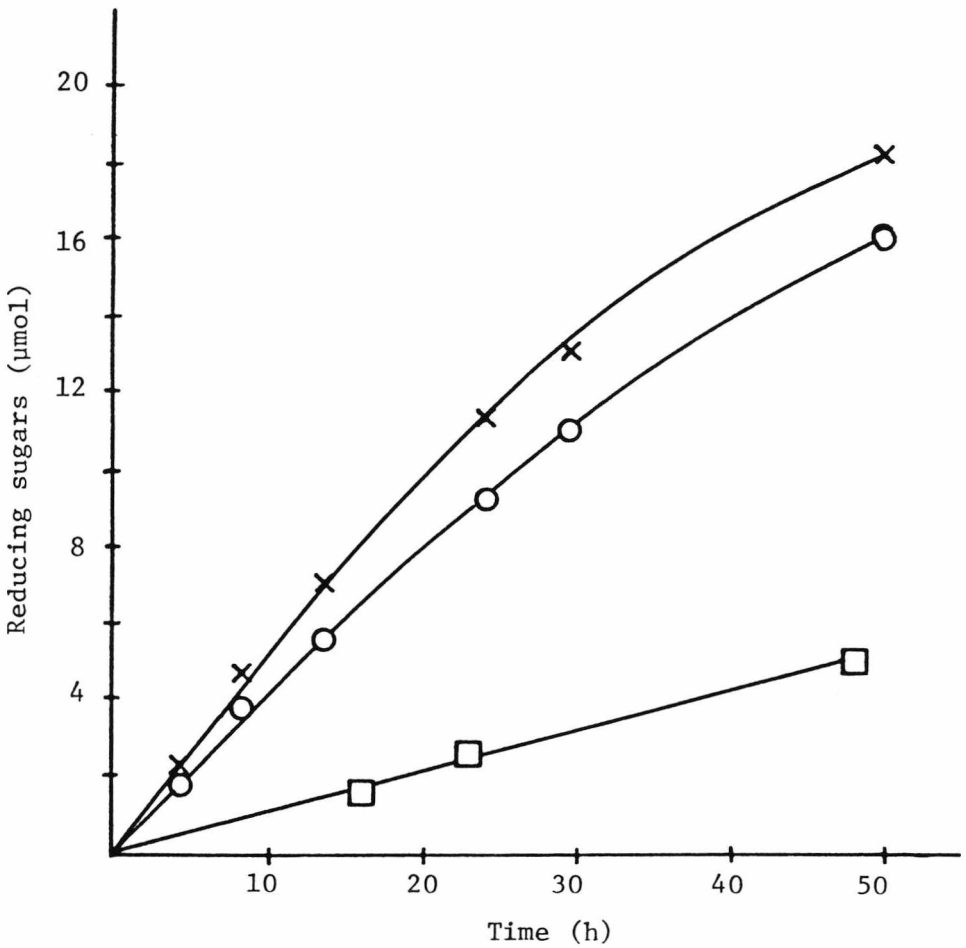
3.3.4 Endoglucanase Activity

Fig. 3.11 shows the release of reducing sugars from CMC in field-moist soil (Hamble 19/10/1982) and air-dried soil (Hamble 20/5/1980). Using a 16 h assay the activity in air-dried soil was 110 nmol $g^{-1}$ $h^{-1}$.

The level of endoglucanase activity in field-moist soil was 490 nmol reducing sugars as glucose equivalents (g dry wt soil)$^{-1}$ $h^{-1}$. Ross and Speir (1979) measured the endoglucanase activity in two field-moist silt loam soils as 112 and 114 nmol reducing sugars as glucose equivalents (g dry wt soil)$^{-1}$ $h^{-1}$ at 30°C. Pancholy and Rice (1973) measured endoglucanase activities over a 9 month period in a field-moist prairie soil, pH 6.6. The measured activities ranged from 76-967 nmol reducing sugars as

FIGURE 3.11

Production of reducing sugars [µmol glucose equivalents (g dry wt soil)$^{-1}$] with time. ×, Field-moist soil pH 5.75; ○, field-moist soil pH 5.0; □, air-dried soil pH 5.75.



glucose equivalents (g dry wt soil)$^{-1}$ h$^{-1}$ at 30$^{o}$C. However, our results are not comparable as both Ross and Speir (1979) and Pancholy and Rice (1973) measured reducing sugars by the Nelson-Somogyi method and removed residual CMC by centrifugation, a process which in our hands removed some of the chromatogen and gave artificially low results.

In Hamble soil we found more reducing sugars produced h$^{-1}$ from CMC than from Avicel, suggesting that endoglucanase is present at higher levels than exoglucanase.

# CHAPTER 4: ENZYME DIFFUSION AND PERSISTENCE

## 4.1 INTRODUCTION

Several microorganisms were isolated from the silt loam, Hamble series soil and were found to be capable of utilizing insoluble cellulose (Avicel) as their sole carbon source (section 3.3.1). To accomplish cellulolysis a microorganism must possess an extracellular enzyme system capable of producing soluble products that can pass into the cell (section 1.3.2). Furthermore, in a soil environment it seems improbable that extracellular enzymes are produced and secreted constitutively (section 1.4.4). The persistence of extracellular cellulases will therefore be an important consideration in determining the rate at which cellulose is degraded in soil and will influence the way in which microbial species and communities respond to intermittent and spatially separated cellulosic substrates.

A low level of cellulase activity and a soil-immobilized β-D-glucosidase activity have been characterized in Hamble soil (Chapter 3). Before ascribing an ecological role to immobilized enzymes, such as that described in section 1.4.4, information was needed on the survival and efficacy (in terms of locating and reacting with substrates) of free (i.e. unbound) cellulases. The first requirement was to develop a technique which would enable us to study under carefully controlled conditions the diffusion of extracellular enzymes through soil and soil-like environments.

Radial diffusion gels or the agar 'cup-plate' diffusion technique (originally developed for antibiotic screening - Kavanagh, 1975), has been applied previously to the semiquantitative determination of enzyme activity. For example, the technique has been used to assay proteases

(Iowenstein and Ingild, 1976) as well as pectin-esterase, amylase, polygalacturonase and cellulase (Dingle *et al.*, 1953). The technique involves the diffusion of an enzyme through a gel containing a macromolecular substrate, resulting in the release of water-soluble low molecular weight products. We used a modified version of this technique in which a ring of solid medium was removed from an agar plate and replaced with various obstacles, such as substrate-free medium or medium containing soil or one of its particulate components (sand, silt, clay). The effect of various 'barrier rings' on the diffusion and activity of extracellular cellulases could thus be assessed.

Cellulase enzymes from different sources have different molecular weights and activities etc. (section 1.3.1). For this reason we used the diffusion plate technique to investigate the properties and activity of cellulases from:

[1] *Trichoderma viride*,

[2] a *Streptomyces* species, and

[3] *Sporocytophaga myxococcoides*.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Characterization of Enzyme Preparations

#### (i) *Trichoderma viride* commercial cellulase

*Trichoderma viride* commercial cellulase preparation (BDH - activity 0.02 unit* $mg^{-1}$) was assayed for activity using Avicel, CMC and pNPG (Table 4.1). The assay used for combined exo-and endoglucanase was the same as that used in the soil (section 3.2.4i) except that 1 g soil was

*BDH define one unit as the quantity of enzyme that liberates 1 µmol glucose from CMC in 1 min at 40°C pH5.0.

replaced with 1 ml enzyme preparation and the amount of buffer added reduced from 5 ml to 4 ml. The DNS method for determining endoglucanase activity is described in section 2.4.2 and the assay for β-D-glucosidase using pNPG was that used in the soil (section 3.2.3) except that 0.2 g soil was replaced with 0.5 ml enzyme preparation, the amount of buffer reduced to 1.0 ml and the $CaCl_2$ omitted.

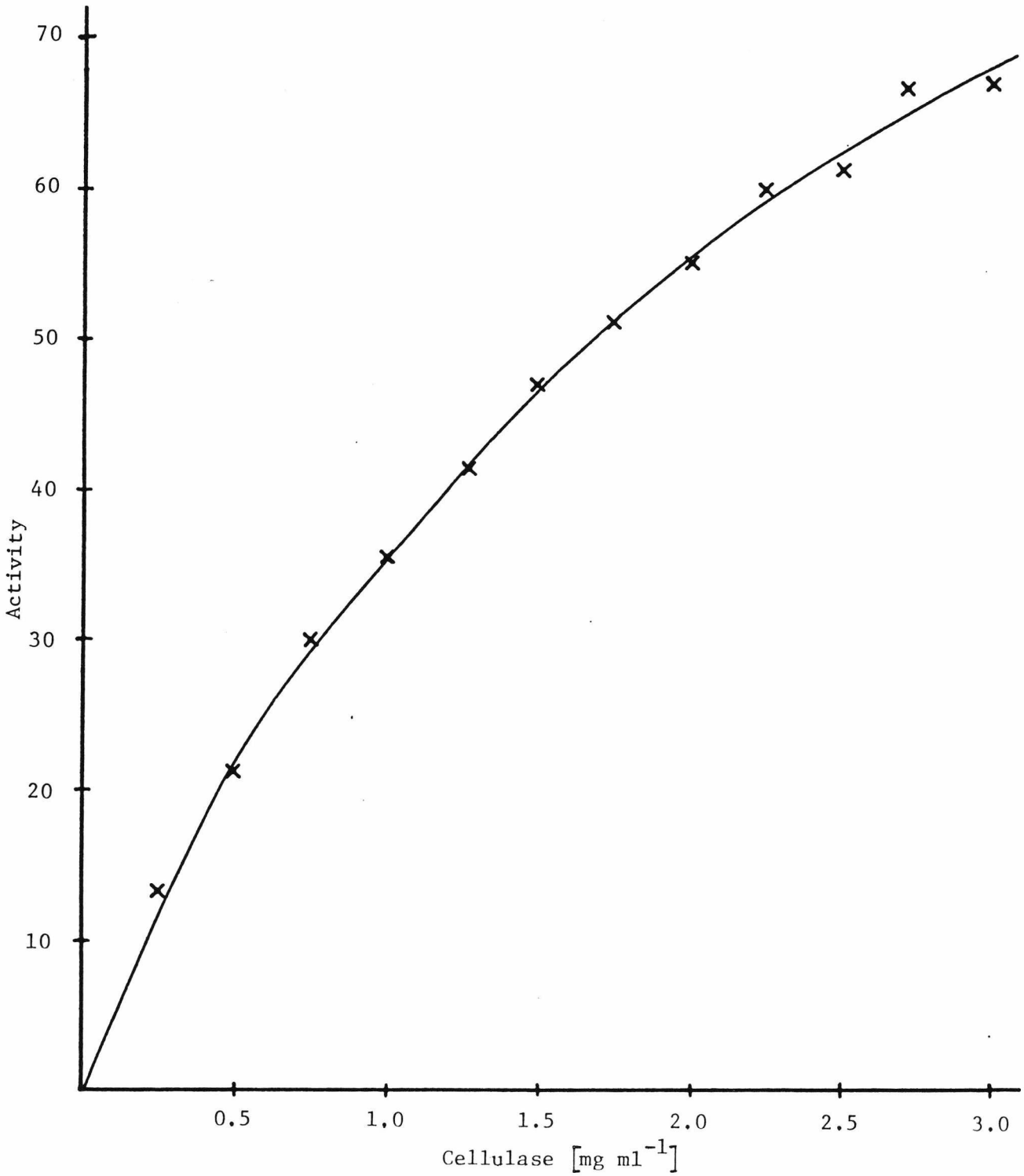
TABLE 4.1 Activities (mg enzyme)$^{-1}$ of *Trichoderma viride* commercial cellulase preparation (BDH) 1 mg ml$^{-1}$

| SUBSTRATE | ACTIVITY |
|---|---|
| Avicel | 0.23 µmol reducing sugars h$^{-1}$ |
| CMC | 35 µmol reducing sugars h$^{-1}$ |
| pNPG | 1.04 µmol pNP h$^{-1}$ |

Fig. 4.1 shows a standard curve of reducing sugars, expressed as glucose equivalents produced h$^{-1}$, versus concentration of cellulase solution (section 2.4.2). Cellulase (1 mg) under our conditions produced 35 µmol reducing sugars h$^{-1}$ at 40°C pH5.0. The discrepancy between the activity reported here and that reported by BDH is because we measured total reducing sugars rather than glucose alone. Despite the non-linear nature of the standard curve obtained using BDH cellulase as compared to that of the *T. viride* cellulase preparation from Sigma (section 2.4.2), the BDH cellulase was preferred for developing the diffusion plate assays because it had greater stability at 25°C. For diffusion studies 1 mg ml$^{-1}$ solutions of *T.viride* cellulase (BDH) in 0.1M-acetate buffer pH 5.0 were

FIGURE 4.1

DNS Endoglucanase Assay.
Standard curve for *Trichoderma viride* commercial cellulase preparation (BDH). Activity expressed as µmol reducing sugars as glucose equivalents produced $ml^{-1}$ $h^{-1}$.



filter sterilized using HT-200 Tuffryn 0.2 µm filters (Gelman). [Millipore Type GS Nitro-cellulose 0.22 µm filters were found to adsorb cellulase]. Filter sterilized cellulase solutions were stable for up to 20 days at 25°C.

(ii) Cellulase preparation from a *Streptomyces* sp. (act 9)

A grey sporulating antinomycete (act 9) isolated from Hamble soil was identified as belonging to the genus *Streptomyces*. Fig. 4.2 shows the time course of growth and cellulase production for this organism.

Culture Conditions

Shake flask cultures were grown at 30°C in 250 ml Erlenmeyer flasks baffled with stainless steel springs to prevent clumping. Flasks containing 49 ml Ishaque medium (see appendix I) + 1% (w/v) CMC 7L (IC) were inoculated with 1 ml of an 8 h culture grown on basal Ishaque medium (IM). Samples (1 ml) were withdrawn at intervals, the $A_{610}$ recorded, and after centifuging in a microfuge (Beckman), the supernatant liquid assayed for endoglucanase using the DNS method. Endoglucanase was not detected in the supernatants of samples taken from cultures grown on IM or on IM supplemented with 0.1% (w/v) or 1% (w/v) glucose. In flasks containing Berg MM + 1% (w/v) CMC 7L or 1% (w/v) CMC 9M8F only limited growth occurred and no cell-free extracellular endoglucanase was detected.

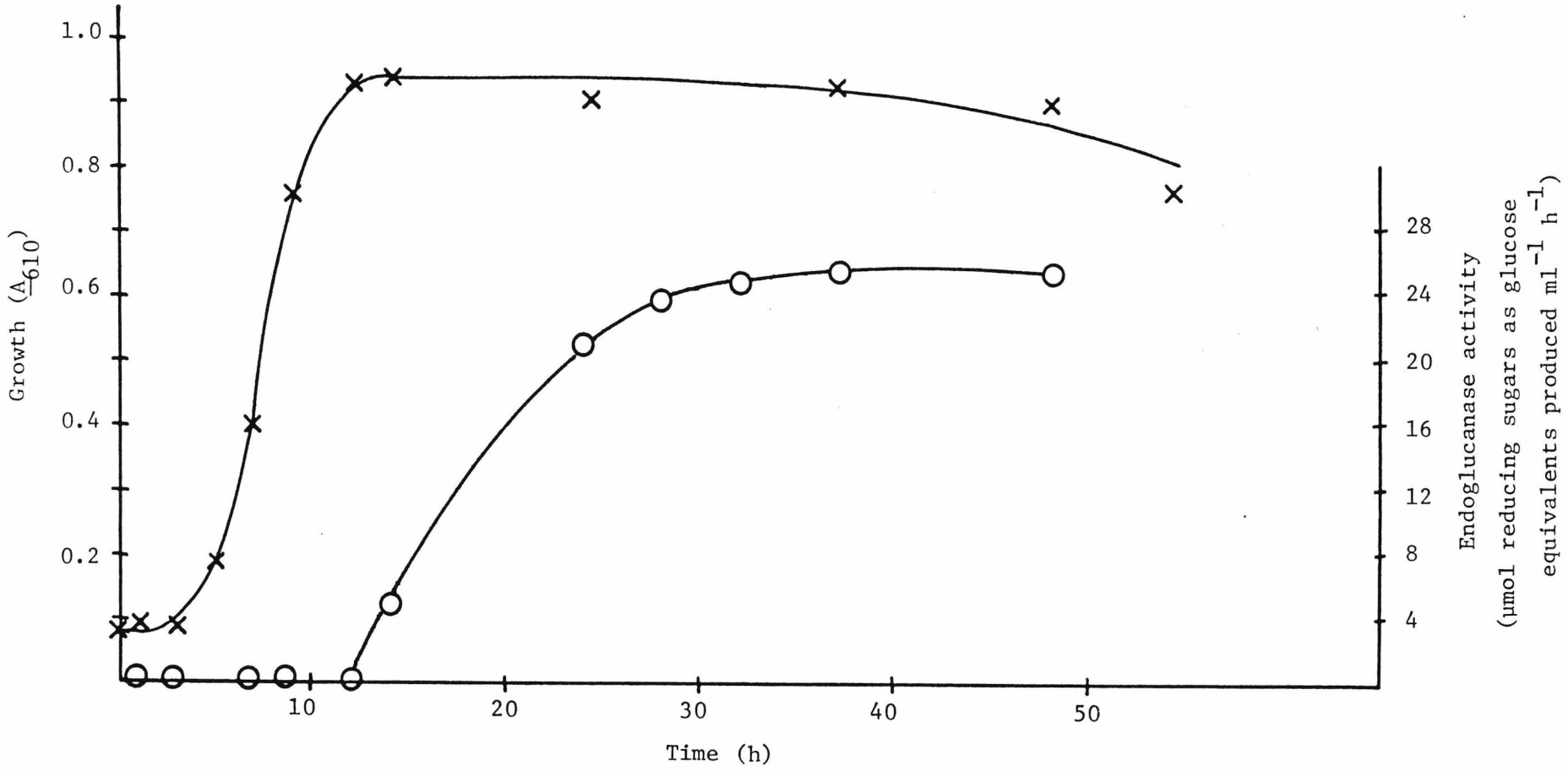
Cellular location of endoglucanase and β-D-glucosidase in act 9

Two IC grown cultures were harvested after 48 h by centrifuging (38 000 x g, 10 min), the supernatant liquid was filtered through a HT-200 Tuffryn 0.2 µm filter (Gelman) under vacuum and stored at 4°C = CULTURE FILTRATE.

The pellet was washed twice by resuspending in 0.01M-phosphate buffer pH 7.0 and centrifuging. The pellet from the second wash was

FIGURE 4.2

Growth (×) and endoglucanase production (○) of isolate act 9.



taken up in 7.5 ml buffer and sonicated 6 x 20 sec at 3 μm amplitude (MSE Ultrasonic disintegrator) = SONICATED PREPARATION.

The sonicated preparation was centrifuged (10 000 x g, 10 min) to remove whole cells and cell debris, the supernatant liquid decanted and centrifuged (50 000 x g, 120 min, MS50 prepspin centrifuge). The supernatant from prepspin = CELL-FREE EXTRACT (CFE). The pellet from the prepspin was washed by resuspending in 5 ml phosphate buffer, centrifuged (50 000 x g, 120 min), and finally taken up in 2 ml buffer = PARTICULATE FRACTION.

The different fractions (i.e. culture filtrate, sonicated preparation, CFE and particulate fraction) were assayed using CMC, pNPG and cellobiose (Table 4.2). The assay methods using CMC and pNPG have been described previously (section 4.2.1i). Fig. 4.3 shows the effect of pH on endoglucanase activity in the culture filtrate from act 9. The optimum pH appears to be in the range 5.5 - 6.25. All subsequent assays were performed at pH 5.75 since this is still within the buffering range of acetate buffer. Protein was measured by the Lowry method using bovine serum albumin as a standard.

β-D-Glucosidase assay using cellobiose

Enzyme preparation (0.5 ml) was incubated with 1 ml 25mM-cellobiose and 1 ml 0.1M-acetate buffer at 30°C. After 4 h, 1 ml of cellobiose was added to the controls and the reaction terminated by heating for 10 min in a boiling water bath at 100°C. After cooling to room temperature 1 ml samples were assayed for glucose using the glucose oxidase/peroxidase method (section 2.5.1).

Endoglucanase activities varied between flasks (22 - 29 EU* $ml^{-1}$).

*1 EU is defined as the quantity of enzyme that liberates 1 μmol reducing sugars measured as glucose equivalents from CMC in 1 h when using the DNS endoglucanase assay method (section 2.4.2).

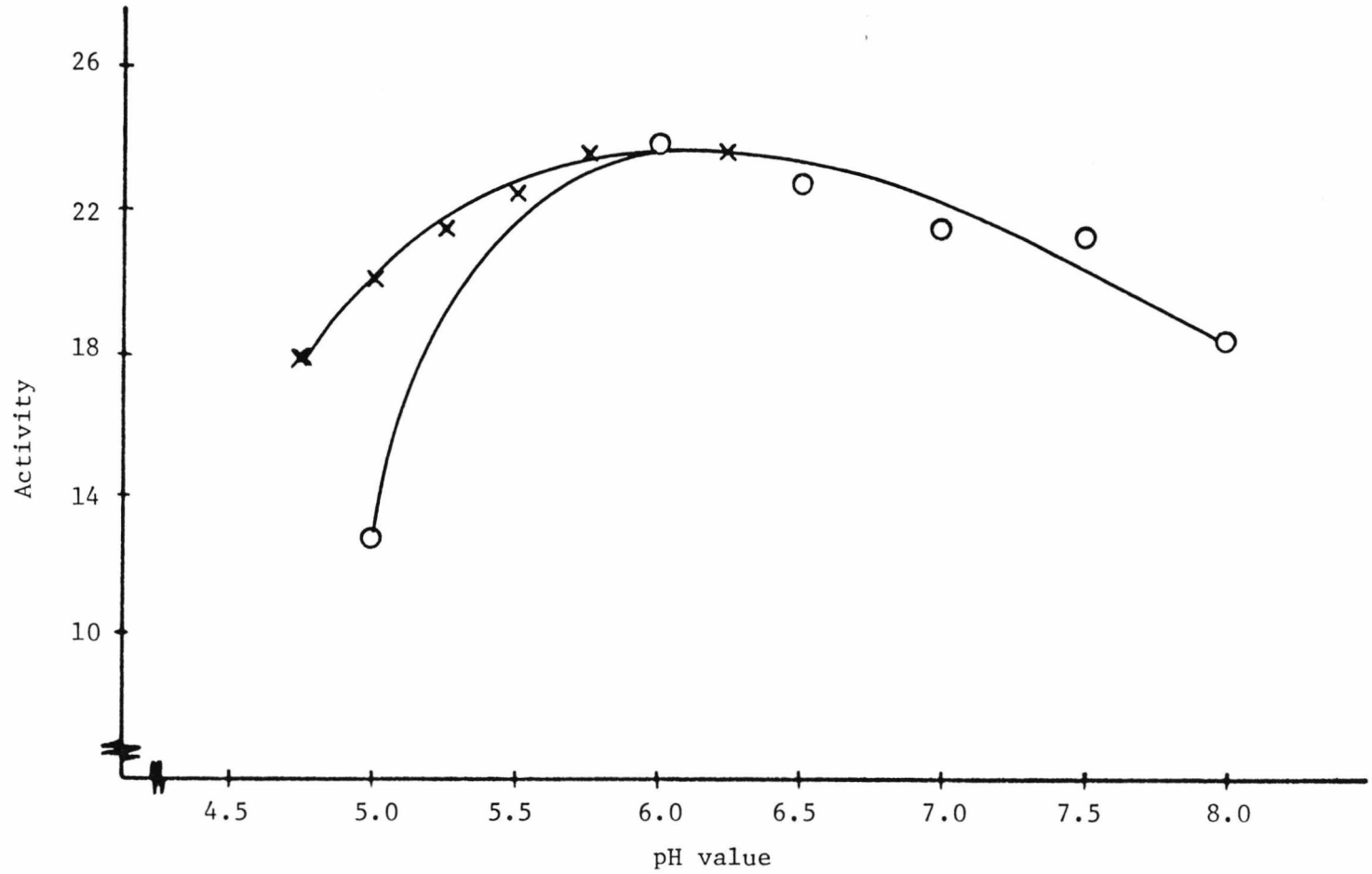
TABLE 4.2

Location of endoglucanase and β-D-glucosidase in IC grown cultures of act 9. Activities are expressed in fraction derived from 100ml: Endoglucanase as μmol reducing sugars $h^{-1}$ at 40°C, β-D-glucosidase as μmol pNP $h^{-1}$ at 30°C and cellobiase as μmol glucose $h^{-1}$ at 30°C.

| | Protein (mg) | Endoglucanase | β-D-Glucosidase (pNPG) | Cellobiase |
|---|---|---|---|---|
| CULTURE FILTRATE | - | 2320 | 2.56 | <0.2 |
| SONICATED PREPARATION | 31.5 | - | 14.98 | 6.0 |
| CELL-FREE EXTRACT | 27.0 | 21 | 13.55 | 5.4 |
| PARTICULATE FRACTION | 3.56 | 4.0 | 0.054 | <0.002 |

FIGURE 4.3

Effect of pH on endoglucanase activity (μmol reducing sugars as glucose equivalents produced $ml^{-1}$ $h^{-1}$). ×, Sodium acetate buffer, ○, tris/maleate buffer.



Culture filtrate was filter sterilized as described in section 4.2.1 (i) and stored frozen at $-4^{o}C$. A loss of activity of 2 - 4% was recorded on thawing. The activity of the culture filtrate against Avicel was *ca.* 0.16 µmol reducing sugars $ml^{-1}$ $h^{-1}$. The low ratio of β-D-glucosidase : exo-and endoglucanase activity, as compared to that for *Trichoderma viride* cellulase (BDH) will affect the type of soluble sugars produced during hydrolysis of cellulose. TLC (section 2.6.1) of Avicel hydrolysates confirmed that cellobiose and some cellotriose were produced by act 9 culture filtrate while glucose and cellobiose were the major sugars present in Avicel hydrolysates produced by *T. viride* cellulase (BDH).

In cultures grown on IM + 1% (w/v) Avicel instead of IC, higher yields of endoglucanase were obtained in the culture filtrate (> 200 EU $ml^{-1}$). We wanted to compare the results from the diffusion experiments with those from experiments in which the microorganisms were inoculated into the centre of CMC plates containg 'barrier rings' of soil or one of its components (Chapter 5). For this reason, crude cellulase preparations (filter-sterilized culture filtrates) from the CMC grown cultures were used for all the diffusion experiments with the exception of the experiment in which the effect of enzyme concentration on the diameter of the hydrolysis zone was investigated (section 4.3.1 ii). For this experiment high endoglucanase activities were needed and therefore the culture filtrate from Avicel grown act 9 was used.

Ultrafiltration of act 9 culture filtrate

Culture filtrate (37.5 ml) from IC grown cells was filtered under pressure (138 KPa) through a Diaflo PM10 ultrafiltration membrane (Amicon Co.). The residue was washed with 2 x 10 ml 0.1M-acetate buffer pH 5.75 and resuspended in buffer. Eighty per cent of the original activity was recovered. A small amount of activity was also found in the filtrate, suggesting the presence of a low molecular weight endoglucanase or

active subunit (section 1.2.1).

(iii) Cellulase preparation from *Sporocytophaga myxococcoides*

A culture of *Sporocytophaga myxococcoides* NCIB 8639 (supplied by Dr. I. Vance, Polytechnic of Central London, London.) was maintained at 4°C on slopes of Berg Minimal Medium (see appendix I) containing 1% (w/v) agar overlaid with strips of Watman No.1 filter paper.

Culture conditions

Shake flask cultures were grown at 30°C in 250 ml Erlenmeyer flasks containing 95 ml Berg MM + 1% (w/v) Avicel PH105. The flasks were inoculated with 5 ml of a 6 day culture of *Sporocytophaga myxococcoides* grown on Berg MM amended with 0.1% (w/v) glucose. Flasks were harvested at 4, 6 and 8 days by centrifuging (18 000 x g, 20 min, 4°C) The supernatants were filtered using an HT-200 Tuffryn 0.2 µm filter (Gelman) and stored frozen prior to assay.

Maximum endoglucanase activity (6.0 EU $ml^{-1}$) was present after 8 days and culture filtrate taken from 8 day flasks was used for the diffusion studies.

(iv) β-D-Glucosidase

Commercial β-D-glucosidase from sweet almond (Kochlight, 100 units* $mg^{-1}$) was assayed for activity using CMC and pNPG (Table 4.3).

TABLE 4.3 Activities (mg enzyme)$^{-1}$ of commercial β-D-glucosidase (Kochlight).

| SUBSTRATE | ACTIVITY |
|---|---|
| CMC | 0.6 µmol reducing sugars $h^{-1}$ |
| pNPG | 212.4 µmol pNPG $h^{-1}$ |

*Kochlight define one unit as the amount of enzyme required to liberate 1 µg glucose from Salicin $min^{-1}$ at 35°C pH5.0.

For diffusion studies β-D-glucosidase solutions in 0.1M-acetate buffer pH5.0 were filter sterilized using HT-200 filters.

4.2.2 Diffusion Plates

(i) Preparation of plates

Diffusion of endoglucanase was investigated using standard 9 cm Petri dishes containing CMC agar. Hankin and Anagnostakis (1977) used solid media containing CMCs of different DP and DS to detect cellulase-producing microorganisms and found that the best clearing zones were produced with 0.5% (w/v) CMC 9M8F. Therefore we used 0.5% (w/v) CMC 9M8F and 1% (w/v) purified agar (Oxoid) in our diffusion plates. The thickness of CMC agar and the amount of moisture in the plate were both found to affect the diffusion of endoglucanase. Standard diffusion plates were prepared by pipetting 20 ml sterilized CMC agar at 48°C into Petri dishes. After the agar had set the plates were dried for 15 min in a laminar airflow cabinet (Microflow Pathfinder Ltd.).

A central well (10 mm diameter) was made in the plates by removing the agar using a sterilized cork-borer, (in some of the experiments a well of diameter 8 mm was used). The enzyme solution (100 µl) was placed in the central well and the plate incubated at 25°C for an appropriate period after which the central well was plugged using a few drops of molten agar. The extent of the diffusion of endoglucanase through the solid media was detected by precipitating any unhydrolysed CMC and measuring the diameter of the cleared zone in two directions at right angles to each other. The reason for plugging the wells was to prevent the uneven precipitation of CMC caused by CETMAB diffusing from the edges of the well into the surrounding agar. The following precipitating agents were evaluated:

[1] 0.5% (w/v) Cetyltrimethylammonium bromide (CETMAB),

[2] 1% (w/v) CETMAB (Hankin and Anagnostakis, 1977),

[3] 5% (w/v) CETMAB, and

[4] 10% (w/v) Cupric acetate (Dingle *et al.*, 1953).

The zone of hydrolysis, as visualized by an area of clearing in the precipitated (unhydrolysed) CMC, was most distinct following flooding of the plates with 10 ml 1% (w/v) CETMAB for 10 min. With Cupric acetate only a very faint zone of clearing was seen.

Initially, it was thought that enzyme solution may leak from the base of the central well between the agar and the plastic of the Petri dish. For this reason the wells were lined with a few drops of 0.5% (w/v) agar. However, it was found that blue dextran diffused from lined and unlined wells at the same rate and the practice of lining the wells was abandoned.



We wanted to compare the results from the enzyme diffusion experiments with measurements of the radial growth rates of microorganisms on CMC agar plates and for this reason the diffusion plates were prepared using either Berg MM for the diffusion of bacterial endoglucanase, or Sternberg MM for the diffusion of fungal endoglucanase. Dingle *et al.* (1953) reported that the pH of the gel had a marked effect on the size of the developed zone in cup-plate assays of polygalacturonase while Musolan *et al.*, (1978), in estimating cellulase by radial diffusion, found no significant variation in the diameter of the hydrolysis zone in the pH range 3.8 - 7.0. Instead, they reported that in gels with a pH near the optimum for the enzyme (pH5.4) the hydrolysis zones were almost clear while in gels with a lower pH(3.8) the hydrolysis zones were stained blue.

To investigate the effect of pH and different media on the extent of the hydrolysis zone the following plates were prepared:

[1] 0.5% (w/v) CMC 9M8F in 0.1M-acetate buffer pH5.0 + 1% (w/v) agar.

[2] 0.5% (w/v) CMC 9M8F in 0.1M-tris/maleate buffer pH7.0 + 1% (w/v) agar.

[3] 0.5% (w/v) CMC 9M8F in Sternberg MM (pH5.45) + 1% (w/v) agar.

[4] 0.5% (w/v) CMC 9M8F in Berg MM (pH7.5) + 1% (w/v) agar.

The central wells were filled with 100 µl *Trichoderma viride* cellulase (50 mg $ml^{-1}$ in 0.1M-acetate buffer pH5.0) and the plates incubated at 25°C. The plates were removed at intervals, flooded with CETMAB and the clearing zones measured and scored for clarity (Table 4.4).

(ii) Alternative methods for detecting endoglucanase in diffusion plates

We attempted to detect and measure endoglucanase activity in different regions of the diffusion plate by removing cores of agar (5 mm diameter) from different parts of the plate and assaying for endoglucanase activity. In an experiment designed to test the sensitivity of this technique *Trichoderma viride* cellulase (BDH) was added to the molten CMC agar immediately before the plate was poured, the agar allowed to set, and cores removed for assay. This revealed that a minimum concentration of 1 mg $ml^{-1}$ endoglucanase was required in the agar core before activity could be measured.

To confirm that the observed clearing zone represented the area in which CMC had been hydrolysed the plates were stained according to the method of Musolan *et al.* (1978). The agar medium was removed from the Petri dishes after the incubation period, the fluid phase removed by pressing the agar disc between filter papers, and the agar stained with 0.3% (w/v) toluidine blue in 2% acetic acid for 10 min. After repeated washings in 2% acetic acid the agar was blotted dry and the diameter of

TABLE 4.4

Effect of pH and media composition on the diameter (mm) and clarity of the hydrolysis zone. +, least distinct, ++++, most distinct.

| Time of Incubation (h) | Sternberg MM | Acetate buffer pH 5.0 | Berg MM | Tris/maleate buffer pH 7.0 |
|---|---|---|---|---|
| 4 | 16 ++++ | 16 ++++ | 16 ++ | 16 ++ |
| 24 | 30 ++++ | 28 ++++ | 32 ++ | 32 ++ |
| 48 | 38 ++++ | 40 +++ | 36 + | 38 + |
| 72 | 49 +++ | 49 ++ | 42 + | 44 + |
| 144 | 66 +++ | 66 ++ | - | - |

the clear zone measured. The dimensions of the clear zone following staining were equivalent to those of the clear zone following CMC precipitation with 1% (w/v) CETMAB confirming that hydrolysis of CMC was occurring within this region.

(iii) Parameters influencing the diameter of the hydrolysis zone

As expected the diameter of the cleared zone increased if: the thickness of the CMC agar was reduced, the volume of enzyme solution added to the central well was increased, and the temperature at which the plates were incubated was increased from 25°C to 30°C.

4.2.3 Diffusion Plates for β-D-Glucosidase

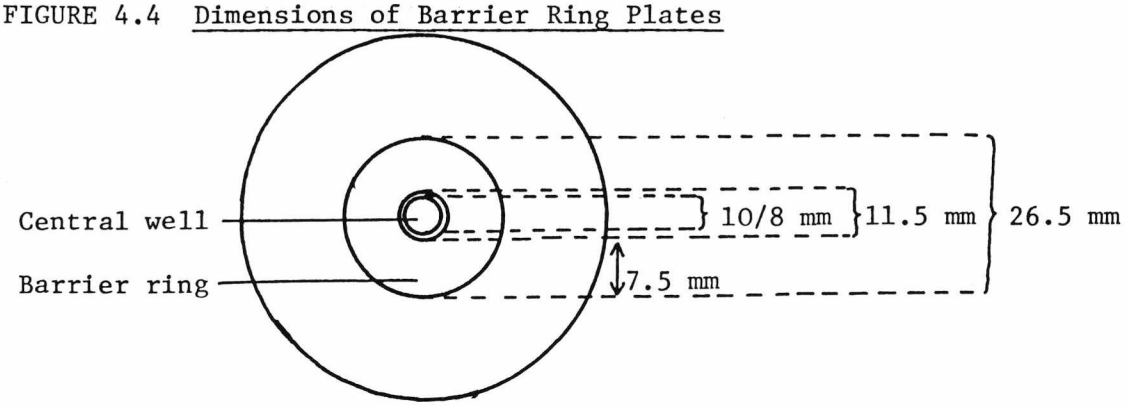
In addition to the diffusion of endoglucanase we also investigated the diffusion of β-D-glucosidase through BC 9M8F plates (20 ml-BC 9M8F plate with 8 mm central well to which 100 µl of filter sterilized β-D-glucosidase was added). β-D-Glucosidase is unable to hydrolyse CMC, and therefore, the extent of enzyme diffusion was measured by brushing the plates with 25 mM pNPG and incubating at 30°C for 1 h. The pH of the plates was then raised by flooding with 0.5 ml 0.5M-NaOH. The region containing active β-D-glucosidase appeared yellow due to the presence of product pNP (see Plate 4.6). The yellow zones were somewhat diffuse and in some of the experiments slices of agar 1 cm wide were cut from the plate and examined using a Gilford 250 gel scanner at 400 nm. However, because of the crude nature of the gel and the rapid diffusion of pNP, this method of detection proved no more accurate than judging the limits of the zone using the naked eye.

4.2.4 Preparation of Diffusion Plates Containing Barrier Rings

The diffusion plate technique was further developed to allow us to investigate the effect of soil on the diffusion of endoglucanase and β-D-glucosidase. A ring of agar surrounding the central well was carefully removed and replaced by substrate-free medium or medium containing entire soil of one of its particulate components (sand, silt, clay). Bentonite and kaolin (Fisons) were used as examples of a smectite and kaolinite clay. Varying concentrations (0.1 - 10% w/v) were suspended in the appropriate media, sterilized by autoclaving 115°C, 15 min and introduced into the ring. [Stotzky and DeMumbrum (1965) examined the effect of autoclaving on the X-ray characteristics of clay minerals and concluded that in neither the natural nor purified soil clay minerals were the X-ray characteristics altered by this form of sterilization treatment]. Plates containing barrier rings of sand and soil were prepared by filling the ring with autoclaved samples of sand (*ca.* 2 g) and soil (*ca.* 1 g) and then adding molten medium to fill the gaps.

By removing agar rings of different diameters both the dimensions and the position of the barrier ring could be changed. For the diffusion experiments a standard barrier ring of inner and outer diameter 11.5 mm and 26.5 mm was used (Fig. 4.4).

FIGURE 4.4 Dimensions of Barrier Ring Plates




11.5 - 26.5 mm barrier ring contained 1.5 ml medium.

4.2.5 Persistence of *Trichoderma viride* and act 9 Endoglucanase in Soil

In a more direct experiment the persistence of endoglucanase activity in soil was further investigated by adding aliquots of the crude cellulase preparations to soil and measuring the activity at intervals.

Experimental procedure

*Trichoderma viride* cellulase preparation (BDH) 0.2 ml containing 0.2% (w/v) $NaN_3$ in 0.1M-acetate buffer pH5.0 filtered (PM-10 ultrafiltration membrane) to remove carbohydrate (section 4.2.1 ii) was added to 0.4 g air-dried soil in sterile 15 ml test tubes. [The endoglucanase activity of the filtered preparation, as measured using the DNS method, was 6.0 µmol reducing sugars produced $(0.2\ ml)^{-1}\ h^{-1}$)]. Control tubes contained 0.2 ml enzyme solution and 0.4 g soil + buffer only. The tubes were incubated at 25°C and assayed for endoglucanase activity at intervals using a modification of the DNS method.

Assay

DNS-CMC reagent (2 ml) was added to the tubes containing soil + enzyme and the mixture incubated for 30 min 40°C. DNS reagent (3 ml) was then added and the tubes heated in a 100°C water bath for 15 min. After cooling the mixtures were centrifuged (38 000 x g, 10 min) and the absorbance of the supernatant liquids at 640 nm recorded. [Zero time controls in which the CMC was added at the end of the 30 min 40°C incubation were also prepared]. Reducing sugars were calculated with reference to a standard glucose curve.

A similar experiment using PM-10 filtered cellulase from act 9 was performed.

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Endoglucanase Diffusion Plates

#### (i) Change in the diameter of the hydrolysis zone with time

Fig. 4.5 shows how the diameter of the hydrolysis zone changes with time when 100 µl of cellulase preparation from isolate act 9 was added to the central well of a BC diffusion plate. Each point represents the mean of two measurements on each of three replica plates. A similar curve was obtained when *Trichoderma viride* cellulase preparation (BDH) was used. Because of the non-linear relationship between the diameter of the hydrolysis zone and the time of incubation, standard times of either 72 h or 120 h were used for the routine assay.

#### (ii) Effect of concentration on the diameter of the hydrolysis zone

Using act 9, *Trichoderma viride* or *Sporocytophaga myxococcoides* cellulase preparations, a linear relationship was observed between the diameter of the hydrolysis zone and the log of the enzyme concentration added to the central well over the range of enzyme concentrations tested; i.e. *T. viride* cellulase preparation (Fig. 4.6) 10 - 50 000 µg $ml^{-1}$, act 9 cellulase preparations 0.23 - 230 EU $ml^{-1}$, and *S. myxococcoides* cellulase preparation 0.06 - 6.0 EU $ml^{-1}$.

A similar linear relationship between the diameter of the hydrolysis zone and the log of the cellulase concentration in the limited range 0.5 - 20 mg $ml^{-1}$ was reported by Musolan *et al.*, (1978) for an *Oxysporous* sp. cellulase preparation (Merck). Our diffusion plate assay is more sensitive than the system of Musolan *et al.* (1978) and has enabled us to test a far greater range of endoglucanase concentrations from different sources.

FIGURE 4.5

Relationship between time and the distance diffused (i.e. diameter of hydrolysis zone) for cellulase preparation from isolate act 9.

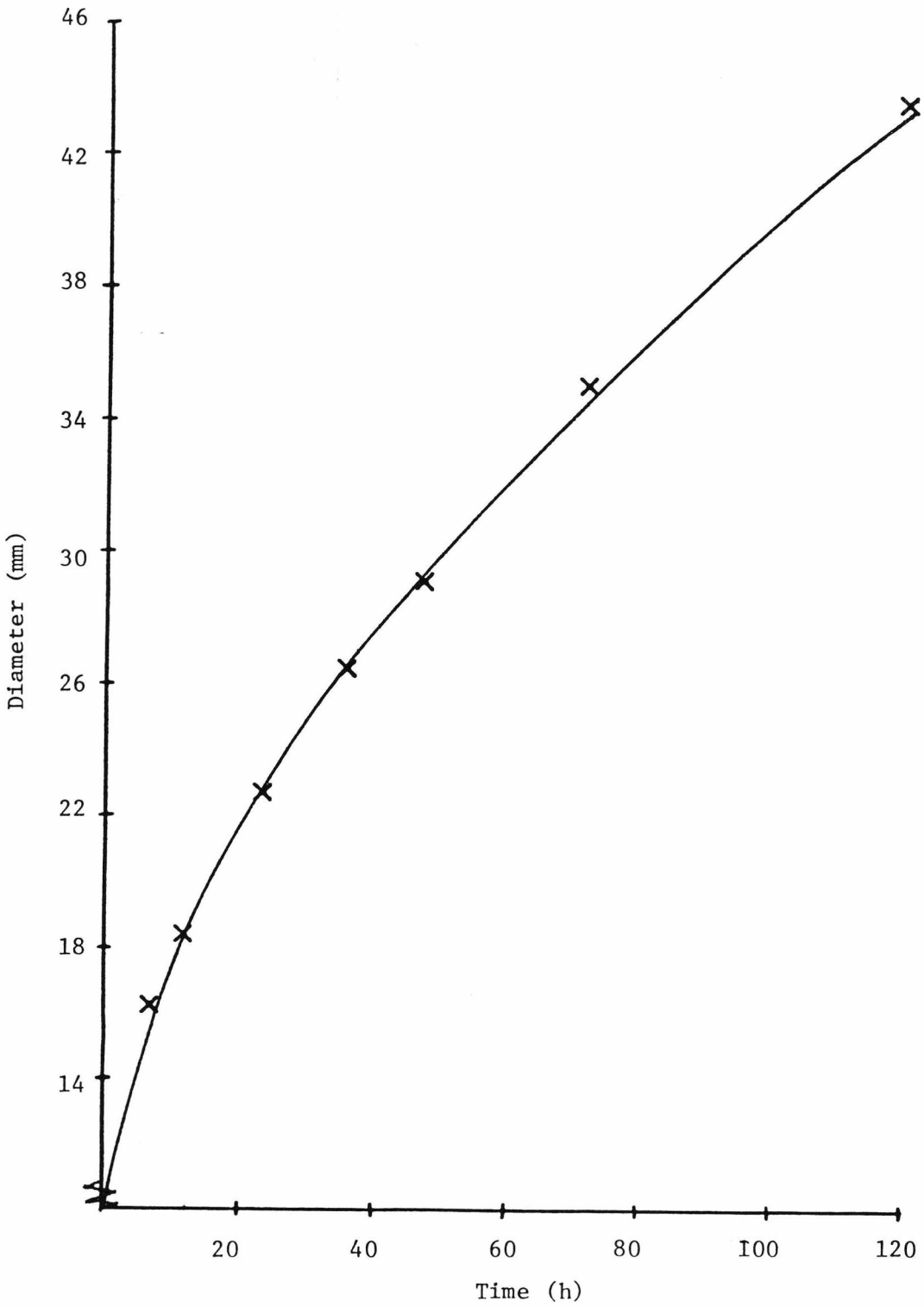
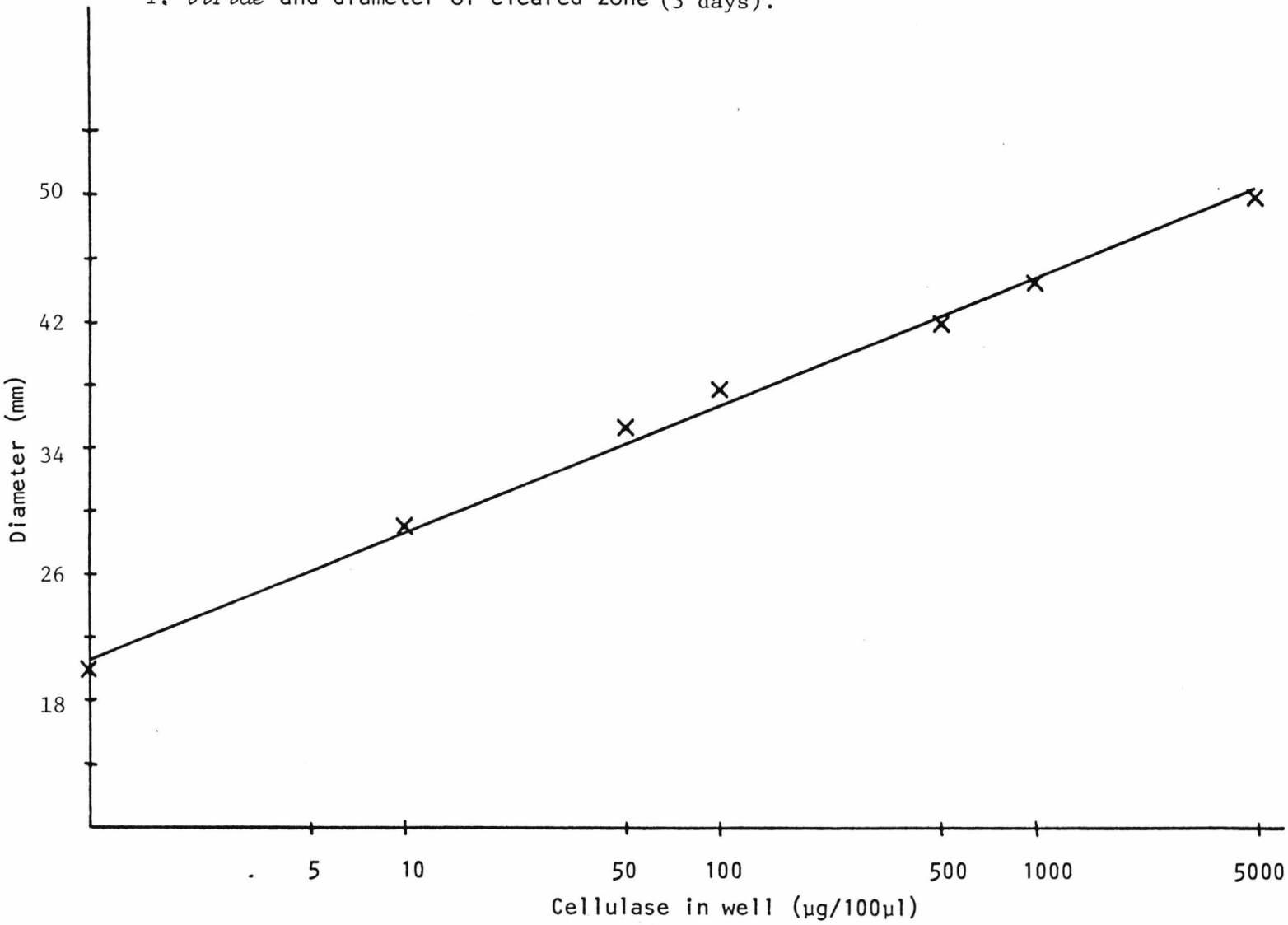


FIGURE 4.6

Relationship between concentration of commercial cellulase preparation from *T. viride* and diameter of cleared zone (3 days).



Ficks second law of diffusion can be written:

$$c = \frac{C}{2\sqrt{\pi Dt}} \exp\left(\frac{-x^2}{4Dt}\right)$$

where; C = initial quantity of solute

D = diffusion constant

t = time

c = concentration at a distance x from origin

rearrangement gives

$$\ln C = K + \frac{1}{4Dt} x^2$$

where K = constant (for fixed t and D)

Therefore, for linear diffusion, the log of the enzyme concentration should be proportional to the square of the distance diffused by the enzyme. The theory of antibiotic diffusion has been discussed by Cooper (1972), and Kavanagh (1960) gives examples of antibiotics and bacteria for which the relation $x^2$ versus log C is a straight line as well as examples for which x versus log C gives a better fit than $x^2$ versus log C. We found a linear relationship between the diameter of the hydrolysis zone and the log of the enzyme concentration. The physical chemistry of the radial diffusion of enzymes in gels is not fully understood. The rate of enzyme diffusion will obviously be influenced by the temperature at which the plates are incubated, the molecular weight of the enzyme and the agar concentration etc. However, in addition the edge of the hydrolysis zone is determined by a 'critical concentration' of enzyme, which, will in turn be determined by such factors as; enzyme activity, pH etc.

(iii) Effect of barrier rings on the diffusion of endoglucanase

The presence of a substrate-free barrier ring caused a small increase in the diameter of the hydrolysis zone. This increase was only significant ($t_{0.975}$) when greater than three replica plates were used. However, when soil components were added to the barrier ring the control plates were prepared with no substrate in the barrier ring.

Diffusion of *Trichoderma viride* cellulase (BDH) and cellulase from act 9 was not affected by the presence of kaolin (up to 5% (w/v)) in the barrier ring (Plate 4.1 shows the hydrolysis zones produced following diffusion of cellulase from act 9 through barrier rings of kaolin (0 - 5% (w/v)). By contrast the presence of bentonite in the barrier ring caused a reduction in the diameter of the hydrolysis zone with both *T. viride* cellulase (BDH) and cellulase from act 9. Plate 4.2 shows hydrolysis zones produced following diffusion of cellulase from act 9 through barrier rings of bentonite (0 - 5% (w/v)). Bentonite is known to adsorb CMC (section 5.3.1) and hence a small zone of clearing around the edge of the ring was seen even in the absence of endoglucanase. Fig. 4.7 shows a plot of the diameter of the hydrolysis zone versus the concentration of bentonite in the barrier ring for *T. viride* cellulase (BDH) and cellulase from act 9. Inferences from the different shapes of these graphs will be discussed in section 4.3.2. Plate 4.3 shows the effect of the width of the bentonite ring (1% w/v) on the diffusion of cellulase from act 9.

The incorporation of sand (*ca.* 2 g) in the barrier ring did not significantly reduce the diameter of the hydrolysis zone (Plate 4.4) although the standard deviation of measurements from replica plates was increased. The inner zone of dense precipitate observed following flooding

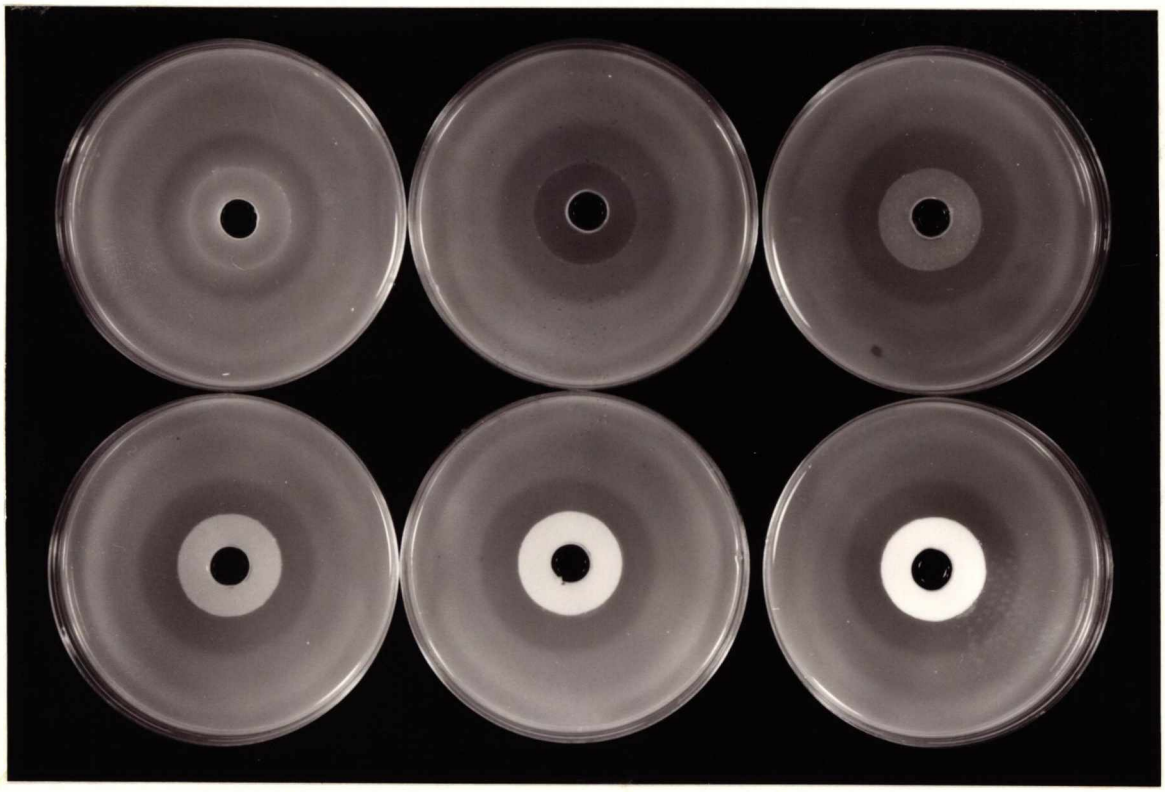
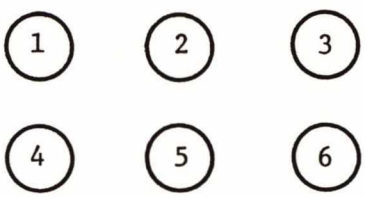


PLATE 4.1

Diffusion of cellulase preparation from act 9 through barrier rings of kaolin (6 days).

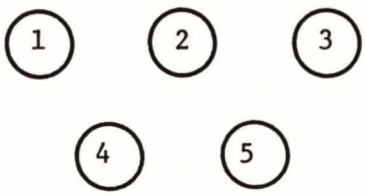



Barrier rings (11.5mm - 26.5mm)

1. BC control
2. Substrate-free
3. 0.1%(w/v) kaolin
4. 0.5%(w/v) kaolin
5. 1.0%(w/v) kaolin
6. 5.0%(w/v) kaolin

PLATE 4.2

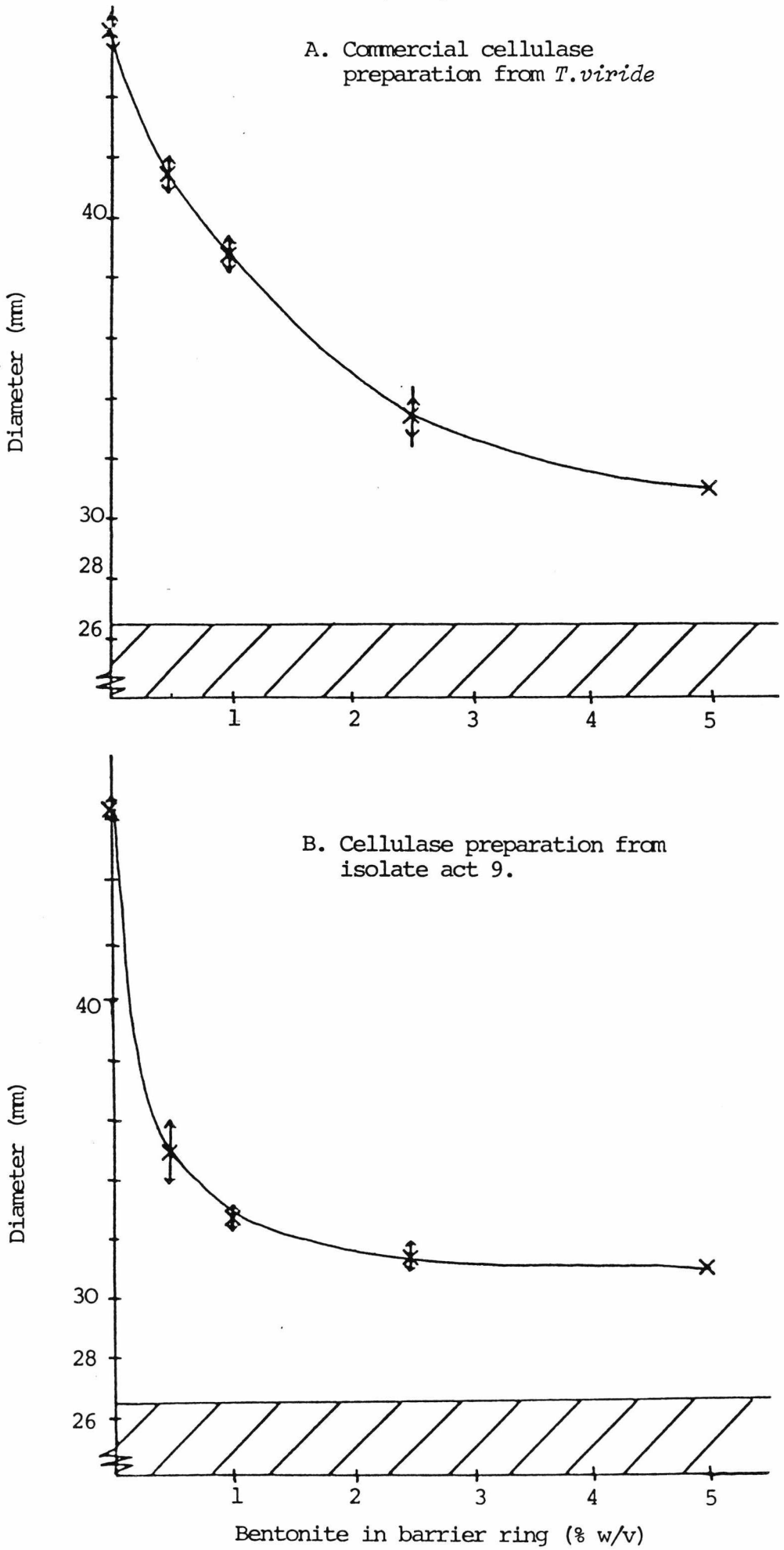
Diffusion of cellulase preparation from act 9 through barrier rings of bentonite (5 days).




Barrier rings (10.0 - 26.5mm)

1. Substrate-free
2. 0.5%(w/v) bentonite
3. 1%(w/v) bentonite
4. 2.5%(w/v) bentonite
5. 5%(w/v) bentonite

FIGURE 4.7 Diffusion of cellulase through bentonite barrier ring (5 days).



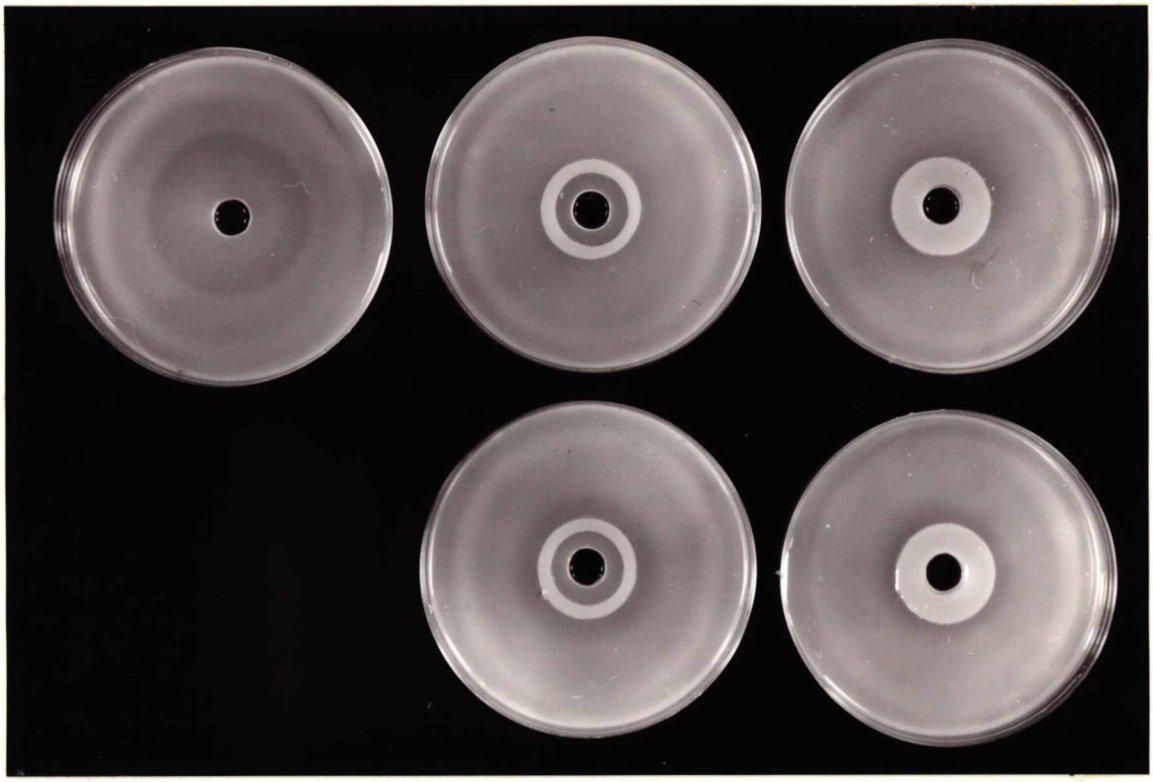
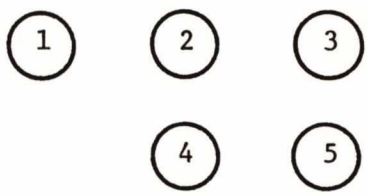


PLATE 4.3

Diffusion of cellulase preparation from act 9 through bentonite (1% w/v) barrier rings of different width (5 days).




1. BC control
2. Barrier ring 11.5 - 20.5mm (i.e. 4.5mm) control plate (no enzyme)
3. Barrier ring 11.5 - 26.5mm (i.e. 7.5mm) control plate (no enzyme)
4. Barrier ring (4.5mm) assay
5. Barrier ring (7.5mm) assay

PLATE 4.4

Diffusion of *Trichoderma viride* commercial cellulase preparation through sand barrier ring (5 days)

(1) (2)

Barrier ring

1. Substrate-free
2. Sand (ca. 2g)

of *Trichoderma viride* cellulase diffusion plates was thought to be due to the precipitation of CMC hydrolysis products.

The effect of soil (*ca.* 1 g) in the barrier ring was difficult to quantify. This was because diffusion of coloured humic compounds from the soil into the surrounding agar masked any hydrolysis zones which may have been produced by the endoglucanase. However, it was apparent that the presence of a soil barrier ring significantly reduced the distance diffused by endoglucanase from act 9. Furthermore, no difference in the appearance of the soil barrier ring plates was seen when cellulase from act 9, heated to 100°C 15 min (inactivated), was added to the central well as compared to active (untreated) cellulase from act 9 (Plate 4.5). *Trichoderma viride* endoglucanse (BDH) appears to penetrate the soil barrier ring more efficiently than the endoglucanase from act 9. Hydrolysis zones on the outer edge of the soil barrier ring were evident when 1 mg $ml^{-1}$ cellulase was added to the central well and if the concentration was increased to 5 mg $ml^{-1}$ or 10 mg $ml^{-1}$ no significant difference in the size of the hydrolysis zones as compared to those in control (no soil) plates was observed. However, there was a large variation between replica plates, presumably due to the large size of the particles in the barrier ring (cf. sand barrier ring plates).

To investigate the effect of the different components of Hamble soil on the diffusion of endoglucanase the colloidal-size fraction separated from whole soil (section 3.2.1) was incorporated into the barrier ring (1 -18 % w/v). Fig. 4.8 shows the effect of the CSF on the hydrolysis zone produced by cellulase from act 9. Fig. 4.9 shows an analysis of variance for the data and illustrates the significance of the data at a 95% confidence level. A pooled (pooled variance) and twosample (does not assume equal variance) test shows the significance of the measurements from the BM control and the 1% (w/v) CSF plates.

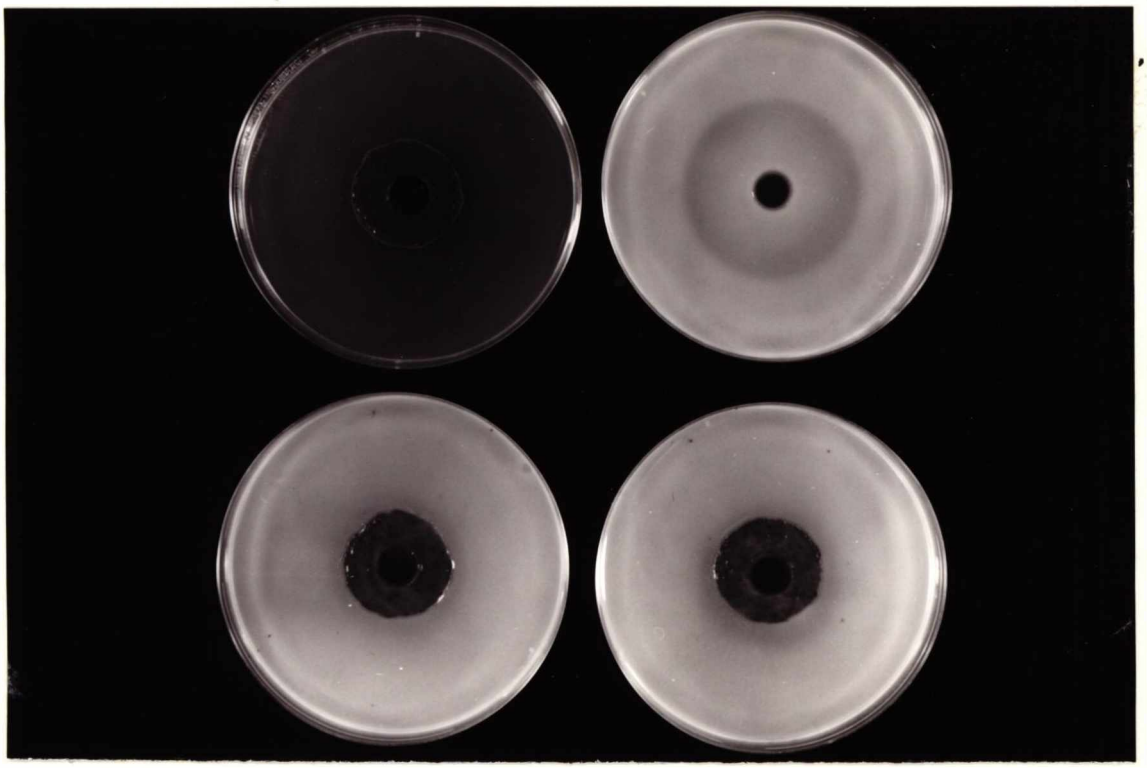
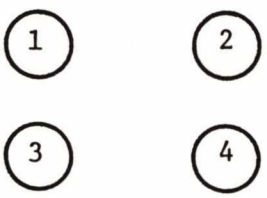


PLATE 4.5

Diffusion of cellulase preparation from act 9 through soil (ca. 1g) barrier ring (7 days).




1. BC soil barrier ring plate prior to precipitation of CMC
2. BC control plate
3. BC soil barrier ring plate - inactivated cellulase preparation
4. BC soil barrier ring plate - assay

FIGURE 4.8 Diffusion of cellulase preparation from isolate act 9 through clay barrier ring (CSF).

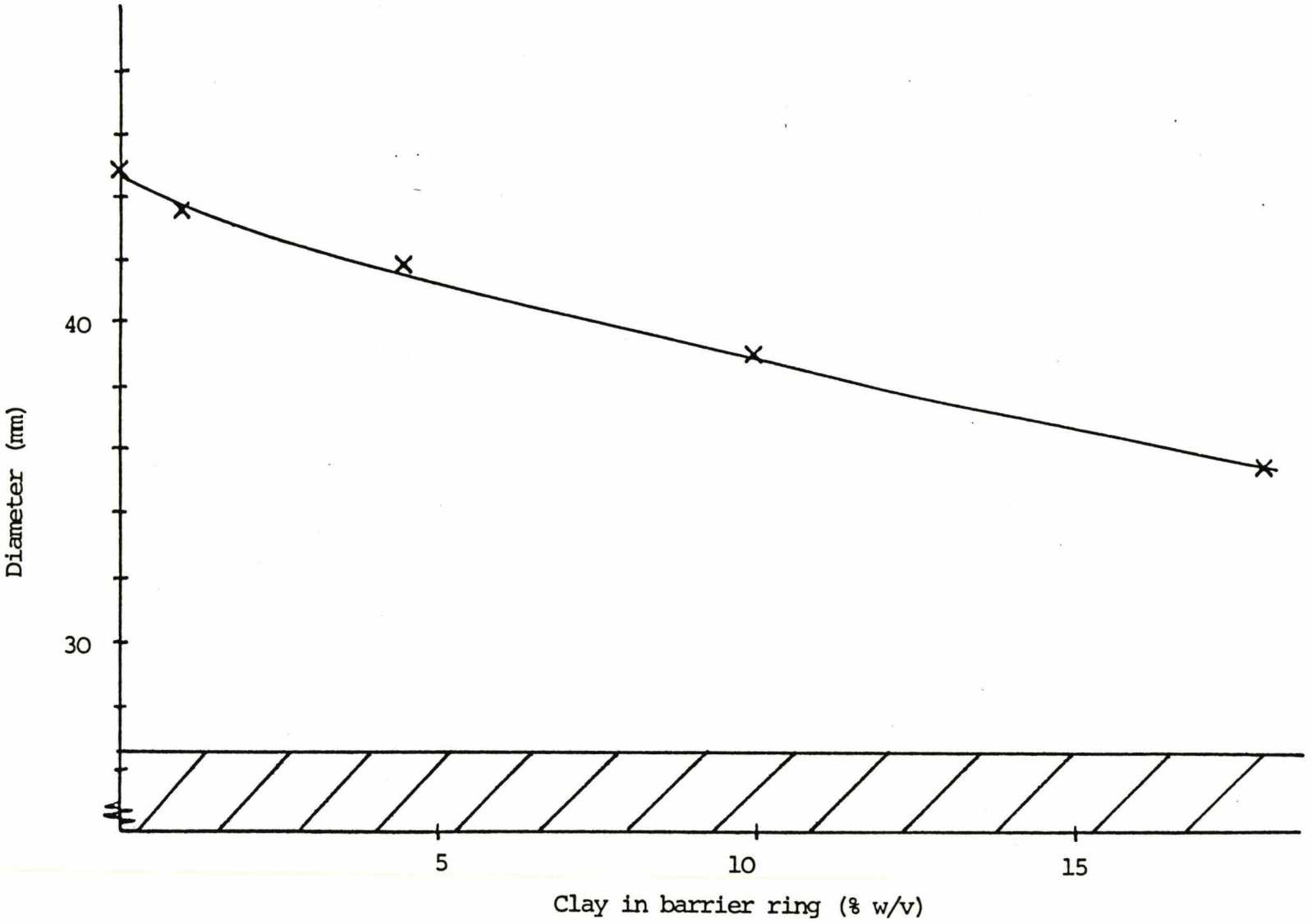


FIGURE 4.9
ANALYSIS OF CELLULASE PREPARATION FROM ACT9 DIFFUSING THROUGH COLLOIDAL-SIZE FRACTION (1-18%W/V) IN BARRIER RING

C1 = BM CONTROL; C2 = 1%(W/V)CSF; C3 = 5%(W/V)CSF;
C4 = 10%(W/V)CSF; C5 = 18%(W/V)CSF.

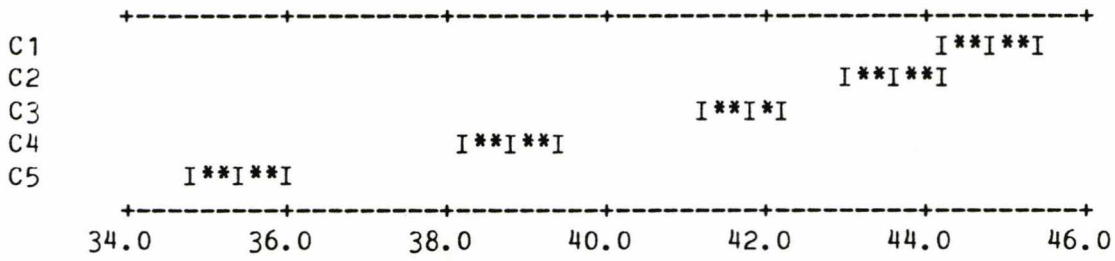
ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF | F-RATIO |
|---|---|---|---|---|
| FACTOR | 4 | 172.889 | 43.222 | 222.03 |
| ERROR | 10 | 1.947 | 0.195 | |
| TOTAL | 14 | 174.836 | | |

| LEVEL | N | MEAN | ST. DEV. |
|---|---|---|---|
| C1 | 3 | 44.767 | 0.473 |
| C2 | 3 | 43.533 | 0.404 |
| C3 | 3 | 41.700 | 0.265 |
| C4 | 3 | 38.867 | 0.416 |
| C5 | 3 | 35.333 | 0.586 |

POOLED ST. DEV. = 0.441

INDIVIDUAL 95 PERCENT C. I. FOR LEVEL MEANS
(BASED ON POOLED STANDARD DEVIATION)




TWOSAMPLE C1,C2

| | | | | | | |
|---|---|---|---|---|---|---|
| C1 | N = | 3 | MEAN = | 44.767 | ST.DEV. = | 0.473 |
| 1% CSF | N = | 3 | MEAN = | 43.533 | ST.DEV. = | 0.404 |

APPROX. DEGREES OF FREEDOM = 3

A 95.00 PERCENT C.I. FOR MU1-MU2 IS ( 0.0908, 2.3759)

TEST OF MU1 = MU2 VS. MU1 N.E. MU2
T = 3.435
THE TEST IS SIGNIFICANT AT 0.0414

POOLED C1,C2

| | | | | | | |
|---|---|---|---|---|---|---|
| C1 | N = | 3 | MEAN = | 44.767 | ST.DEV. = | 0.473 |
| 1% CSF | N = | 3 | MEAN = | 43.533 | ST.DEV. = | 0.404 |

DEGREES OF FREEDOM = 4

A 95.00 PERCENT C.I. FOR MU1-MU2 IS ( 0.2363, 2.2304)

TEST OF MU1 = MU2 VS. MU1 N.E. MU2
T = 3.435
THE TEST IS SIGNIFICANT AT 0.0264

Analysis of the clay fraction of Hamble soil (G. Brown, Rothamsted Experimental Station.) revealed that the major components were discrete mica, kaolin and an interstratified smectite-mica. However the breadth of the X-ray reflections suggested that the mica and kaolin were present in much larger crystals than the interstratified smectite-mica and because of this the surface properties of the clay were likely to be dominated by those of the smectite-mica.

### 4.3.2 Persistence of *Trichoderma viride* and act 9 Cellulase in Soil

Figs. 4.10-11 show the results of experiments in which *Trichoderma viride* cellulase preparation (BDH) and cellulase from act 9 were added directly to soil (section 4.2.5). The endoglucanase activity in the *T. viride* cellulase preparation showed an initial decline following addition to soil but could nonetheless be detected at nearly 30% its original activity after 20 days incubation. PM-10 filtration appeared to somewhat reduce the stability of endoglucanase activity in the cellulase preparation from act 9 grown in both IM + CMC and IM + Avicel media. A rapid decline in activity was observed for both cellulase preparations from act 9 following addition to soil. A pilot experiment suggested that a similar inactivation occurred when cellulase from act 9 was added to autoclaved soil. These results, taken in conjunction with the results from the diffusion experiments in which soil or bentonite was present in the barrier ring suggest that endoglucanase inactivation results from adsorption of the enzyme by the smectite components of the soil and that endoglucanase from act 9 was more susceptible to this form of inactivation than endoglucanase from *T. viride*. It is important to remember that the crude cellulase preparationsused in this work most likely contain more than one endoglucanase and without an extensive purification procedure it is not possible to say whether all the *T. viride* endoglucanases are being adsorbed to a limited degree or whether some are

FIGURE 4.10

Persistence in soil of *Trichoderma viride* commercial cellulase preparation. Endoglucanase activity expressed as µmol reducing sugars as glucose equivalents $h^{-1}$. ×, cellulase (0.2ml); ○, soil (0.4g) + cellulase (0.2ml).

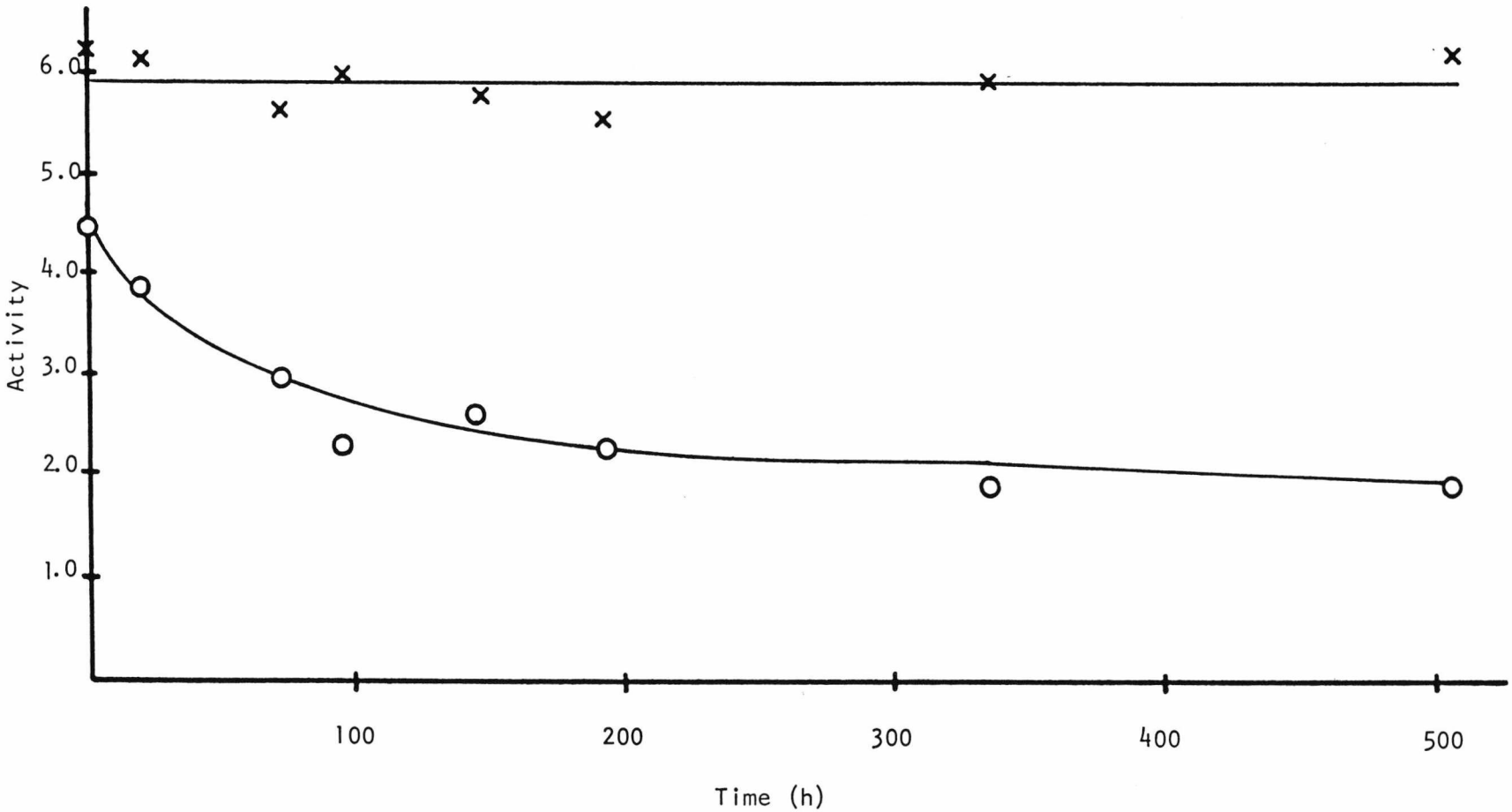
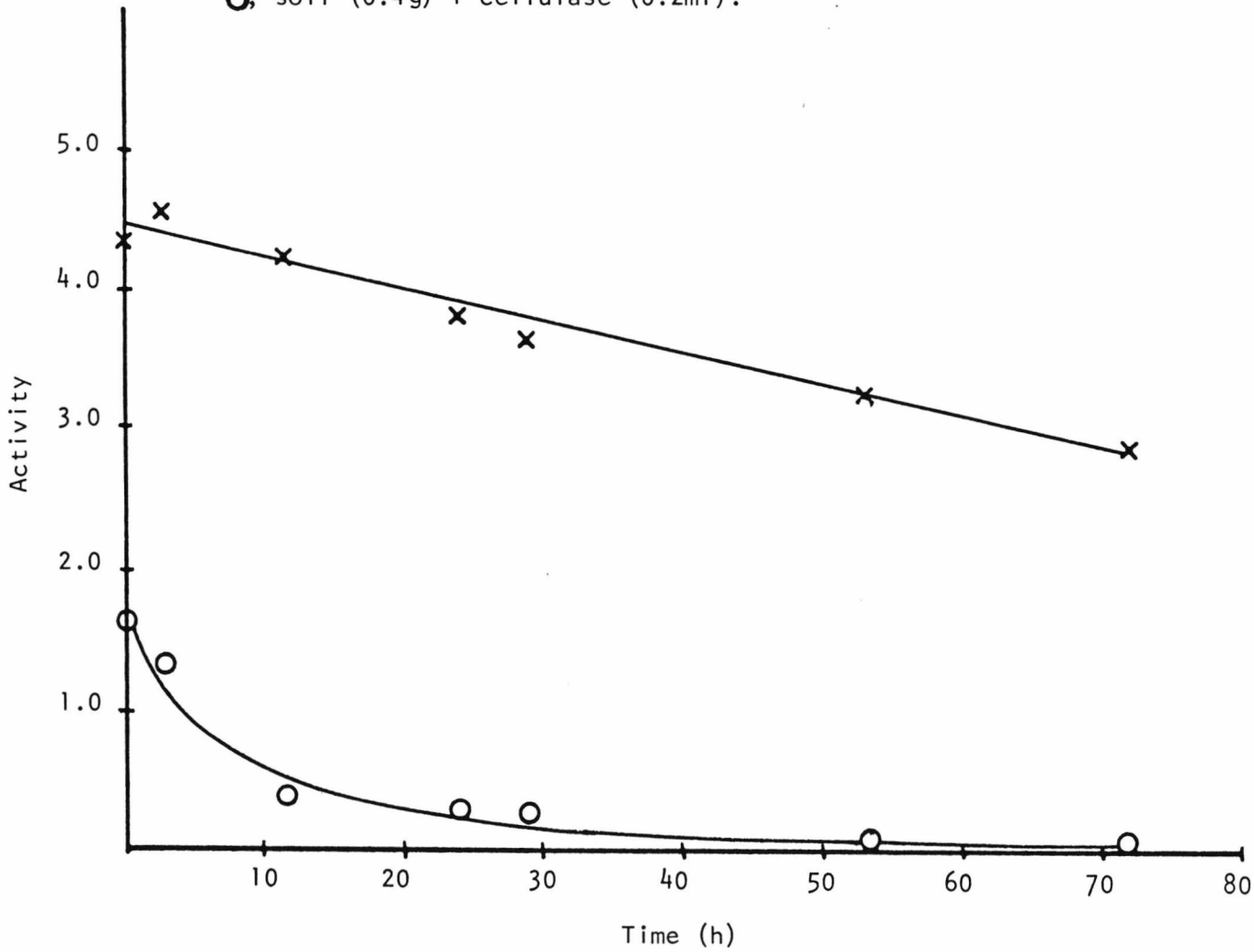


FIGURE 4.11

Persistence in soil of cellulase preparation from isolate act 9 grown on IM + Avicel. Endoglucanase activity expressed as µmol reducing sugars as glucose equivalents $h^{-1}$. ×, cellulase (0.2ml); ○, soil (0.4g) + cellulase (0.2ml).



resistant to adsorption and inactivation.

### 4.3.3 β-D-Glucosidase Diffusion Plates

#### (i) Effect of concentration and development time on the diameter of the diffusion zone

Fig. 4.12 shows a semi-log plot of concentration of β-D-glucosidase in the central well versus the diameter of the diffusion zone and also illustrates the effect of different development times (i.e. 30 min and 60 min incubation with pNPG) on the diameter of the diffusion zone. The increased diameter of the diffusion (yellow) zone following incubation with pNPG for 60 min as compared with 30 min suggests that a concentration gradient of diffused β-D-glucosidase exists and in selecting specific parameters in the detection method we are selecting an arbitrary lower concentration level below which the presence of diffused enzyme is not detected.

#### (ii) Effect of soil and bentonite barrier rings on the diffusion of β-D-Glucosidase

Bentonite at concentrations of 0.5 - 5.0% (w/v) in the barrier ring prevented the diffusion of β-D-glucosidase (20 $\mu g\ 100\ \mu l^{-1}$) into the surrounding agar. In plates containing 0.1% (w/v) bentonite in the barrier ring a small amount of pNP was seen on the outside of the barrier ring following development of the plate.

In an experiment where various concentrations of β-D-glucosidase were added to the central well of 1% (w/v) bentonite barrier ring plates, only in the plates containing $>100\ \mu g\ 100\ \mu l^{-1}$ β-D-glucosidase was a yellow zone observed on the outer edge of the barrier ring (Fig. 4.13). Similar results were observed when soil (*ca.* 1 g) was added to the barrier ring (Fig. 4.14 and Plate 4.6).

FIGURE 4.12

Radial diffusion of β-D-glucosidase (3 days). Effect of concentration of β-D-glucosidase on diameter of yellow zone for development times of; O, 30min; and ×, 60min.

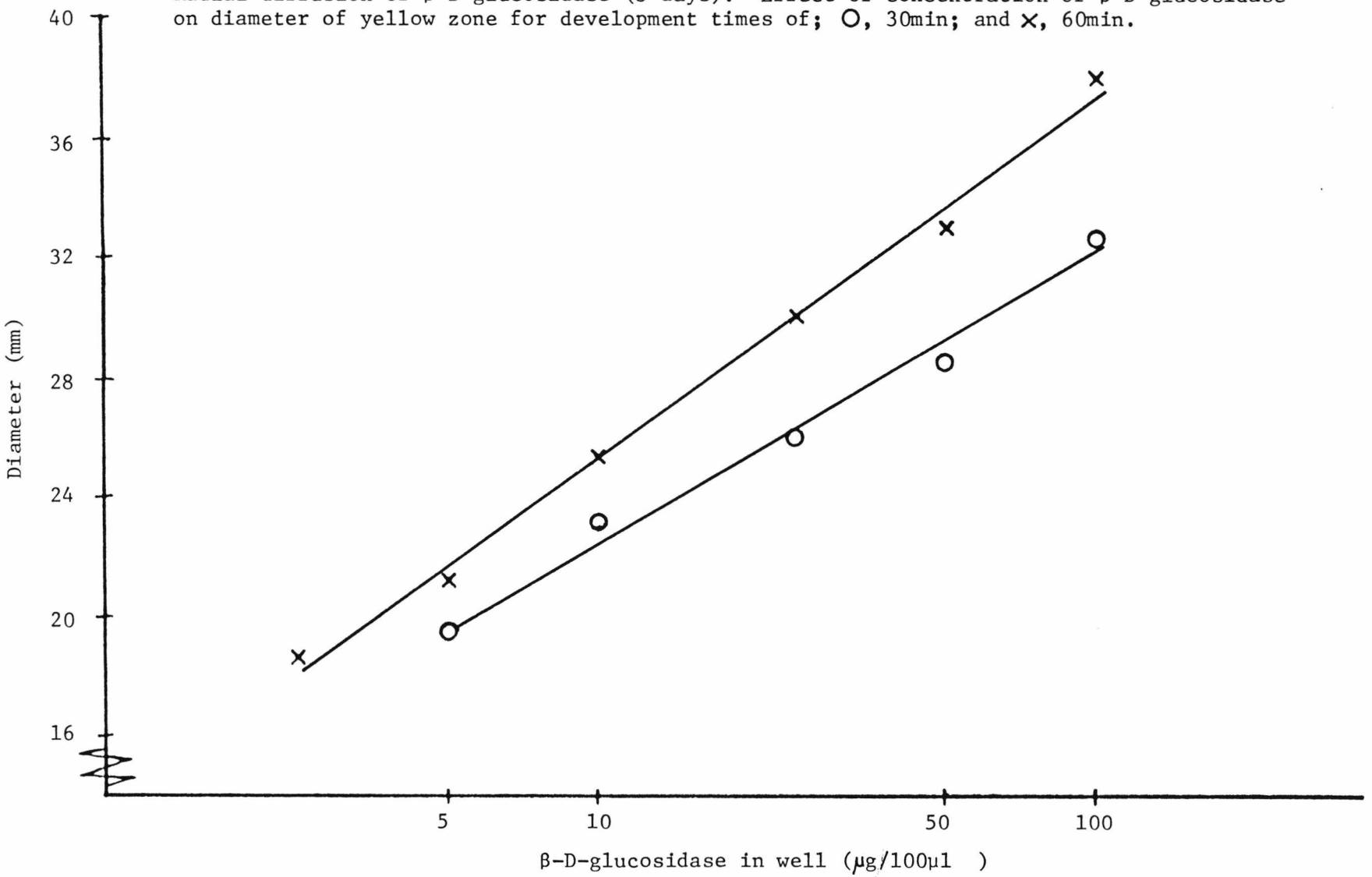


FIGURE 4.13 Effect of bentonite (1% w/v) in barrier ring on radial diffusion (5 days) of β-D-glucosidase. O, Bentonite in ring; ×, control plates.

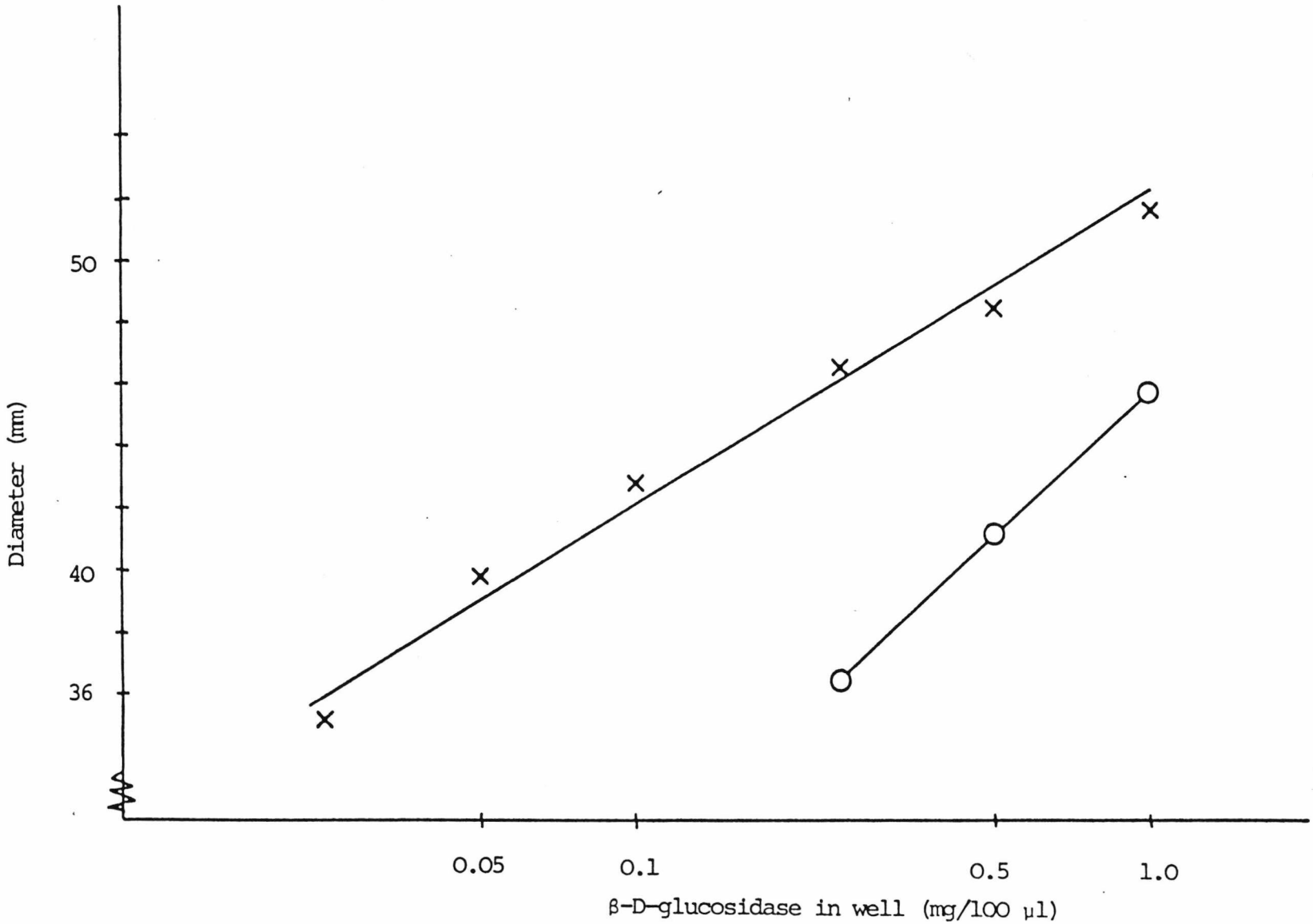
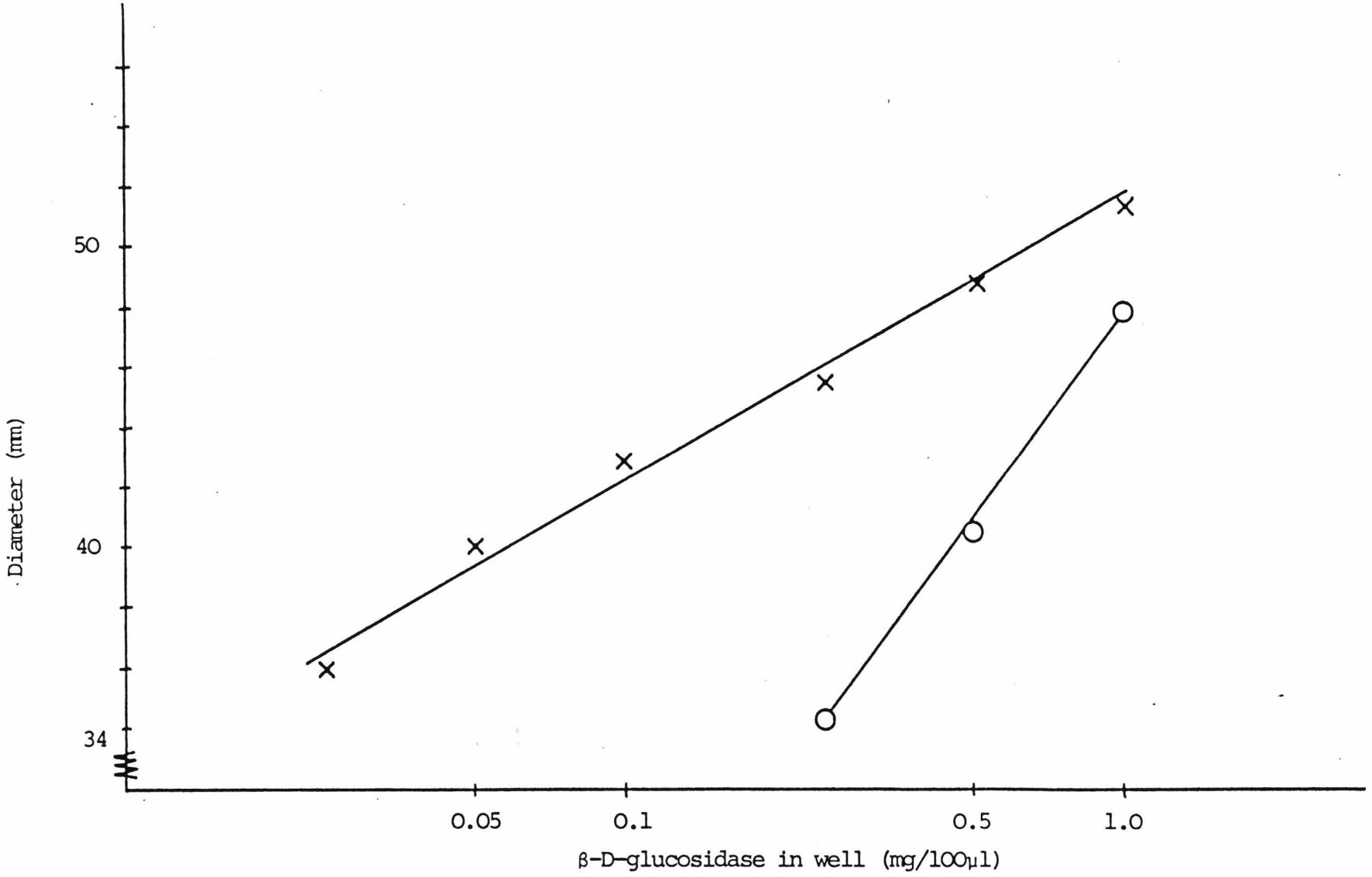


FIGURE 1.14 Effect of soil (1.0g) in barrier ring on radial diffusion (5 days) of β-D-glucosidase. O, Soil in ring; X, control plates.



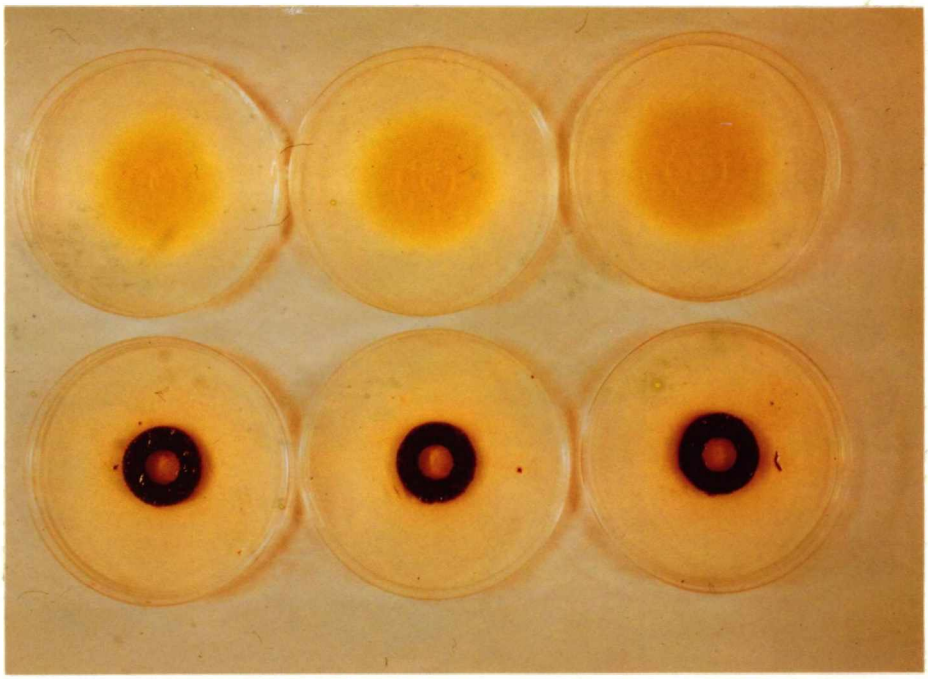
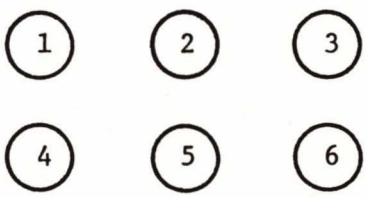


PLATE 4.6

Diffusion of β-D-glucosidase through soil barrier ring (5 days).




β-D-Glucosidase

1. 0.025 mg $100\mu l^{-1}$
2. 0.05 mg $100\mu l^{-1}$
3. 0.1 mg $100\mu l^{-1}$

} control plates

4. 0.025 mg $100\mu l^{-1}$
5. 0.05 mg $100\mu l^{-1}$
6. 0.1 mg $100\mu l^{-1}$

} soil (ca. 1g) barrier ring plates

# CHAPTER 5 : RADIAL GROWTH OF SOIL ISOLATES

## 5.1 INTRODUCTION

Fungal counts on dilution plates are normally considerably lower than bacterial counts. However, fungal biomass dominates the metabolism of many agricultural and forest soils (Anderson and Domsch, 1975) and as long ago as 1924 Waksman and Starkey stated that fungi are the main decomposers of the principal macromolecular substrate, cellulose. However, even at the time of these pioneer studies, Kalnins (1930) isolated a variety of aerobic cellulose degrading bacteria from 28 samples of English soils and more recently the involvement of actinomycetes in soil cellulose decomposition has been recognized (Hankin *et al.*, 1976; Lamot and Voets, 1976; Baeker and King, 1980). It is well known that environmental factors, such as C:N ratio and pH, influence not only the rate of cellulose decomposition in soil (section 1.4.1) but also the composition of the cellulolytic microflora (Alexander, 1977). However, even for a given set of environmental conditions, a complex network of poorly-understood interactions makes it difficult to determine the in situ roles of individual organisms in cellulose decay. Indeed, it is probably unrealistic to think of cellulose degradation in terms of individual microbial species. Lynch *et al.*, (1981) have looked at microbial populations involved in the breakdown of a crude cellulose fraction extracted from straw. Their studies, using a perfusion column, implicated a nine membered community comprised of five fungi, two yeasts and two bacteria (one an actinomycete). The yeasts and bacteria did not exhibit cellulase activity but may have been members of stable communities (Slater and Bull, 1978) with the fungi and contributed to overall cellulolysis indirectly by depolymerizing the associated xylan and arabinan which represented 11% of the cellulose extract. Another example of the

interactions between different microbial species involved in soil cellulose decomposition is provided by the work of Sato (1981). Sato (1981) studied the changes in the microbial population and in the rate of $CO_2$ evolution following addition of powdered cellulose to soil. A correlation was found between the rates of $CO_2$ evolution and total Gram-negative bacteria but not between the rates of $CO_2$ evolution and the number of cellulose (i.e. filter paper) - decomposing microorganisms. He suggested that the Gram-negative bacteria were utilizing the simple sugars produced during cellulose decomposition although he did not determine what proportion of these bacteria produced endoglucanase enzymes.

The problems facing soil microorganisms which rely on extracellular enzymes were discussed in section 1.4.4 and an ecological relationship involving soil immobilized enzymes was proposed. In this model, the products from a substrate - soil immobilized enzyme interaction could initiate the direct involvement of cellulolytic microorganisms in the decomposition of substrate by one, or a combination of, the following means:

[1] promoting germination of microbial spores or stimulating resting cells (section 1.4.4);

[2] stimulating a chemotactic or chemotropic response in microorganisms located at a distance from the substrate; and

[3] inducing, or mediating in the induction of the microbial extracellular cellulolytic enzymes (section 1.3.3).

As a first step in assessing the relevance of this hypothesis we have used the model soil environment developed here to study microbial growth and to investigate the ability of different microbial species to cross barrier rings containing various soil components and to respond to concentration gradients of soluble sugars. Plates similar to those used

in the diffusion studies (section 4.2.4) were centrally inoculated with a cellulolytic species and the growth pattern (i.e. increases in the diameter of the colony) observed.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Soil Isolates

The fungal, actinomycete and bacterial isolates from Hamble soil (section 3.3.1) were grown on plates containing either filter paper, Avicel or CMC as the sole carbon source. The fungal and actinomycete isolates decomposed insoluble cellulose more rapidly than any of the bacterial isolates. Three fungal isolates identified as; *Trichoderma viride* Pers. ex S.F. Gray aggr. (Rifai, 1969), *Trichoderma koningii* Oud. aggr. (Rifai, 1969) and *Botryotrichum piluliferum* Sacc. March. (Commonwealth Mycological Institute) and an unidentified *Streptomyces* species (act 9) were selected for further study. These organisms were chosen because of their capacity to degrade insoluble cellulose and because these genera are frequently described as involved in cellulose decomposition in soil (Daniels, 1961; Tribe, 1966; Griffin, 1972; Lamot and Voets, 1976; Lynch *et al.*, 1981).

*Sporocytophaga myxococcoides* was used to illustrate how the barrier ring plates can be used for the study of cellulolytic bacteria. *S. myxococcoides* has been isolated from several soils (Walker and Warren, 1937; Christensen, 1977) and strain NCIB 8639 (supplied by Dr. I. Vance, Polytechnic of Central London, London) is able to cause extensive degradation of filter paper.

5.2.2 CMC Barrier Ring Plates

(i) Preparation of plates

Sternberg MM + 0.5% (w/v) CMC agar plates (SC plates) and Berg MM + 0.5% (w/v) CMC agar plates (BC plates) were prepared as described in section 4.2.2. Barrier rings devoid of substrate or containing entire soil or one of its particulate components were introduced into the plates as described in section 4.2.4. The dimensions of the barrier ring were not the same in all experiments in order to allow radial growth rates to be measured both before and after the microorganism traversed the ring.

(ii) Inoculation of plates

The centre of BC plates were inoculated with a 10 µl drop of an act 9 spore suspension prepared by flooding a 14 day Berg MM + 1% Avicel agar slope with 5 ml Berg MM. The SC plates were inoculated with a small core (3 mm diameter) taken from just behind the margin of a colony of the relevant fungal species growing on a Sternberg MM plate. All the plates were incubated at 25°C in a humid atmosphere to prevent dehydration. Three replica plates for each treatment were normally used and radial growth was monitored by measuring the colony diameter at intervals in two directions at right angles to one another. Measurements were made using calipers and in the case of the fungi a low power binocular microscope. Endoglucanase production and diffusion was revealed by flooding the plates with 10 ml 1% (w/v) CETMAB (section 4.2.2i).

5.2.3 Avicel Barrier Ring Plates

Avicel (1% w/v) barrier ring plates were constructed in the same way as the SC and BC barrier ring plates.

5.2.4 Filter Paper Plates

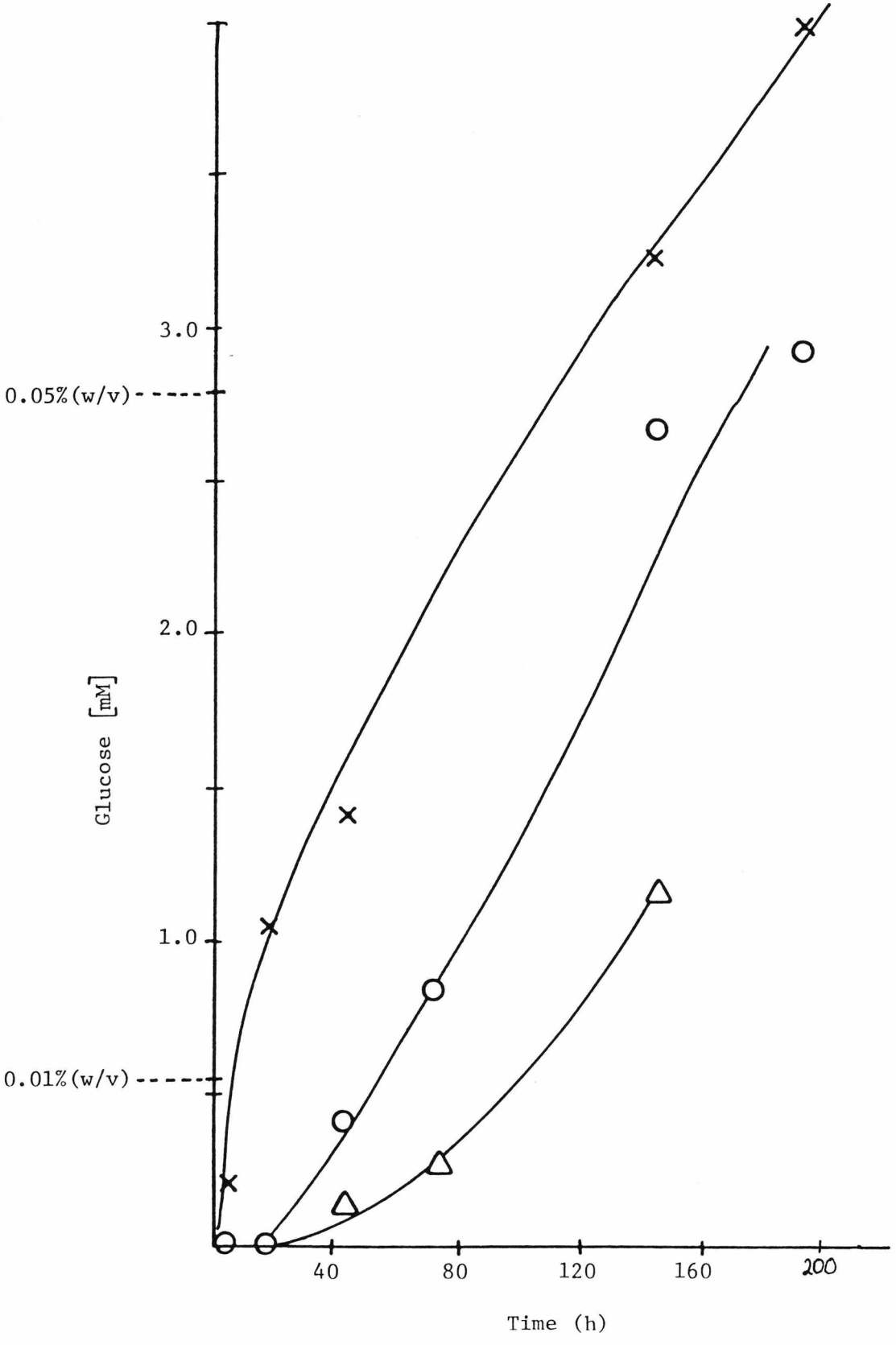
Bacteria normally form small discrete colonies on solid media and hence cannot readily be used in the barrier ring plates. However, *Sporocytophaga myxococcoides* spreads over moistened filter paper in a way which permits radial growth measurements to be made. Berg MM agar plates, with pieces of sterilized Watman No.1 filter paper arranged as in Plate 5.6 so as to provide barriers (1-5 mm) in which there was no substrate, were centrally inoculated with a suspension of *S. myxococcoides* in Berg MM. The plates were incubated at 25°C.

5.2.5 Glucose Gradient Plates

In order to investigate the response of cellulolytic microorganisms to a concentration gradient of soluble reducing sugars it was necessary to find a means of restricting substrate diffusion since due to the small size of, for example, the glucose molecule, the differential in a standard glucose gradient plate would be lost within 24 h. It is known that cellulases are strongly adsorbed to cellulose (section 1.3.2) and this characteristic was utilized to achieve a gradient of soluble sugars. A ring of filter paper (inner and outer diameter 70 mm and 85 mm), to which 0.5 ml, 10 mg $ml^{-1}$ filter sterilized commercial cellulase (BDH) in 0.1M-acetate buffer pH 5.0 had been added, was placed at the edge of the agar plate. The release of glucose with time was monitored by removing agar cores (6.5 mm diameter) from different parts of the plates at intervals. The agar cores were melted in a 100°C water bath, the tubes cooled to 40°C and 0.4 ml distilled water added. The samples were then assayed for glucose using the Benefield glucose oxidase/peroxidase method (section 2.5.1) and the amount of glucose in the agar core (0.1 ml) calculated by reference to a glucose standard curve. Fig. 5.1 shows the establishment of a glucose gradient with time by this means.

FIGURE 5.1

Establishment of a glucose gradient. Concentration of glucose in cores taken from the edge of a central circle of diameter. △, 20mm; ○, 40mm; ×, 60mm.



### 5.2.6 Statistical Analysis

Regression analysis of the data was performed using the Minitab release 81.1 Copyright - Penn State University, 2900 series version, on an ICL 2960 main frame computer (see appendix II).

## 5.3 RESULTS AND DISCUSSION

All the fungal and actinomycete isolates tested were able to grow across zones devoid of substrate on both CMC* and Avicel plates. With these organisms the rate of radial growth was measured.

### 5.3.1 Radial Growth of Isolate act 9

No difference in radial growth was observed when isolate act 9 was grown over a substrate-free barrier ring or over a barrier ring containing kaolin (0.5 - 5% w/v). Plate 5.1 shows act 9 on BC-kaolin barrier plates after 5 weeks. However, a reduction in the radial growth rate was observed (Fig. 5.2) if bentonite (0.5 - 5% w/v) was present in the barrier ring. By growing act 9 on plates in which the barrier ring was moved outwards (i.e. inner and outer diameter 19.5 mm and 38.0 mm) it was possible to show that the reduction in growth rate was limited to the region of the barrier ring (Fig. 5.3).

There are a number of factors which may contribute to the reduced radial growth rate (rgr) of act 9 on bentonite barrier ring plates. Smectite clays are known to adsorb dextrans (Olness and Clapp, 1975) and CMC (A.J. Desmarais, Hercules Inc., Delaware. personal communication). Evidence for adsorption of CMC by bentonite in the barrier ring plates

---

*In the SC and BC plates the CMC diffused slowly into the substrate-free ring. The presence of a small amount of CMC in the substrate-free ring could be seen on flooding control, uninoculated plates after 7 days. After 28 days, although a differential still existed, CMC was seen throughout the substrate-free barrier ring.

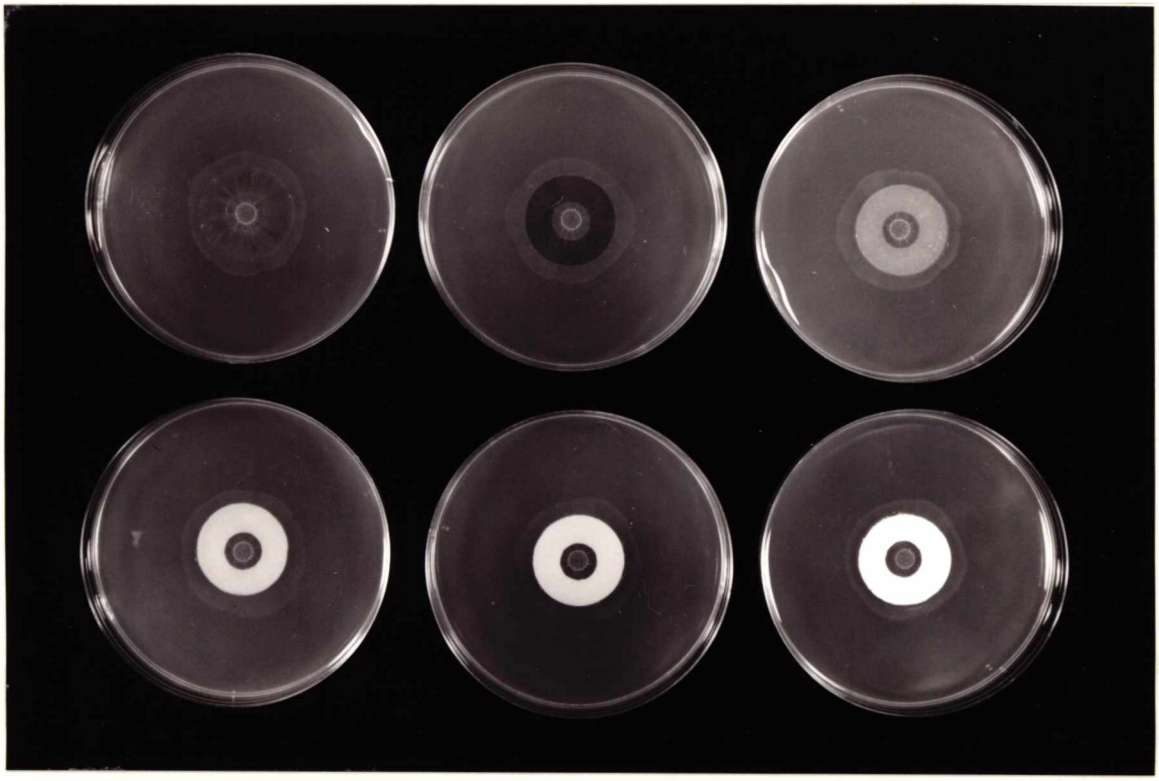
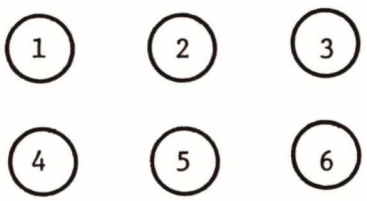


PLATE 5.1

Growth of isolate act 9 on BC-kaolin barrier ring plates (5 weeks).




Barrier rings (11.5mm - 26.5mm)

1. BC control
2. Substrate-free
3. 0.1%(w/v) kaolin
4. 0.5%(w/v) kaolin
5. 1.0%(w/v) kaolin
6. 5.0%(w/v) kaolin

FIGURE 5.2

Effect of bentonite barrier ring on radial growth of isolate act 9. ✕ , control plate; △ , 0.5%(w/v) bentonite ○ , 5.0%(w/v) bentonite.

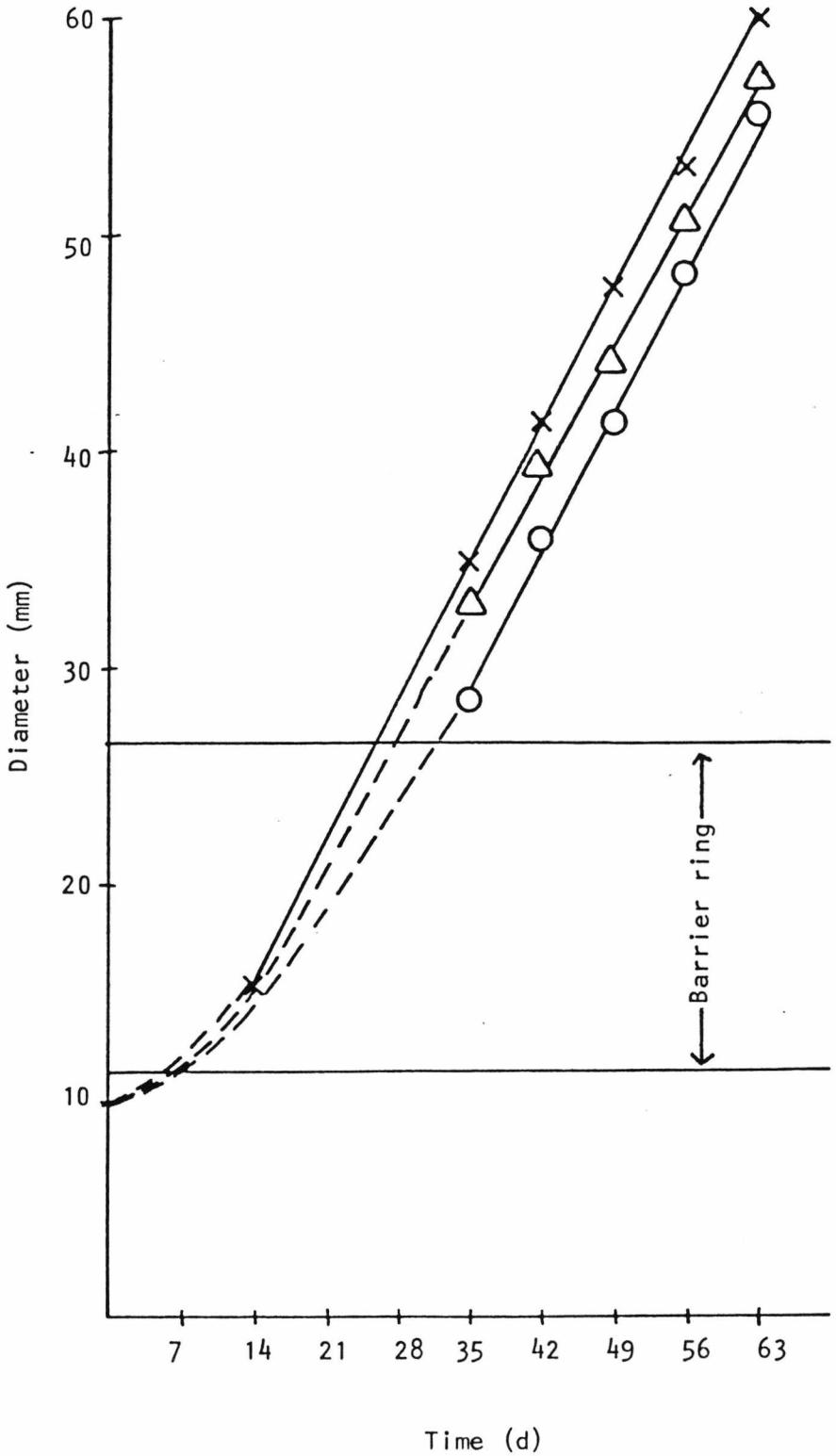
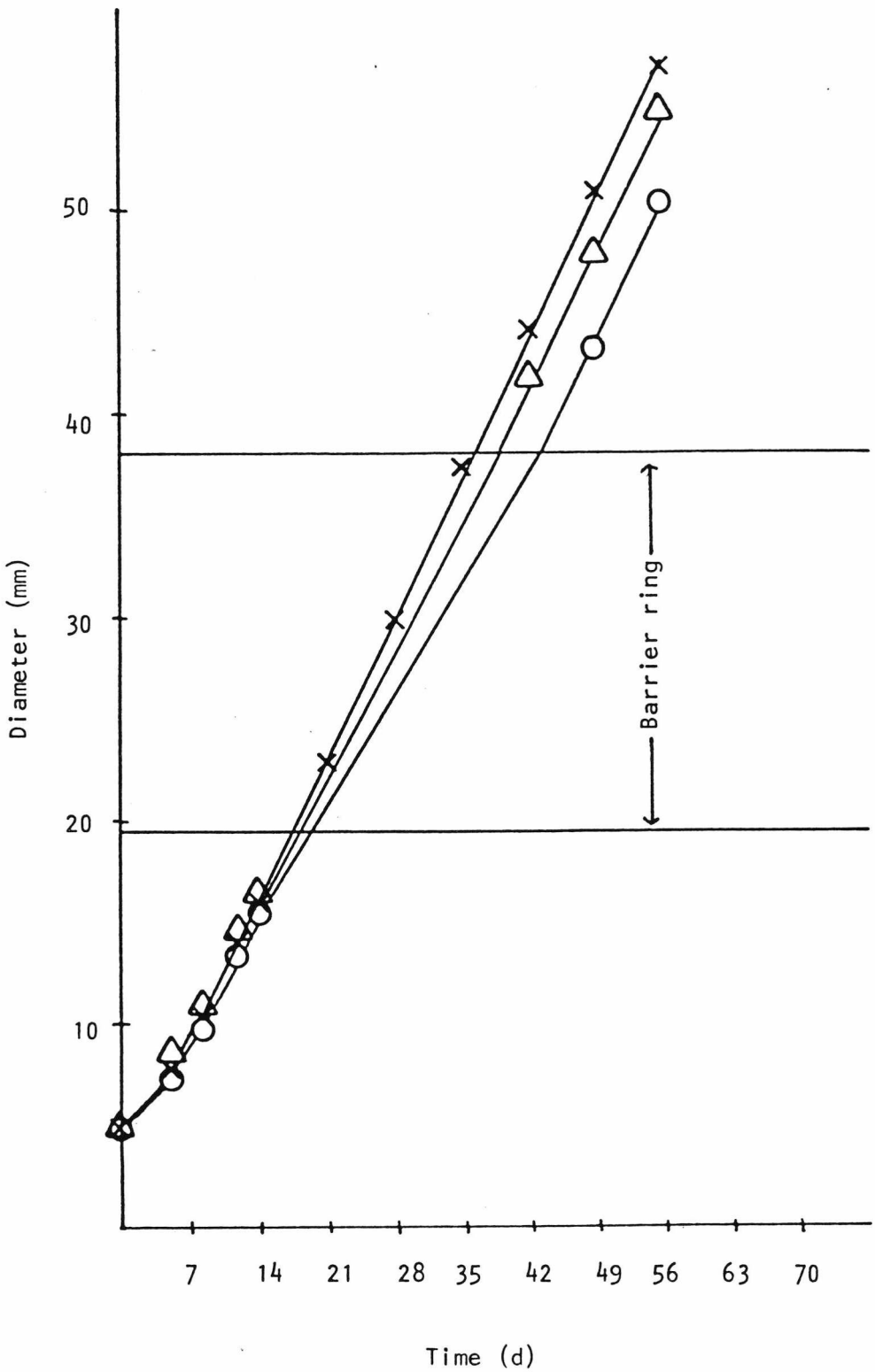


FIGURE 5.3

Effect of bentonite barrier ring on radial growth of isolate act 9. ×, control plate; △,0.5%(w/v) bentonite; ○,5.0%(w/v) bentonite.



comes from the slight clearing seen around the barrier ring in the control enzyme diffusion plates (section 4.3.1iii). Furthermore, strong adsorption of methyl sugars (Greenland 1956a; 1956b) and cellulose dextrin (Lynch *et al.*, 1956) by smectite clays has been reported.

In section 4.3.1 (iii) we showed that a bentonite barrier ring (> 1% w/v) prevented the diffusion of act 9 cellulase from a central well into the surrounding medium. Therefore, the adsorption of extracellular endoglucanases is likely to be another factor contributing to the observed reduction in rgr. Indeed the diameter of the hydrolysis zone (visualized by flooding the plates with 1% (w/v) CETMAB), which on control plates extended some distance beyond the colony margin, was considerably reduced on plates containing bentonite barrier rings.

Martin *et al.*, (1976) showed that in well-aerated cultures, 0.25% (w/v) Ca-montmorillonite actually increased the growth and metabolic activity of various *Streptomyces*, *Micromonospora* and *Nocardia* species and it was suggested that the stimulatory effect was due to the clay adsorbing one or more metabolic inhibitors. We attempted to investigate the effect of bentonite (sealed in dialysis tubing) on the growth of act 9 in shake flask cultures. By using dialysis tubing to separate the bentonite from the cells we had hoped to monitor growth by measuring increases in the turbidity of the culture and to eliminate any adsorption of cells and enzymes by the bentonite. Unfortunately, the cellulose containing dialysis tubing was itself degraded by the actinomycete before the influence of bentonite on growth could be assessed! An alternative approach would be to monitor $CO_2$ evolution from cultures grown in CMC with or without bentonite.

When entire soil was added to the barrier ring the radial growth of act 9 was stimulated. This stimulation of radial growth was independent

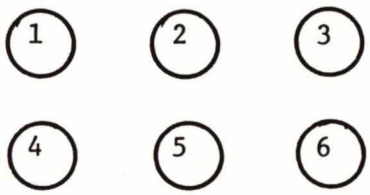
of the method of sterilization, occurring with both autoclaved and gamma-irradiated (10Mrad) soil and was probably due to an increase in soluble nutrients due to the sterilization process (Lynch, 1982). Radial growth was also stimulated if the colloidal-size fraction from Hamble soil was incorporated (0.5 - 10% w/v) in the barrier ring (Plate 5.2 and Fig. 5.4). However, if the carbonate and organic matter were removed from the CSF (section 3.2.1 iii) prior to its incorporation in the barrier ring a reduction in the radial growth rate was observed (Fig. 5.5). An alternative explanation is that the humic (organic) and not the clay fraction adsorbed an unknown growth inhibitor. The two graphs (Fig. 5.4/5) obtained using CSF illustrate the difficulties encountered when analysing the data but will be used as examples to illustrate the statistical methods applied. The presence of CSF in the barrier ring appears to influence radial growth in two ways.

[1] The CSF causes an increase in the average rgr of act 9 over the whole plate (as determined by the slope of the regression line fitted to <u>all</u> points for a given plate - Fig. 5.4). The regression analysis of the data from the measurements together with the appropriate tests for significance are given in appendix II.

[2] The CSF, from which the organic matter has been removed, when present in the barrier ring (10% w/v) caused a temporary reduction in the rgr as act 9 grew across the actual barrier ring (Fig. 5.5). The rgr of act 9 after emergence from the barrier ring was the same as for the control plates (the slopes of the regression line fitted to the points x = 4-8 (weeks) were not significantly different - appendix II). During the time the organism is growing across the barrier ring it is impossible to make enough accurate measurements to justify a regression analysis. This is not only because of the short distance

PLATE 5.2

Growth of isolate act 9 on BC-CSF barrier ring plates (4 weeks).




Barrier rings (11.5mm - 26.5mm)

1. Substrate-free
2. 0.5%(w/v) CSF
3. 1.0%(w/v) CSF
4. 2.5%(w/v) CSF
5. 5.0%(w/v) CSF
6. 10.0%(w/v) CSF

FIGURE 5.4

Effect of colloidal-size fraction (CSF) on radial growth of isolate act 9. ×, Control plates; △, 0.5%(w/v) CSF; ○, 10%(w/v) CSF.

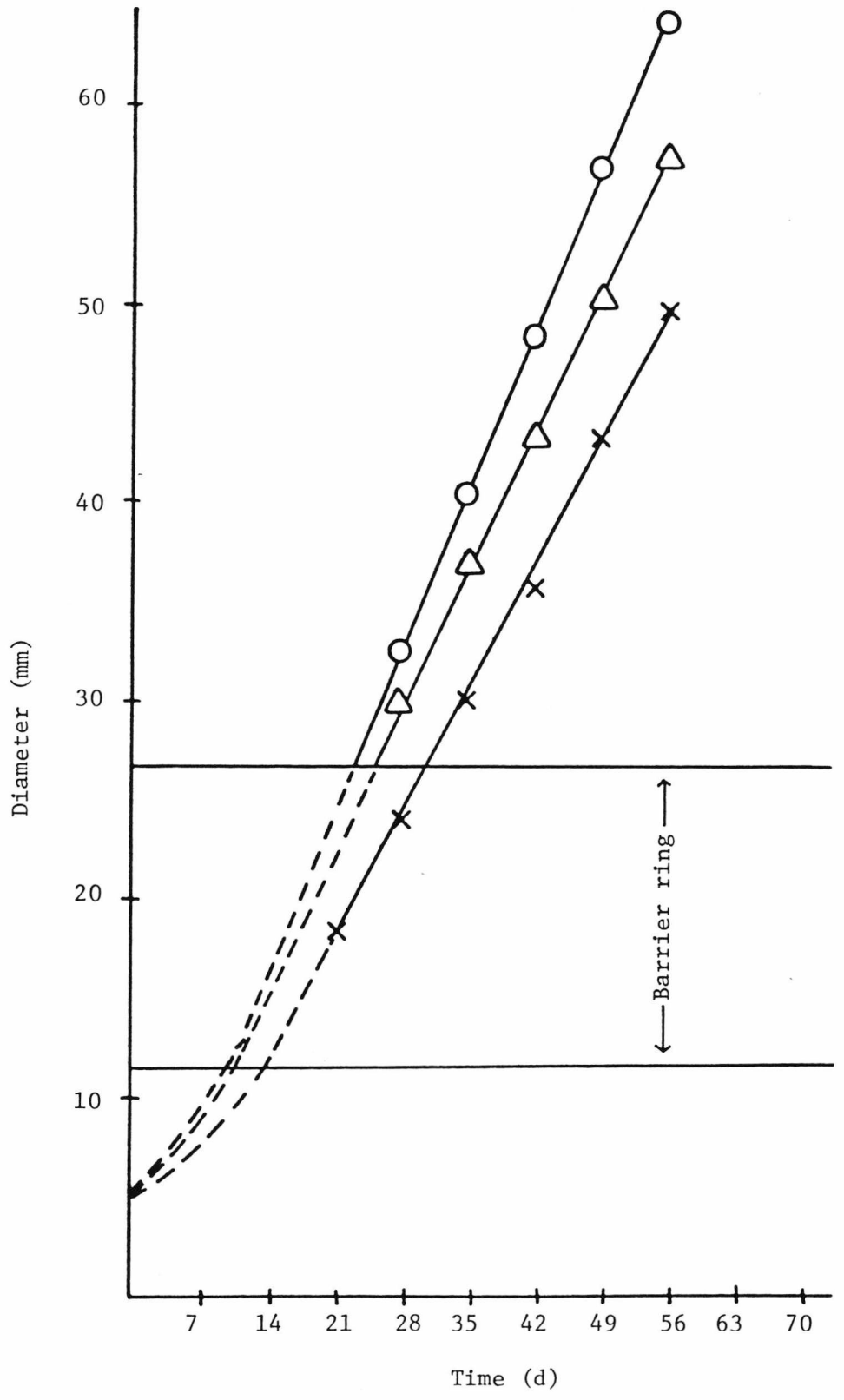
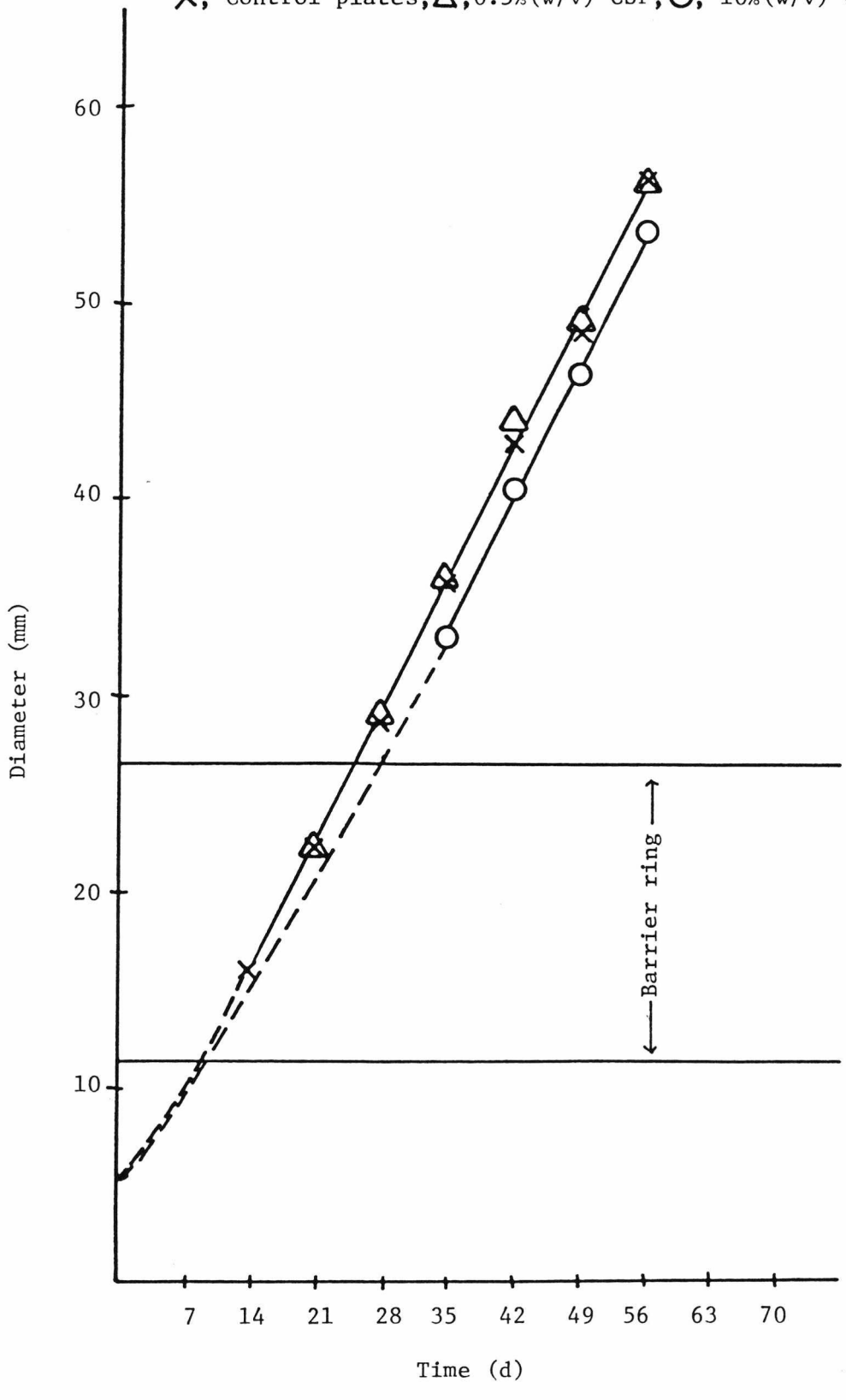


FIGURE 5.5

Effect of colloidal-size fraction (CSF) - free from organic matter, on radial growth of isolate act 9. ×, Control plates; △, 0.5%(w/v) CSF; ○, 10%(w/v) CSF.



involved but also because it is difficult to measure the extent of growth through a barrier ring containing dense or particulate components. The alternative approach is to test for parallelism in the lines before entry to and after emergence from the ring and, given parallelism, to then test for differences in the $\alpha$ value in the equations.

$$y = \alpha_1 + \beta x$$

$$y = \alpha_2 + \beta x$$

fitted to the lines representing growth after emergence from the ring.

These two ways in which the components in the barrier ring can influence radial growth, one being localized and the other general, require that the data be analysed in two distinct ways. The difficulty arises when radial growth is affected in both ways at the same time, as appears to be the case with the fungi (section 5.3.2).

Radial growth of isolate act 9 on Avicel barrier ring plates was unpredictable. In one experiment act 9 failed to cross a 1% (w/v) bentonite barrier ring on all four replica plates (Plate 5.3) while in a separate experiment in which bentonite at a range of concentrations was included in the barrier ring growth was indistinguishable from that on the control plates. The unpredictable nature of act 9 growth on Avicel barrier ring plates can be partially explained by the discrete nature of the Avicel and the difficulty encountered in obtaining an even distribution of the Avicel microcrystals throughout the agar. On the control plates discrete clumps of heavy growth and sporulation were seen in contrast to the evenly spread mycelial growth seen on CMC agar plates.

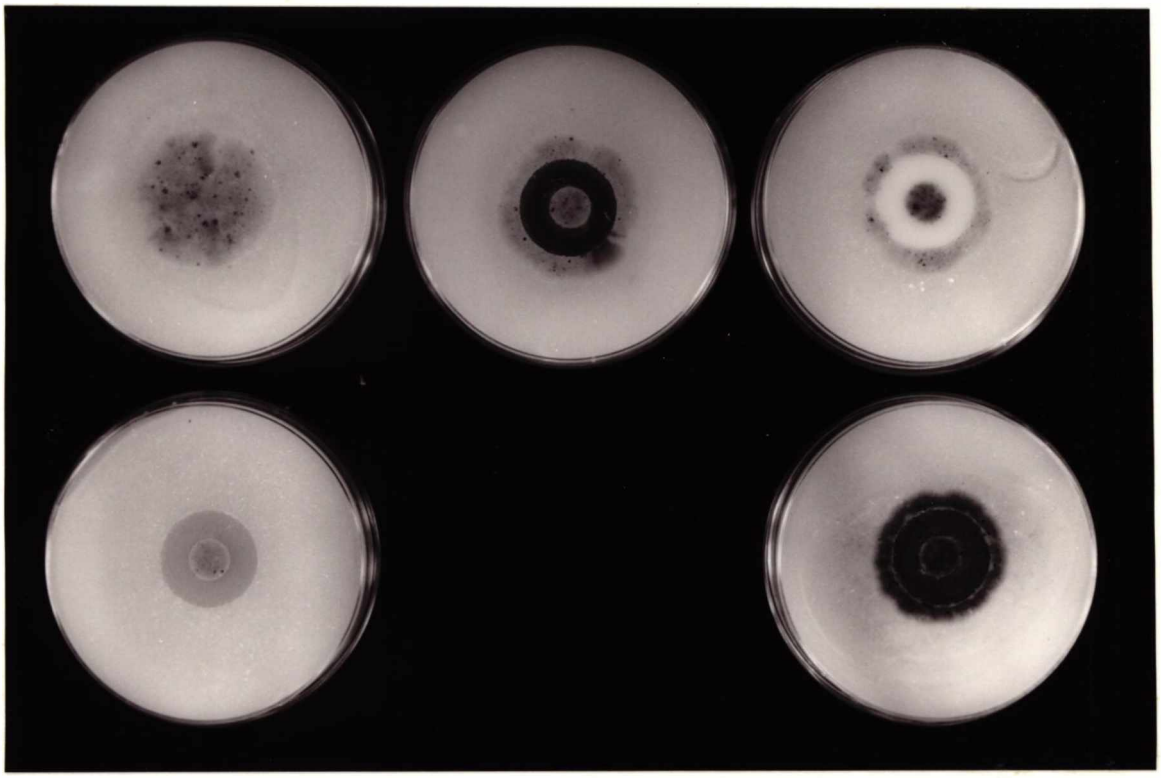


PLATE 5.3

Growth of act 9 on BA barrier ring plates (6 weeks).


Barrier rings (11.5mm - 26.5mm)

1. BA control
2. Substrate-free
3. 1%(w/v) kaolin
4. 1%(w/v) bentonite
5. Soil

5.3.2 Radial Growth of Fungal Isolates

(i) *Trichoderma viride*

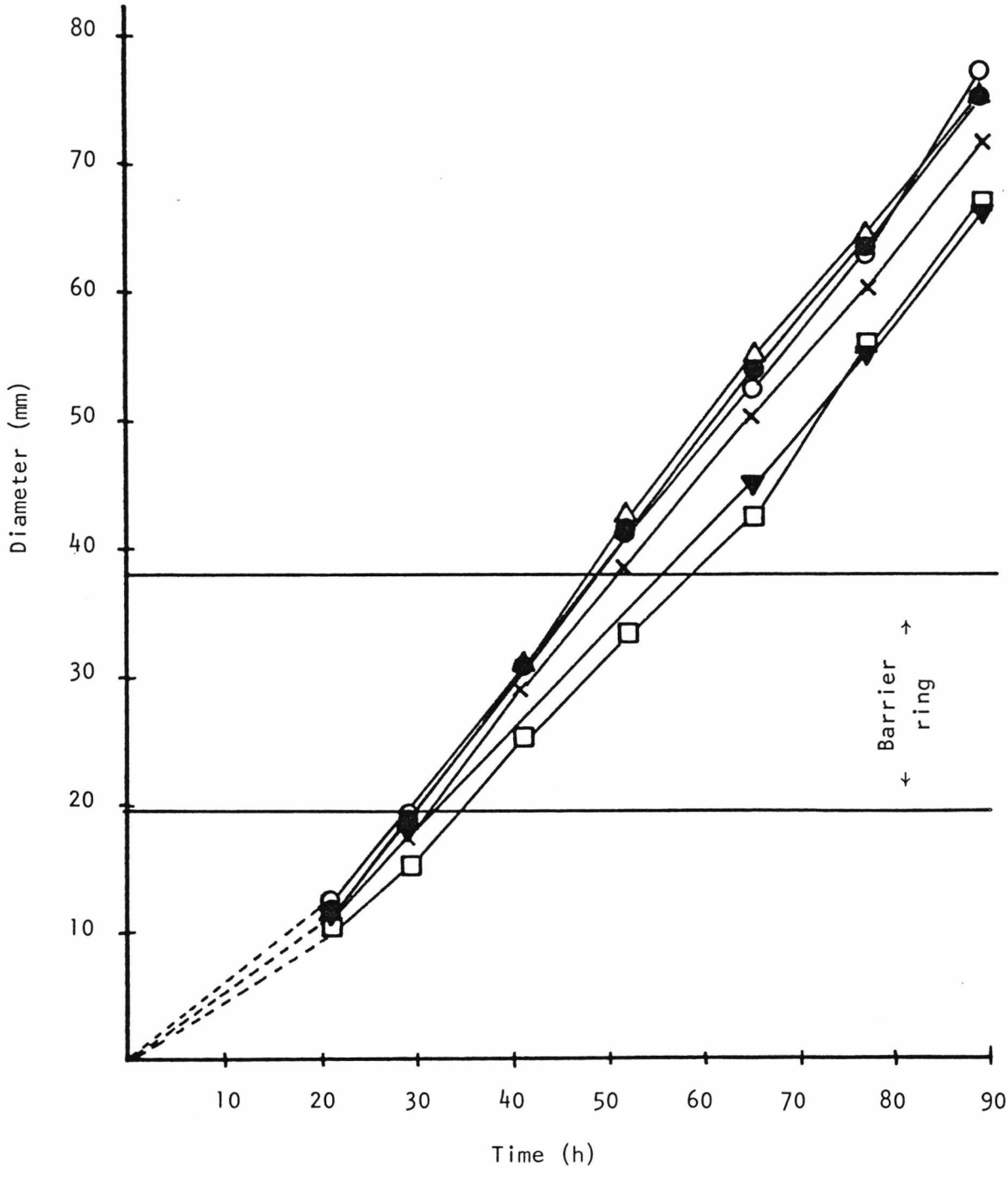
Bentonite and sand in the barrier ring caused a reduction in the radial growth rate (rgr) of the *Trichoderma viride* isolate on both CMC and Avicel barrier ring plates (Fig. 5.6). Soil caused an increase in the hyphal density of the colony (Plate 5.4) and a small stimulation in rgr. Increased hyphal density is a result of increased branching so that there are only very small areas of the medium which remain uncolonized. The difficulties encountered when statistically analysing this data have been outlined (section 5.3.1).

Stotzky and Rem (1967) showed that montmorillonite at concentrations greater than 2% (w/v) reduced the respiration rate of *Trichoderma viride* grown in liquid culture. At 2% (w/v)montmorillonite a reduction in respiration was observed when the glucose concentration in the medium was below 1% (w/v). However, no decrease occurred if the glucose concentration was 1, 2 or 4% (w/v). Kaolinite up to 40% (w/v) was reported as having no effect on respiration. From experiments in which the effect of pH, shaking and the viscosity of the medium were investigated it was concluded that inhibition of fungal respiration by montmorillonite at concentrations above 2% (w/v) was due to impaired $O_2$ diffusion. It is unlikely that depletion of $O_2$ is affecting fungal growth on our barrier ring plates. Adsorption of endoglucanase by the bentonite will reduce the amount of soluble sugars available for uptake. In addition, the adsorption of products from CMC hydrolysis (section 5.3.1) is a factor likely to contribute to the observed reduction in rgr.

No clearing (hydrolysis) zone was seen on flooding the plates with 1% (w/v) CETMAB. This result was not unexpected since the radial growth rate of *Trichoderma viride* on BC plates was *ca.* 0.45 mm $h^{-1}$, while the

FIGURE 5.6

Effect of various soil components in the barrier ring on the radial growth of *Trichoderma viride* on SC plates. ×, SC control; ●, substrate-free; △, 10% (w/v) kaolin; □, 10% (w/v) bentonite; ▼, sand; ○, soil.



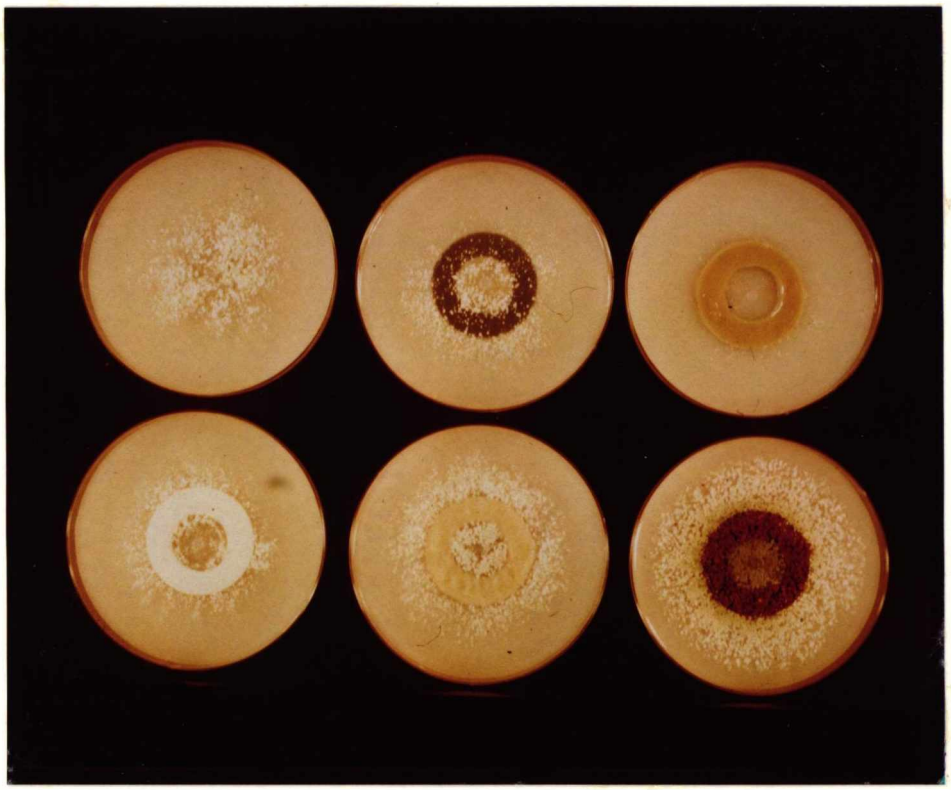
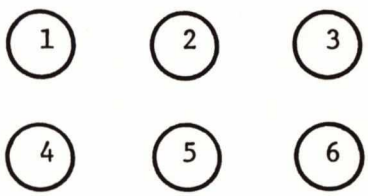


PLATE 5.4

Growth of *Trichoderma viride* on SA barrier ring plates (6 days).




Barrier rings (19.5mm - 38.0mm)

1. SA control
2. Substrate-free
3. 10%(w/v) bentonite
4. 10%(w/v) kaolin
5. Sand
6. Soil

rate of diffusion of endoglucanase from *T. viride* commercial cellulase preparation averaged 0.28 mm $h^{-1}$ for the first 20 h (section 4.3.1 i). Therefore, even if the advancing hyphae were producing large quantities of endoglucanase one would not expect to see a clearing edge. In passing it is of interest to note that the rgr of colonies is very seldom taken into consideration when screening fungi for cellulase production by measuring the size of clearing or hydrolysis zone they produce. A fungus which produces high levels of endoglucanase may, as a result, be overlooked if it has a high rgr.

On the Avicel barrier ring plates any clearing of the Avicel was masked by the dense sporulating mycelium.

(ii) *Trichoderma koningii*

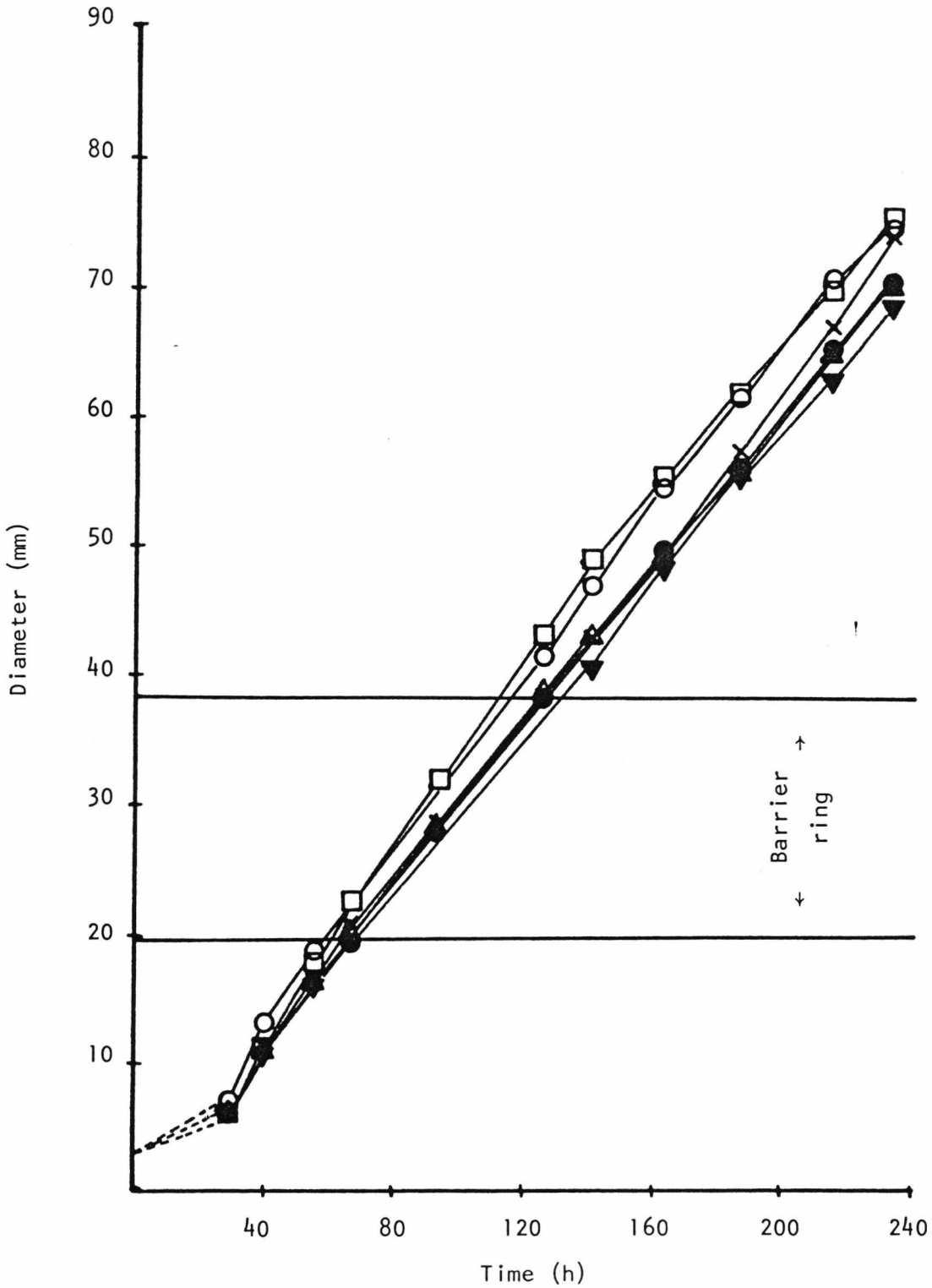
The behaviour of *Trichoderma koningii* on CMC and Avicel barrier ring plates was similar to that of *T. viride*.

(iii) *Botryotrichum piluliferum*

In contrast to the *Trichoderma* spp., the average rgr of *Botryotrichum piluliferum* was, if anything, stimulated by the presence of bentonite (10% w/v) in the barrier ring of SA and SC plates. The rgr of *B. piluliferum* appeared to decrease with increasing glucose concentration (section 5.3.4) and it is possible that this fungus responds to a reduced nutrient supply by, at least in the short term, increasing its rgr. An apparent stimulation was also observed when soil was present in the barrier ring (Fig. 5.7). However, the variation between replica plates and the localized effect of the barrier ring (section 5.3.1) makes statistical analysis of these data difficult. In addition there appears to be a slight stalling effect (Robinson, 1978) as the colony margin reaches the edge of the plate.

FIGURE 5.7

Effect of various soil components in the barrier ring on the radial growth of *Botryotrichum piluliferum* on SA (Avicel) plates. ×, SA control; ●, substrate-free; △, 10% (w/v) kaolin; □, 10% (w/v) bentonite; ▼, sand; ○, soil.



If the *Botryotrichum piluliferum*, SA plates were retained in the incubator after the colony had reached the edge of the plate a gradual clearing of the Avicel occurred. This clearing originated in the centre of the control plates, while on those plates containing bentonite or kaolin, clearing appeared to start from the ring itself (Plate 5.5). This suggests that there was a high concentration of active cellulase either in the vicinity of, or adsorbed to, the clay. An obvious experiment suggested by these results would be to compare the diffusion properties of cellulase from *Botryotrichum piluliferum* with those of the *Trichoderma viride* cellulase preparation.

### 5.3.3 Radial Growth of *Sporocytophaga myxococcoides*

Measurements of the diameter of the yellow zone, in which degradation of the filter paper had occurred, were used as a measure of the radial growth rate of *Sporocytophaga myxococcoides* growing on the BM plates overlayed with filter paper. The rgr was constant for zone sizes 20 - 55 mm. *S. myxococcoides* was unable to cross greater than a 1 - 2 mm gap in the filter paper (Plate 5.6). Attempts at inducing *S. myxococcoides* to cross a larger gap by adding 0.01% (w/v) or 0.1% (w/v) glucose to the underlying basal medium were unsuccessful.

### 5.3.4 Radial Growth in Response to a Glucose Gradient

The response of the two *Trichoderma* species to a glucose gradient (section 5.2.5) was analysed by plotting the radial growth rate (mm $h^{-1}$) against time. Both *T. viride* and *T. koningii* responded in a similar way to the glucose gradient and hence this discussion will be restricted to *T. koningii* (Fig. 5.8). When growing on SM (control) plates the rgr is constant at *ca.* 0.375 mm $h^{-1}$. On plates containing 0.01% (w/v), 0.1% (w/v) and 1.0% (w/v) glucose, the rgr had increased to *ca.* 0.6 mm $h^{-1}$ by 30 h after

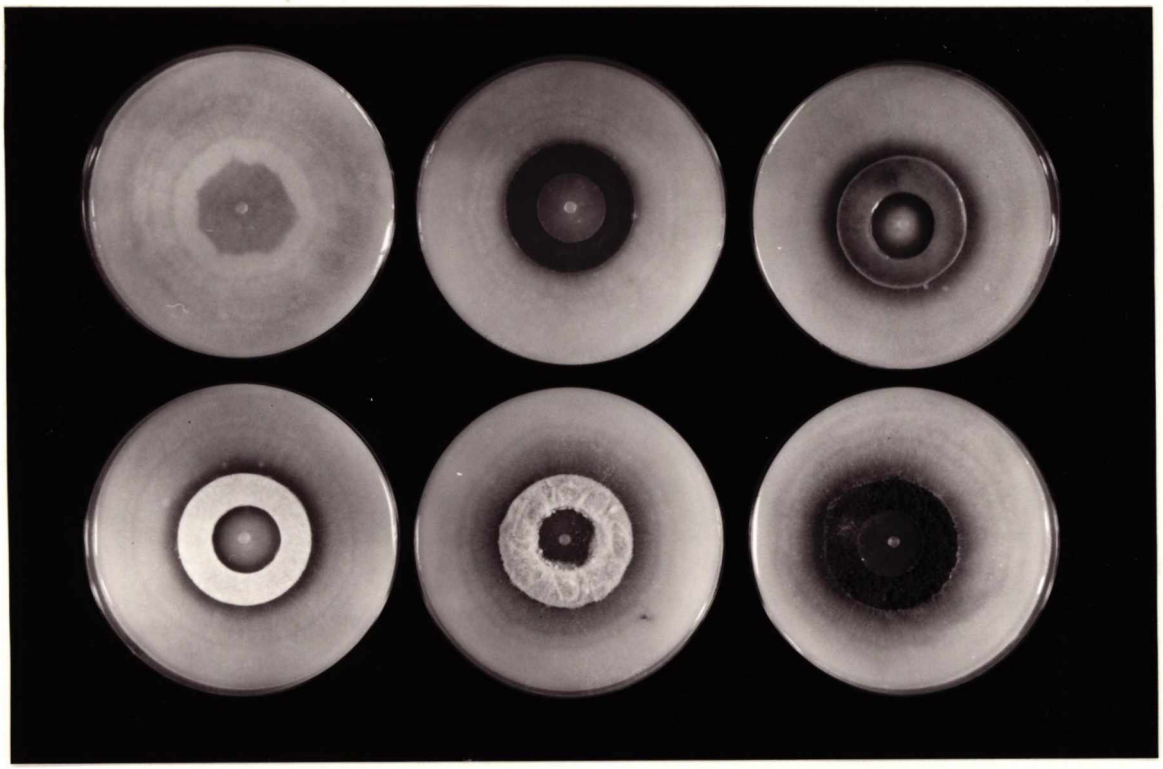


PLATE 5.5

Development of clearing zones following growth of *Botryotrichum piluliferum* on SA barrier ring plates (27 days).


Barrier rings (19.5mm - 38mm)

1. SA control
2. Substrate-free
3. 10%(w/v) bentonite
4. 10%(w/v) kaolin
5. Sand
6. Soil

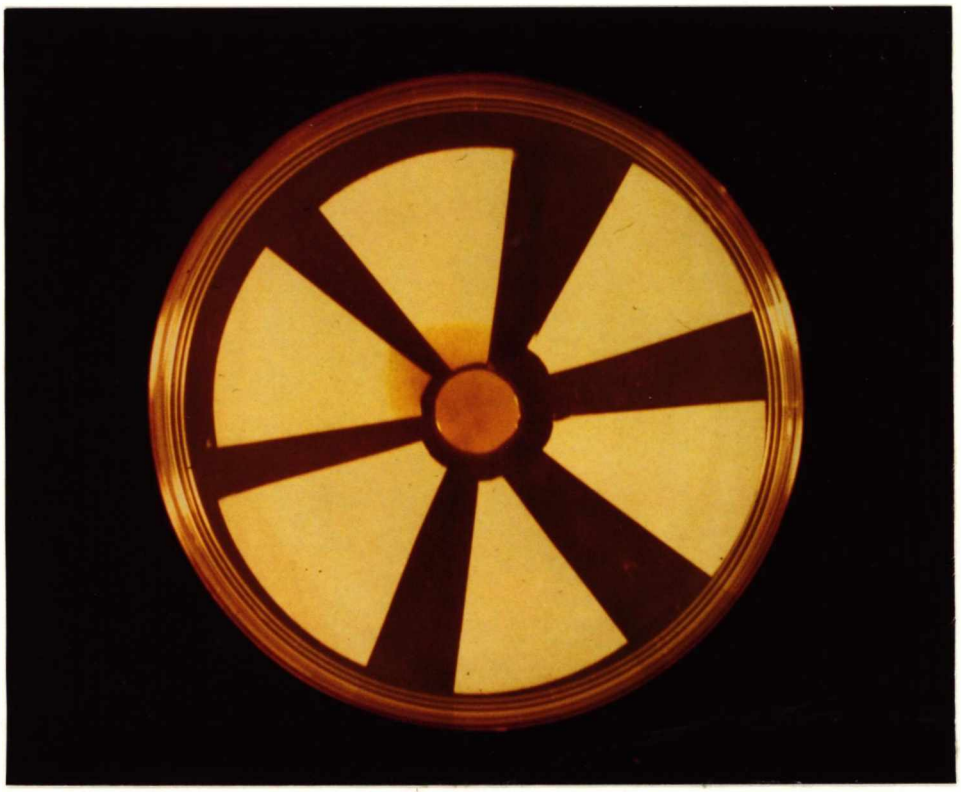
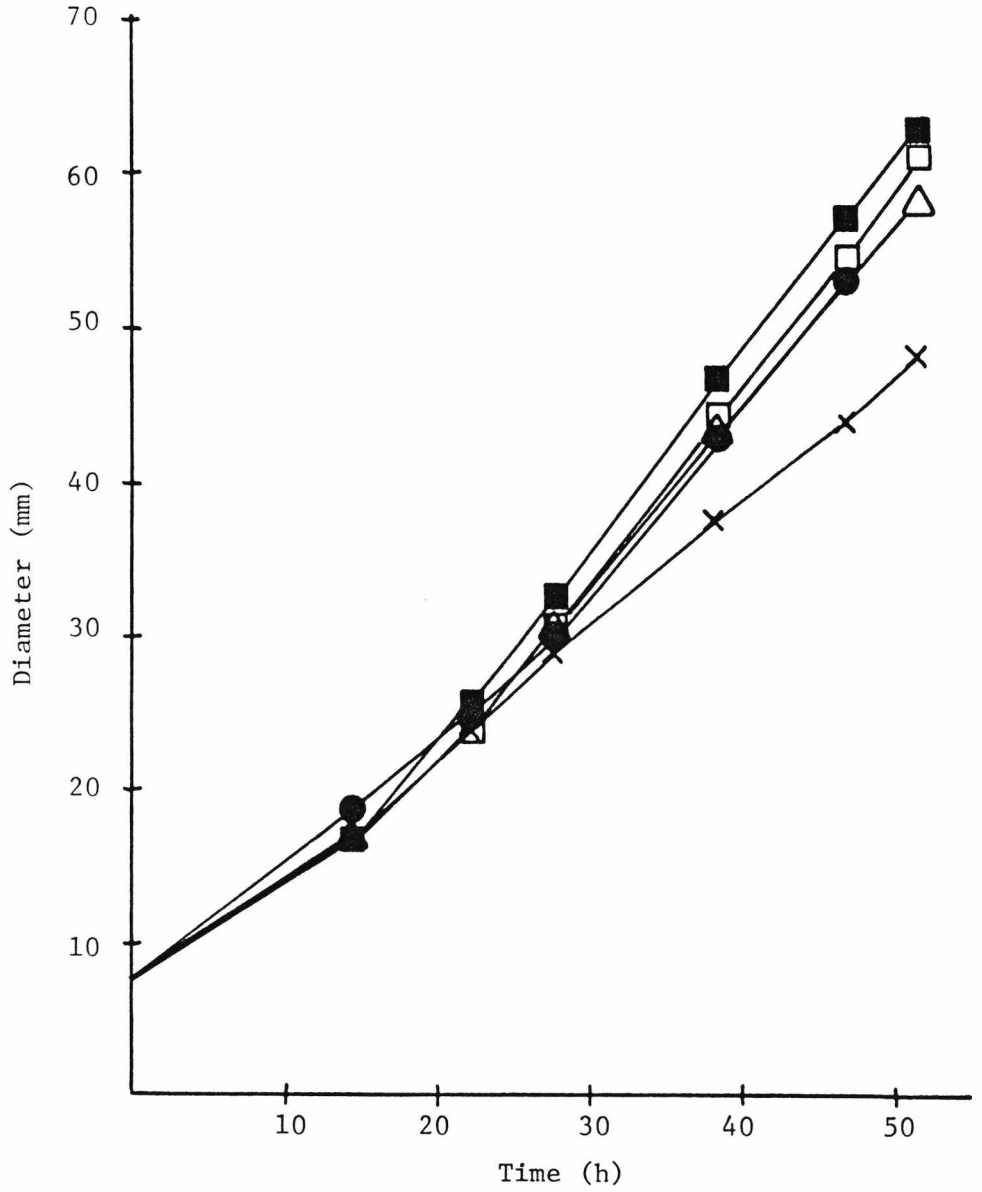


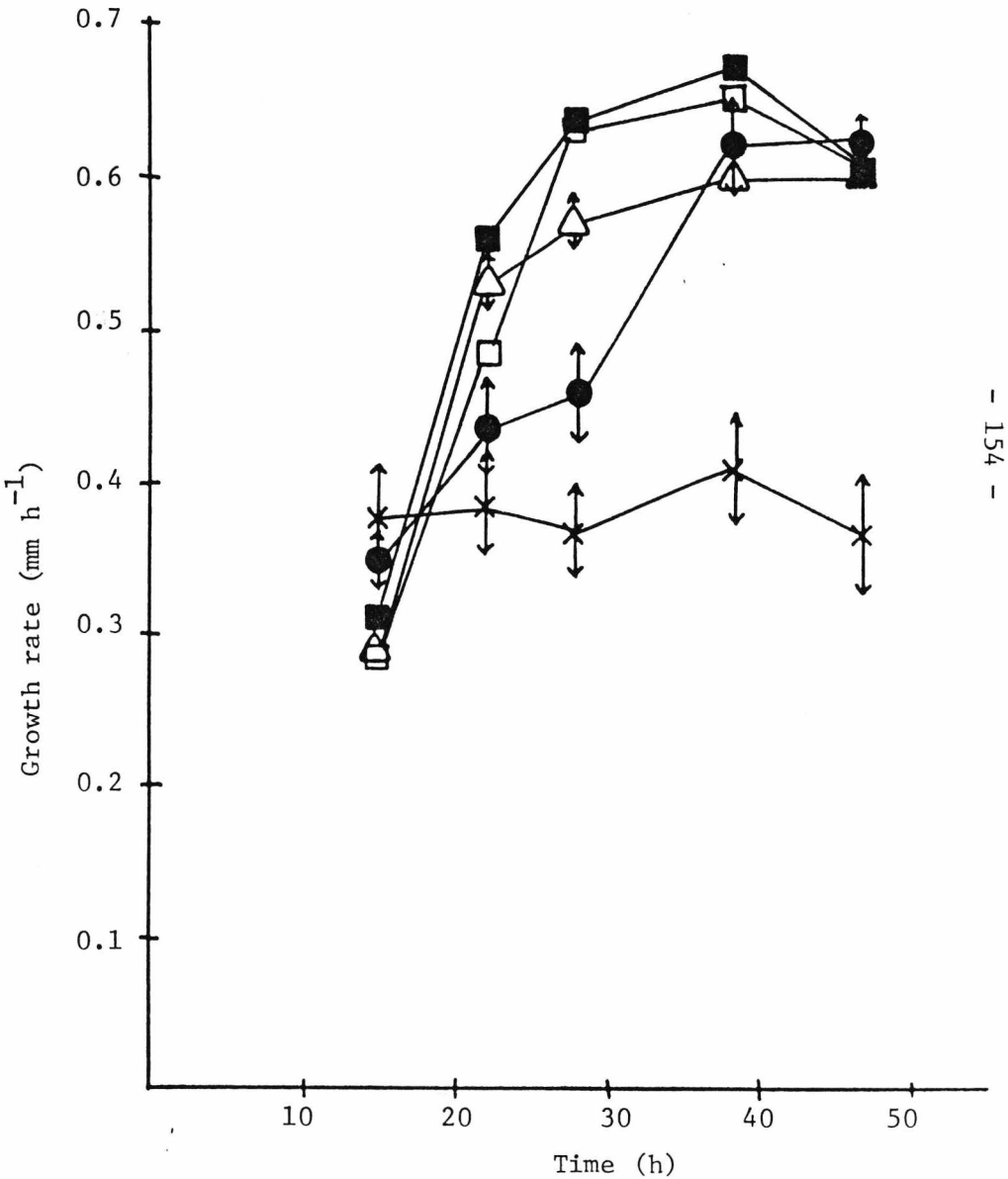
PLATE 5.6

Growth of *Sporocytophaga myxococcoides* on Berg MM plates overlayed with filter paper (14 days).

FIGURE 5.8

Radial growth of *Trichoderma koningii* in response to different glucose concentrations and a glucose gradient. ×, Control, △, 0.01%(w/v) glucose; □, 0.1%(w/v) glucose; ■, 1.0%(w/v) glucose; ●, glucose gradient. Radial growth rate given with standard deviation ($\sigma_{n-1}$).


70
60
50
40
30
20
10
Diameter (mm)
10
20
30
40
50
Time (h)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
Growth rate (mm h$^{-1}$)
10
20
30
40
50
Time (h)

inoculation and remained at this level until the colony margin neared the edge of the plate. By contrast, on the glucose gradient plates, the increase in rgr occurred between 30 h and 40 h after inoculation The factors which influence the rate of radial growth and differentiation within the developing fungal colony are not fully understood and for this reason it is not possible to conclude, at this stage, that the *Trichoderma* spp. are responding to a glucose gradient *per se* by increasing their rgr.

Robinson (1978) showed that if an agar core containing L-cysteine were placed behind the colony margin of a *Saprolegnia* sp. growing on malt agar, some of the leader hyphae would turn towards the core and lateral branch formation would be stimulated in the region of the core. However not all fungi respond in this way and when *Trichoderma koningii* was grown on SM plates and agar cores, containing 0.01% (w/v), 0.1% (w/v) and 1.0% (w/v) glucose, placed behind the colony margin an increase in lateral branching in the vicinity of the cores was observed but none of the leader hyphae turned towards the cores. Similarly, attempts to demonstrate a chemotropic response with germinating *T. koningii* conidiospores up a glucose gradient were inconclusive. A higher percentage of the conidia germinated at the higher glucose concentrations but the germ tubes appeared to grow in random directions. However, negative autotropism (Robinson *et al.*, 1968) and the early branching of the germ tubes may have obscured any chemotactic response.

*Botryotrichum piluliferum* and the *Streptomyces* isolate (act 9) responded to a glucose gradient by an increase in the hyphal density of the colony (Plate 5.7) and a reduction in the rgr. Trinci (1969) studied the effect of glucose concentration (0.009 - 90 g $\ell^{-1}$) on the rgr of *Aspergillus nidulans*, *Mucor hiemalis* and *Penicillium chrysogenium*. A plot of the

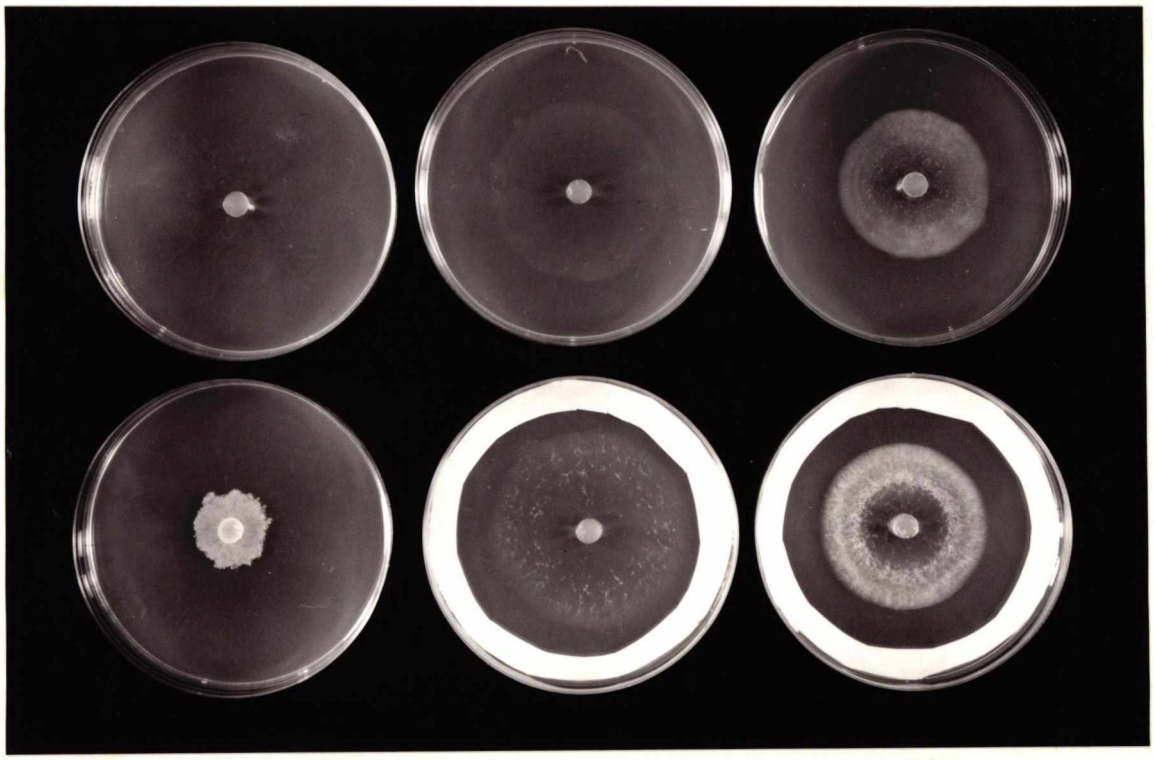
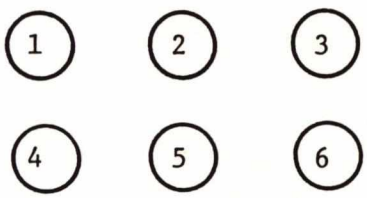


PLATE 5.7

Growth of *Botryotrichum piluliferum* in response to different glucose concentrations and a glucose gradient (148h).




1. Sternberg MM
2. 0.01%(w/v) glucose
3. 0.1%(w/v) glucose
4. 1.0%(w/v) glucose
5. Filter paper
6. Filter paper + cellulase (= glucose gradient)

rgr versus glucose concentration was different for each fungus. With *Mucor hiemalis* the maximum rgr occurred at 0.075 g $\ell^{-1}$ glucose while with *Aspergillus nidulans* the rgr increased with increasing glucose concentrations up to 0.2 g $\ell^{-1}$ glucose, it then decreased with increasing glucose concentrations to 10 g $\ell^{-1}$ glucose after which it continued increasing with increasing glucose concentration. These differences in response demonstrate the difficulty in interpreting rgr data.

The mechanism of radial growth on solid media is not fully understood and this, in turn, limits the application of our model soil environment in the study of interactions between soil microorganisms and potential substrates.

# CHAPTER 6 : DISCUSSION AND CONCLUSIONS

The aim of the research in this thesis was to investigate the dynamics of cellulose breakdown in soil at the level of the soil microenvironment. To this end, the objectives were fourfold:

[1] to design a novel technique for studying the dynamics of substrate hydrolysis and microbial growth in model soil environments;

[2] to develop methods for the quantitative measurement and characterization of cellulase activities in soil;

[3] to investigate the dynamics of cellulose decomposition in both soil and model soil environments; and

[4] to evaluate the suggestion that cellulase enzymes are immobilized on soil colloids and that this form of enzyme has a significant role in substrate decomposition.

The many experimental and interpretative difficulties involved in quantifying mircoorganisms and their activities (including enzymes) in natural environments such as soil have been well documented ( e.g. Stotzky, 1974; Burns, 1978; Jenkinson and Ladd, 1981; Atlas, 1982). Most traditional studies have concentrated on the microbiology of large volumes of well-mixed soil. However, in this work we have attempted to develop a new approach to soil microbiology by taking into account the spatial and temporal discontinuity which occurs in the microenvironment where microorganisms, enzymes and substrates interact. The process of cellulose decomposition was chosen as the subject of this investigation since cellulose is deposited in enormous quantities in terrestrial environments (section 1.4.1) and an understanding of the factors controlling cellulose decomposition in soil is of obvious importance with reference to carbon recycling in the biosphere. In addition, biotechnological

interests now view cellulose as a renewable resource that can be used for the production of not only methane and fermentable sugars (Spano *et al.*, 1979; Laube and Martin, 1981) but also microbial biomass for use as single cell protein (Chahal and Wang, 1978; Sadana *et al.*, 1979; Han, 1982).

The efficacy of 'free' or soluble extracellular endoglucanases and β-D-glucosidase, in terms of their ability to resist inactivation and to locate high concentrations of distant (i.e. mm away) substrate in soil, was investigated using a novel technique - the barrier ring diffusion plate. Barrier ring diffusion plates provide a simple and flexible system which permits the research worker to monitor the effects of soil components on the diffusion of enzymes. For instance, the concentration and type of soil component in the barrier ring can be varied as can the ring's dimensions and the location of the ring itself in relation to the source of the enzyme(s).

Using barrier ring diffusion plates we have shown that bentonite, a high unit surface area, high cation exchange capacity clay, restricts the diffusion of endoglucanase from both *Trichoderma viride* commercial cellulase preparation and a *Streptomyces* sp. cellulase preparation when compared to control (i.e. no barrier ring) plates. In contrast, kaolin a low unit surface area, low cation exchange capacity clay had no effect on the diffusion of endoglucanase from either microbial source. Of the two preparations, *T. viride* endoglucanase appeared better able to diffuse through barrier rings containing low concentrations of bentonite (0.1-1.0% w/v) than did endoglucanase from the *Streptomyces* sp. (section 4.3.1). Furthermore, *T. viride* endoglucanase showed greater resistance to inactivation following its addition to whole soil than did endoglucanase

from the *Streptomyces* sp. (section 4.3.2). The differences in behaviour of the endoglucanase from the different sources probably reflects inherent differences in the enzymes (section 1.3.1).

It is important to remember that, whilst most enzymes are inactivated following adsorption to smectite clays some enzymes show residual activity, e.g. glucose oxidase (Morgan and Corke, 1976), amylase (Filip, 1973) and catalase (Stotzky, 1974). Using the barrier ring diffusion plates it was not easy to assess whether the endoglucanase adsorbed to the bentonite retained activity or if it was reversibly or irreversibly inactivated. This was because of difficulties in assaying low levels of enzyme activity in the presence of bentonite. However, Lynch and Cotnoir (1956) reported a reduction in the endoglucanase activity of a commercial cellulase preparation following addition of montmorillonite. They estimated endoglucanase activity by monitoring changes in the effluent time (i.e. viscosity) of a CMC solution from a pipette, a method which precludes the expression of activity as a percentage of the original. However, even this more conventional system will not reveal whether the reduced activity is a result of enzyme inactivation or of substrate being adsorbed and rendered unavailable for hydrolysis. Smectite clays are known to adsorb both cellulose dextrin and soluble cellulose derivatives (Lynch *et al.*, 1956).

The outward diffusion of β-D-glucosidase at concentrations up to 100 μg 100 $\mu\ell^{-1}$ was prevented by the inclusion of 1% (w/v) bentonite (= 15 mg of evenly distributed bentonite) or 1 g of soil in the barrier ring. The adsorptive capacity of the same bentonite for β-D-glucosidase is known to be *ca.* 10 mg $g^{-1}$ (J.M. Sarkar, U.K.C., personal communication). Barrier rings containing 15 mg bentonite would be expected therefore to adsorb a maximum of 150 μg β-D-glucosidase. However, the adsorptive

capacity of bentonite and other soil colloids for β-D-glucosidase is dependent on the pH, the ionic strength of the medium and the nature of the resident surface ions. Therefore, adsorption studies using the same media as used in the diffusion plates and under the same physicochemical conditions must be performed before a detailed comparison is made.

Barrier ring diffusion plates provide a versatile technique which could be adapted to investigate, for example:

[1] a range of extracellular enzyme - macromolecular substrate interactions, such as those involving the decomposition of hemicellulose, chitin, starch and pectin;

[2] the interactions between plants and the soil microbial (i.e. rhizosphere) populations, whereby the influence of different soils and soil components on the development of the rhizosphere could be investigated by monitoring the effect of plant root exudates, added to the central well, on the growth and metabolism of individual species and communities of microorganisms inoculated outside the barrier ring; and

[3] the influence of different soil components on the metabolic and chemotactic responses of specific groups of microorganisms e.g. *Rhizobium* spp. to leguminous plants or phytopathogenic microorganisms.

It is envisaged that the technique could be used in conjunction with conventional adsorption studies since it allows the experimenter to investigate how soils and soil components impede the diffusion of 'free' enzymes. This 'impedance' value, although obviously related to

the adsorptive and desorptive capacity of the soil or soil component will, in addition be related to pore size. [Particles diffusing through pores of diameter less than 10x that of the particle will experience a drag effect (P. Nye, Oxford University, personal communication)]. Furthermore, it has been suggested (Low, 1962) that the orderliness of the water molecules adjacent to clay surfaces form a quasi-crystalline structure with a viscosity greater than that of normal water which would thus retard solute (e.g. enzyme) diffusion. The advantages of the technique over conventional adsorption studies include the potential for investigating realistic soil conditions in the absence of slurry conditions. However, the main feature of the technique is that it facilitates the study of the spatial interactions between microorganisms, enzymes, substrates and effector molecules in a soil-like but carefully controlled environment.

The potential exists for improving the sensitivity of the barrier ring diffusion plate technique by reducing the scale of the gels to that used in immunodiffusion studies (i.e. reducing the thickness of gel, well size and thickness of the barrier ring). However, the smaller the size of the gel the more difficult becomes the manipulations required to introduce the various barrier rings. One of the main reasons for using relatively thick gels in Petri dishes was to allow us to compare the results directly with those from experiments in which microbial growth over various barrier rings was observed. [With thin gels, despite a humid incubator, the plates tended to dry out before the colonies had grown large enough for the required growth rate determinations to be made].

The observations made on three fungal isolates and a *Streptomyces* sp. have demonstrated how soil components differentially affect the radial growth of soil microorganisms. Bentonite when present in the barrier ring reduced the radial growth rate (rgr) of both the *Streptomyces* sp. and the two *Trichoderma* spp. while the radial growth of *Botryotrichum piluliferum* was stimulated (section 5.3.2). The most likely explanation for the observed changes in rgr is a depletion of sugars because, not only did the bentonite adsorb the cellulolytic enzymes, but also any soluble sugars produced from CMC hydrolysis (section 5.3.1). However, it must be remembered that there are a number of other ways in which clay minerals can affect microbial growth e.g. by adsorbing toxic metabolites, by acting as a source of nutrients, by altering the pH (section 3.3.3i) and by serving as a buffer (Marshman and Marshall, 1981). In addition to its effect on radial growth, whole soil in the barrier ring affected the hyphal density of the colonies (section 5.3.2i). Changes in hyphal density were not quantified because time was limited and because the environmental and genetic factors which influence colony morphology and radial growth are only poorly understood. Gull (1975) investigated branching in *Thamnidium elegans* and provided evidence for a definite and consistent pattern of branching. It has been suggested that an internal control of branch pattern may be exerted due to the existence of a maximum rate of hyphal-tip extension (Katz *et al.*, 1972) while an alternative hypothesis is that branch pattern is influenced by the concentration gradients of particular metabolites in the immediate external environment of the colony (Robinson, 1978). Because of this uncertainty, results obtained from the growth experiments both on barrier ring plates and in response to glucose gradients must be interpreted with caution. The use of cellulase preparations adsorbed to filter paper provides a useful method for producing and maintaining a glucose gradient and may have applications in the study of chemotactic

and chemotropic responses in organisms other than those which are cellulolytic.

A colony of *Sporocytophaga myxococcoides* was unable to spread across a gap in the filter paper of greater than 2 mm. This was surprising as many strains of the genus *Cytophaga*, to which the *Sporocytophaga* are closely related, show a marked ability to spread rapidly over the surface of agar plates. However, there are some *Cytophaga* strains that form compact colonies which never show spreading ability or else spread only under certain nutrient conditions (Christensen, 1977). Barrier ring plates could be used with these organisms to investigate the effect of different soils and soil components on their spreading ( = growth and motility) ability. Furthermore, on contaminated Avicel plates, which were originally inoculated with a cellulolytic fungus, bacteria were seen in close association with the extending fungal hyphae and it is probable that, in a mixed culture study, *S. myxococcoides* (and other non-motile bacteria) would be carried across the gap in the filter paper by the fungal hyphae.

With regards to our second and third objectives : β-D-glucosidase activity in the silt loam Hamble Series soil was measured and characterized by J.M. Alexander and R.J. Geraghty in this laboratory and shown to be independent of microbial proliferation. The assay used was adapted from that used for soil phosphatase (T.W. Speir, Soil Bureau N.Z., personal communication). The β-D-glucosidase activity, which was resistant to added protease and largely unaffected by air-drying, was suspected as being bound to the soil colloids. This was confirmed when about 10% of the original stable activity was detected in a filter-sterilized humic-enzyme extract and was shown to have properties similar to those of the whole soil activity. As a result, we have been able to describe a proportion of the β-D-glucosidase activity in Hamble soil as immobilized

on or within the humic colloids and hence as an enzyme belonging to categories 9 and 10 of enzymes active in soil (Burns, 1982b - section 1.4.4).

A combined exo- and endoglucanase activity was measured in Hamble soil using Avicel as the microcrystalline cellulose substrate. The difficulties and limitations inherent in the measurement of cellulase activity have been previously discussed (section 2.1.2). Because there is no unified model to describe the kinetics of the cellulase-cellulose system we have not attempted a kinetic analysis of the soil cellulase activities. However, the assay we developed does allow us to express our results in standard units. The combined exo- and endoglucanase activity measured in field-moist Hamble soil (*ca.* 110 nmol reducing sugars as glucose equivalents (g dry wt)$^{-1}$ h$^{-1}$ at 40°C) is broadly comparable to that reported by Benefield (1971) for an agricultural soil (3 200 nmol glucose g$^{-1}$ 48h$^{-1}$ at 50°C) and that reported by Ross and Speir (1978) for two silt loam New Zealand soils (25.2 and 50.4 nmol reducing sugars as glucose equivalents (g dry wt)$^{-1}$ h$^{-1}$ at 30°C). A 60 - 70% drop in the combined exo and endoglucanase activity was observed following air-drying and storage (section 3.2.4ii) which contrasts with the 10 - 15% drop (R.G. Geraghty, M.Sc. thesis, U.K.C., 1983) in β-D-glucosidase activity observed following identical treatment. Enzymes immobilized on or within soil colloids are able to withstand air-drying (Lethbridge, 1978). Therefore, the large decrease in exo- and endoglucanase activity observed on air-drying Hamble soil suggests that a significant percentage of the activity measured in field-moist soil is either associated with soil microorganisms which are not resistant to air-drying, or free in the soil solution. The residual activity in air-dried soil was resistant to added protease applied at levels high enough to inactivate a *Trichoderma viride* commercial cellulase preparation.

The level of endoglucanase activity alone, measured in air-dried Hamble soil was *ca.* 3x that of the combined exo- and endoglucanase activity. Measurements of the different activities in culture filtrates from cellulolytic microorganisms invariably show higher activity against CMC than insoluble cellulose derivatives (section 4.2.1), while many microorganisms are able to produce endoglucanases but not exoglucanases (section 1.2.2).

In previous work (Lethbridge, 1978; Pettit, 1978) the location of enzymes in soil has been deduced from comparative studies between those activities found in air-dried soil and those of commercial enzyme preparations. The increased resistance of the enzyme activities in whole soil and humic extracts to long-term storage, gamma irradiation, temperature inactivation, and proteolytic attack when compared with the commercial enzyme activities led these workers to conclude that the enzyme activities being measured (i.e. urease and β-1,3-glucanase) were immobilized on or within the soil humic colloids and belonged to categories 9 and 10 of enzymes active in soil (section 1.4.4).

With the combined exo- and endoglucanase activity; the low level of activity measured in air-dried soil (*ca.* 35 nmol reducing sugars as glucose equivalents (g dry wt)$^{-1}$ h$^{-1}$) as compared to β-1,3-glucanase (0.4 μmol reducing sugars as glucose equivalents (g dry wt)$^{-1}$ h$^{-1}$ - Lethbridge, 1978) combined with the stability and variety (Goksoeyr *et al.*, 1975) of 'free' extracellular endoglucanases (section 1.2.1) makes deductions concerning the location of these enzymes in soil difficult. The fact that 30% of the endoglucanase activity from a *Trichoderma viride* cellulase preparation could be detected 21 days after its addition to soil clearly illustrates the ability of some endoglucanases to withstand inactivation due to adsorption, denaturation and degradation. Drozdowicz (1971) measured both endoglucanase and the

combined exo- and endoglucanase activity of an *Aspergillus niger* commercial cellulase following its addition to soil. He found that while endoglucanase activity declined slowly over a 40 day period the combined exo- and endoglucanase activity of the added preparation disappeared within 24 h. In studies where the in vitro stability of the different components of the cellulase complex have been investigated (e.g. Mandels, 1975; Hagerdal *et al.*, 1980) it appears that the activity of the endoglucanase component has greater stability that that of the combined exo- and endoglucanase activity. The comparative instability of the combined exo- and endoglucanase activity most likely reflects the instability of the exoglucanase component. However, measurements of purified exoglucanase are needed before this can be confirmed.

The hydrolysis of insoluble substrates by immobilized enzymes poses certain conceptual difficulties in terms of substrate - enzyme interaction although the colloidal nature and hence mobility of the enzyme support goes some way towards overcoming these difficulties. With this in mind, the results presented in this thesis lead us to propose that the major portion of the cellulase (combined exo- and endoglucanase) activity in field-moist soil is 'free' in the soil solution (categories 4 and 7 - section 1.4.4), attached to the outer surfaces of cellulolytic microorganisms (categories 3 and 6), or associated in enzyme - substrate complexes (category 8). The residual activity measured in air-dried soil may owe its stability to an association with the soil colloids. However, unlike the β-D-glucosidase activity characterized in Hamble soil, we do not believe that the cellulase activity in field-moist soil includes enzymes which are <u>permanently</u> entrapped by or co-polymerized with the humic material (category 10). Without a further characterization of the soil endoglucanase activity (in terms of pH-activity optimum,

thermostability and the examination of an active humic-endoglucanase extract) it is not possible to define the location of this activity in soil. However, it is likely that the initial response of soil to cellulose additions is very much a function of the extant microbial population rather than due to the soil immobilized enzyme fraction. However, it appears that a significant proportion of the soil β-D-glucosidase activity belongs to this latter immobilized fraction and is likely to be important in the hydrolysis of cellobiose to glucose.

The microbial decomposition of cellulose is in itself a complex process and the necessity for cellulolytic microorganisms to employ extracellular enzymes adds another dimension to the problems facing these organisms living in soil. The results presented in this thesis do not allow a full evaluation of the hypothesis that soil-immobilized cellulase enzymes play an ecological role in the decomposition of insoluble substrates by mediating the induction of microbial extracellular enzymes or stimulating a chemotactic response (section 5.1). The cellulase-cellulose system is not an ideal system to study due to the complexity of the enzyme interactions, the absence of definitive assays for the individual enzyme components, and the low levels of immobilized exo- and endoglucanase activity. Another problem with the cellulase-cellulose system is the uncertainty as to the natural inducer of the cellulase enzymes in different microorganisms (section 1.3.3). It has been proposed (Gritzali and Brown, 1979; Vaheri *et al.*, 1979) that β-D-glucosidase transglycosylation products play a role in the induction of the cellulase enzymes in *Trichoderma viride*, and it is possible that products of a soil immobilized β-D-glucosidase - substrate catalysis act as inducers of cellulase enzymes in soil. This could be shown by studying any transglycosylation products on the soil β-D-glucosidase in terms of their ability to diffuse away from the site of production and induce soil microbial cellulases.

As has been previously stated, there are very few techniques for the direct investigation of the relative importance of the various enzyme fractions in soil. One way to circumvent this problem is to use model soil systems to assess the contribution of the individual enzyme fractions. The model system used in this work has proved useful in investigating the scavenging ability (in terms of resisting inactivation and locating substrate) of various enzymes. Bentonite and whole soil were shown to impede the diffusion of endoglucanase from both fungal and actinomycete sources while the study of β-D-glucosidase diffusion demonstrates how the technique can be extended to study enzymes whose substrates are soluble and of low molecular weight. There remain unresolved problems in the application of the barrier ring technique to the study of filamentous microbial growth through various soil components and in response to substrate gradients produced by immobilized enzymes. These problems arise because of our as yet, poor understanding of the factors which influence colony morphology. Furthermore, it must be remembered that the purified cellulose used in these experiments represents a gross oversimplification. In the environment cellulose is associated with other polymeric organic materials such as hemicellulose, pectin and lignin. Hydrolysis of the cellulose component of organic substrates will therefore be an integrated component of the overall mineralization of substrate by mixed microbial communities (Burns, 1982a). The use of simplified model systems such as the barrier ring plates can however be justified in the absence of the means to study the in situ decomposition process in any detail. Through the use of model systems a view of the overall process may be constructed from an investigation of the component parts and their interactions with one another.

# APPENDIX I

## MICROBIOLOGICAL MEDIA

1) Berg Minimal Media (Berg *et al.*, 1972a)

| | |
|---|---|
| $NaNO_3$ | 2.0g |
| $K_2HPO_4$ | 0.5g |
| $MgSO_4.7H_2O$ | 0.2g |
| $CaCl_2.2H_2O$ | 0.02g |
| $MnSO_4.H_2O$ | 0.02g |
| $FeSO_4.7H_2O$ | 0.02g |

Distilled water ⟶ 1ℓ
The pH after autoclaving (121°C, 20min) was 7.5

2) Ishaque Minimal Media (from Ishaque and Kluepfel, 1980)

| | |
|---|---|
| $KH_2PO_4$ | 1.5g |
| $K_2HPO_4$ | 2.0g |
| $(NH_4)_2SO_4$ | 1.4g |
| Yeast extract (Difco) | 2.0g |
| Proteose peptone (Difco) | 1.0g |
| Tween 80 | 2.0ml |
| $FeSO_4.7H_2O$ | 5.0mg |
| $MnSO_4.H_2O$ | 1.6mg |
| $CoCl_2$ | 2.0mg |
| $ZnSO_4$ | 2.0mg |

Distilled water ⟶ 1ℓ
After autoclaving (121°C, 20min)

| | |
|---|---|
| $MgSO_4.7H_2O$ | 30mg |
| $CaCl_2.2H_2O$ | 30mg |

3) Phage (Ø) buffer

| | |
|---|---|
| $Na_2HPO_4$ | 7g |
| $KH_2PO_4$ | 3g |
| NaCl | 5g |

Distilled water to 1ℓ

4) Sternberg Minimal Media (Sternberg, 1976)

| | |
|---|---|
| $(NH_4)_2SO_4$ | 1.4g |
| $KH_2PO_4$ | 2.0g |
| Urea | 0.3g |
| $CaCl_2$ | 0.3g |
| $MgSO_4$ | 0.3g |
| $FeSO_4.7H_2O$ | 5.0mg |
| $MnSO_4.H_2O$ | 1.6mg |
| $CoCl_2$ | 2.0mg |
| $ZnSO_2$ | 2.0mg |

Distilled water to 1ℓ

| | | |
|---|---|---|
| Proteose peptone | 0.075% | (omitted in diffusion and growth |
| Tween 80 | 0.2% | plates chapter 4 and 5) |

The pH after autoclaving (121°C, 20min) was 4.6

## APPENDIX II

### Statistical Analysis of Radial Growth Measurements

The statistical methods employed here follow from discussions with E. Henness, ARC Letcombe Laboratory, Wantage, Oxon, and with Dr. Bryon Morgan, Statistics Dept., UKC. Regression analysis of the colony diameter measurements (y) against time(x) were obtained using the Minitab release 81.1 Copyright - Penn State University, 2900 series version, on the ICL 2960 main frame computer.

To compare the two regression lines

$$y = \beta_1 x + \alpha_1$$

$$y = \beta_2 x + \alpha_2$$

obtained from $n_1$ and $n_2$ observations, a combined estimate of $\hat{\sigma}^2$ (population variance) was obtained from the sums of the squares Dev $SS_1$ (y,x) and Dev $SS_2$ (y,x) using the equation

$$\hat{\sigma}^2 = \frac{\text{Dev } SS_1 \text{ (y,x)} + \text{Dev } SS_2 \text{ (y,x)}}{(n_1-2 + n_2-2)}$$

$$\text{Then } V(\beta_1 - \beta_2) = \hat{\sigma}^2 \left\{ \frac{1}{CS_1(x,x)} + \frac{1}{(CS_2(x,x)} \right\}$$

where CS = total sum of squares for x.

A test of significance for $\beta_1 = \beta_2$ was provided by comparison of:

$$t = \frac{\beta_1 - \beta_2}{\sqrt{V(\beta_1-\beta_2)}}$$ with a t-distribution for $n_1 + n_2 - 4$ d.f.

Given that the lines being compared were parallel the intercept values ($\alpha$) were compared using the equation

$$t = \frac{\alpha_1 - \alpha_2}{\sqrt{\frac{(SE_{\alpha 1})^2 + (SE_{\alpha 2})^2}{2}}} \qquad \text{for } n_1 + n_2 - 4 \text{ d.f.}$$

EXAMPLE 1

From Fig. 5.4

The t values obtained from comparing the gradients of the lines were:

for BM vs 0.5% CSF t = 2.239 26 d.f.

for BM vs 10% CSF t = 8.522 26 d.f.

for 0.5% CSF 10% CSF t = 7.575 26 d.f.

all of which are significant at the $\alpha = 0.025$ level.

EXAMPLE 2

From Fig. 5.5

The t values obtained from comparing the gradients of the lines were:

for BM vs 10% CSF t = 0.258 28 d.f.

which is not significant at the $\alpha = 0.1$ level.

Comparison of $\alpha_1$ and $\alpha_2$ for BM and 10% CSF gives t = 1.640 28 d.f.

which is significant at the $\alpha = 0.1$ level.

ANALYSIS OF RADIAL GROWTH MEASUREMENTS OF ACT 9 ON CSF BARRIER RING PLATES (FIGURE 5.4)
C10 = TIME(WEEKS)
C1 = CONTROL
C2 = 0.5%W/V CSF
C3 = 10%W/V CSF
T#CTM0001 is a copy of CUR088.CTMY TTYROUTE
-- PRINT C1-C3, C10

| COLUMN | C1 | C2 | C3 | C10 |
|---|---|---|---|---|
| COUNT | 15 | 15 | 15 | 15 |
| ROW | | | | |
| 1 | 23.3000 | 30.2000 | 32.2000 | 4. |
| 2 | 24.2000 | 30.0000 | 32.5000 | 4. |
| 3 | 24.1000 | 29.5000 | 32.7000 | 4. |
| 4 | 29.9000 | 36.9000 | 39.5000 | 5. |
| 5 | 29.9000 | 37.0000 | 40.6000 | 5. |
| 6 | 30.2000 | 36.8000 | 40.5000 | 5. |
| 7 | 35.8000 | 43.5000 | 47.9000 | 6. |
| 8 | 36.0000 | 43.0000 | 48.7000 | 6. |
| 9 | 35.7000 | 43.1000 | 47.6000 | 6. |
| 10 | 43.5000 | 51.0000 | 56.5000 | 7. |
| 11 | 43.1000 | 49.6000 | 56.9000 | 7. |
| 12 | 43.0000 | 49.8000 | 56.8000 | 7. |
| 13 | 51.0000 | 58.0000 | 62.7000 | 8. |
| 14 | 48.6000 | 57.0000 | 64.1000 | 8. |
| 15 | 49.5000 | 57.1000 | 65.4000 | 8. |

REGRESS C1 1 C10

THE REGRESSION EQUATION IS
Y = - 2.40 + 6.49 X1

| | COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|---|
| | -- | -2.4002 | 0.7412 | -3.24 |
| X1 | C10 | 6.4867 | 0.1202 | 53.95 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 0.6586
WITH ( 15- 2) = 13 DEGREES OF FREEDOM

R-SQUARED = 99.6 PERCENT
R-SQUARED = 99.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF |
|---|---|---|---|
| REGRESSION | 1 | 1262.318 | 1262.318 |
| RESIDUAL | 13 | 5.639 | 0.434 |
| TOTAL | 14 | 1267.957 | |

| ROW | X1 C10 | Y C1 | PRED. Y VALUE | ST.DEV. PRED. Y | RESIDUAL | ST.RES. |
|---|---|---|---|---|---|---|
| 13 | 8.00 | 51.000 | 49.493 | 0.295 | 1.507 | 2.56R |

R DENOTES AN OBS. WITH A LARGE ST. RES.

DURBIN-WATSON STATISTIC = 2.31

-- REGRESS C2 1 C10

```
THE REGRESSION EQUATION IS
Y =    2.60 +  6.82 X1

                                          ST. DEV.     T-RATIO =
       COLUMN      COEFFICIENT          OF COEF.     COEF/S.D.
       --              2.5998            0.5271          4.93
X1     C10             6.81669           0.08551        79.72

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 0.4684
WITH (  15- 2) =  13 DEGREES OF FREEDOM

R-SQUARED = 99.8 PERCENT
R-SQUARED = 99.8 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1      1394.026        1394.026
RESIDUAL     13         2.852           0.219
TOTAL        14      1396.878

            X1           Y     PRED. Y    ST.DEV.
ROW        C10          C2       VALUE   PRED. Y   RESIDUAL      ST.RES.
 13       8.00      58.000      57.133     0.209      0.867        2.07R

R DENOTES AN OBS. WITH A LARGE ST. RES.

DURBIN-WATSON STATISTIC = 2.47
-- REGRESS C3 1 C10

THE REGRESSION EQUATION IS
Y =   0.466 +  7.97 X1

                                          ST. DEV.     T-RATIO =
       COLUMN      COEFFICIENT          OF COEF.     COEF/S.D.
       --              0.4664            0.7790          0.60
X1     C10             7.9734            0.1264         63.10

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 0.6922
WITH (  15- 2) =  13 DEGREES OF FREEDOM

R-SQUARED = 99.7 PERCENT
R-SQUARED = 99.6 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS        MS=SS/DF
REGRESSION    1      1907.245        1907.245
RESIDUAL     13         6.228           0.479
TOTAL        14      1913.473

            X1           Y     PRED. Y    ST.DEV.
ROW        C10          C3       VALUE   PRED. Y   RESIDUAL      ST.RES.
 13       8.00      62.700      64.253     0.310     -1.553       -2.51R

R DENOTES AN OBS. WITH A LARGE ST. RES.

DURBIN-WATSON STATISTIC = 2.23
```

```
-- REGRESS C10 1 C1

THE REGRESSION EQUATION IS
Y =   0.395 + 0.153 X1

                                          ST. DEV.     T-RATIO =
       COLUMN      COEFFICIENT          OF COEF.     COEF/S.D.
       --             0.3951             0.1071          3.69
X1     C1           0.153476           0.002845         53.95

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 0.1013
WITH (  15- 2) =  13 DEGREES OF FREEDOM

R-SQUARED = 99.6 PERCENT
R-SQUARED = 99.5 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

 DUE TO      DF           SS       MS=SS/DF
REGRESSION    1      29.86664       29.86664
RESIDUAL     13       0.13341        0.01026
TOTAL        14      30.00005

           X1           Y     PRED. Y    ST.DEV.
ROW        C1         C10      VALUE    PRED. Y   RESIDUAL     ST.RES.
 13      51.0      8.0000     8.2223     0.0488    -0.2223      -2.50R

R DENOTES AN OBS. WITH A LARGE ST. RES.

DURBIN-WATSON STATISTIC = 2.29
```

ANALYSIS OF RADIAL GROWTH MEASUREMENTS OF ACT 9 ON CSF(LESS ORGANIC MATTER) BARRIER RING PLATES (FIGURE 5.5)
C10 = TIME (WEEKS)
C2 = CONTROL
C3 = 0.5%W/V CSF
C5 = 10%W/V CSF
-- PRINT C2,C3,C5,C10

| COLUMN | C2 | C3 | C5 | C10 |
|---|---|---|---|---|
| COUNT | 16 | 16 | 16 | 16 |
| ROW | | | | |
| 1 | 36.8000 | 36.1000 | 34.0000 | 5. |
| 2 | 36.0000 | 35.6000 | 32.9000 | 5. |
| 3 | 35.7000 | 36.9000 | 33.4000 | 5. |
| 4 | 34.1000 | 36.3000 | 32.5000 | 5. |
| 5 | 41.4000 | 44.6000 | 41.6000 | 6. |
| 6 | 43.1000 | 43.4000 | 41.0000 | 6. |
| 7 | 42.9000 | 44.5000 | 40.5000 | 6. |
| 8 | 44.8000 | 44.8000 | 40.5000 | 6. |
| 9 | 47.8000 | 50.1000 | 47.9000 | 7. |
| 10 | 48.5000 | 48.3000 | 47.6000 | 7. |
| 11 | 48.7000 | 49.8000 | 45.9000 | 7. |
| 12 | 49.6000 | 50.3000 | 45.4000 | 7. |
| 13 | 54.7000 | 55.3000 | 55.6000 | 8. |
| 14 | 57.9000 | 55.1000 | 54.7000 | 8. |
| 15 | 54.8000 | 56.0000 | 52.7000 | 8. |
| 16 | 57.5000 | 58.1000 | 53.1000 | 8. |

-- REGRESS C2 1 C10

THE REGRESSION EQUATION IS
Y = 2.13 + 6.73 X1

| | COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|---|
| | -- | 2.132 | 1.889 | 1.13 |
| X1 | C10 | 6.7325 | 0.2865 | 23.50 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 1.281
WITH ( 16- 2) = 14 DEGREES OF FREEDOM

R-SQUARED = 97.5 PERCENT
R-SQUARED = 97.4 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF |
|---|---|---|---|
| REGRESSION | 1 | 906.547 | 906.547 |
| RESIDUAL | 14 | 22.978 | 1.641 |
| TOTAL | 15 | 929.526 | |

DURBIN-WATSON STATISTIC = 2.40
-- REGRESS C3 1 C10

THE REGRESSION EQUATION IS
Y = 4.32 + 6.50 X1

| | COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|---|
| | -- | 4.325 | 1.573 | 2.75 |
| X1 | C10 | 6.5000 | 0.2385 | 27.25 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 1.067
WITH ( 16- 2) = 14 DEGREES OF FREEDOM

R-SQUARED = 98.1 PERCENT
R-SQUARED = 98.0 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF |
|---|---|---|---|
| REGRESSION | 1 | 845.016 | 845.016 |
| RESIDUAL | 14 | 15.930 | 1.138 |
| TOTAL | 15 | 860.947 | |

DURBIN-WATSON STATISTIC = 1.44
-- REGRESS C5 1 C10

THE REGRESSION EQUATION IS
Y = - 0.673 + 6.83 X1

| | COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|---|
| | -- | -0.673 | 1.511 | -0.45 |
| X1 | C10 | 6.8275 | 0.2291 | 29.80 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS
S = 1.024
WITH ( 16- 2) = 14 DEGREES OF FREEDOM

R-SQUARED = 98.4 PERCENT
R-SQUARED = 98.3 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF |
|---|---|---|---|
| REGRESSION | 1 | 932.312 | 932.312 |
| RESIDUAL | 14 | 14.694 | 1.050 |
| TOTAL | 15 | 947.005 | |

DURBIN-WATSON STATISTIC = 1.90
-- REGRESS C10 1 C1

THE REGRESSION EQUATION IS
Y = - 0.402 + 0.151 X1

| | COLUMN | COEFFICIENT | ST. DEV. OF COEF. | T-RATIO = COEF/S.D. |
|---|---|---|---|---|
| | -- | -0.4022 | 0.2539 | -1.58 |
| X1 | C1 | 0.150764 | 0.005477 | 27.53 |

THE ST. DEV. OF Y ABOUT REGRESSION LINE IS

S = 0.1610
WITH ( 16- 2) = 14 DEGREES OF FREEDOM

R-SQUARED = 98.2 PERCENT
R-SQUARED = 98.1 PERCENT, ADJUSTED FOR D.F.

ANALYSIS OF VARIANCE

| DUE TO | DF | SS | MS=SS/DF |
|---|---|---|---|
| REGRESSION | 1 | 19.63726 | 19.63726 |
| RESIDUAL | 14 | 0.36279 | 0.02591 |
| TOTAL | 15 | 20.00003 | |

DURBIN-WATSON STATISTIC = 2.10

ALEXANDER, M. (1977). *Introduction to Soil Microbiology*, 2nd Edition. New York : John Wiley and Sons.

ALMIN, K.E. and ERIKSSON, K-E. (1968). Influence of carboxymethyl cellulose properties on the determination of cellulase activity in absolute terms. *Archives of Biochemistry and Biophysics* 124, 129-134.

ALMIN, K.E., ERIKSSON, K-E. and JANSSON, C. (1967). Enzymatic Degradation of Polymers. II Viscometric determination of cellulase activity in absolute terms. *Biochimica et Biophysica Acta* 139, 248-253.

AMBROŽ, Z. (1973). On the study of the cellulase complex in soils. *Rostlinna Vyroba* (Praha) 19, 207-212.

AMEMURA, A. and TERUI, G. (1965). Studies on Fungal Cellulases. II kinetics of cellulolytic action of *Penicillium variabile* cellulase. *Journal of Fermentation Technology* 43, 281-285.

ANDERSON, J.P.E. and DOMSCH, K.H. (1975). Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. *Canadian Journal of Microbiology* 21, 314-322.

ATLAS, R. (1982). Enumeration and estimation of microbial biomass. In *Experimental Microbial Ecology*, pp. 84-102. Edited by R.G. Burns and J.H. Slater. Oxford : Blackwell Scientific Publications.

ATLAS, R.M. and BARTHA, R. (1981). *Microbial Ecology : Fundamentals and Applications*. Reading, Massachusetts : Addison-Wesley Publishing Company.

AVERY, B.W. and BASCOMB, C.L. (1974). *Soil Survey Laboratory Methods*. Soil survey technical monograph No.6 Rothamstead Experimental Station, Harpenden, Herts.

BAECKER, A.A.W. and KING, B. (1980). Decay of wood by actinomycetales. In *Biodeterioration*, pp. 53-58. Edited by T.A. Oxley, G. Becker and D. Allsopp. London : Pitman.

BAILEY, R.W. and PRIDHAM, J.B. (1962). The separation and identification of oligosaccharides. *Chromatography Reviews* 4, 114-136.

BASCOMB, C.L. (1974). Physical and chemical analyses of <2 mm samples. In *Soil Survey Laboratory Methods*, p. 5. Edited by B.W. Avery and C.L. Bascomb. Soil survey technical monograph No.6. Rothamstead Experimental Station, Harpenden, Herts.

BARRAS, D.R., MOORE, A.E. and STONE, B.A. (1969). Enzyme-substrate relationships among β-glucan hydrolases. *Advances in Chemistry Series* 95, 105-138.

BATISTIC, L., SARKAR, J.M. and MAYAUDON, J. (1980). Extraction, purification and properties of soil hydrolases. *Soil Biology and Biochemistry* 12, 59-63.

BÉGUIN, P. and EISEN, H. (1978). Purification and partial characterization of three extracellular cellulases from *Cellulomonas* sp. *European Journal of Biochemistry* 87, 525-531.

BÉGUIN, P., EISEN, H. and ROUPAS, A. (1977). Free and cellulose-bound cellulases in *Cellulomonas* species. *Journal of General Microbiology* 101, 191-196.

BENEFIELD, C.B. (1971). A rapid method for measuring cellulase activity in soils. *Soil Biology and Biochemistry* 3, 325-329.

BERG, B. (1975). Cellulase location in *Cellvibrio fulvus*. *Canadian Journal of Microbiology* 21, 51-57.

BERG, B. and v. HOFSTEN, B. (1976). The ultrastructure of the fungus *Trichoderma viride* and investigation of its growth on cellulose. *Journal of Applied Bacteriology* 41, 395-399.

BERG, B., v. HOFSTEN, B. and PETTERSSON, G. (1972a). Growth and cellulase formation by *Cellvibrio fulvus*. *Journal of Applied Bacteriology* 35, 201-214.

BERG, B.,v. HOFSTEN, B. and PETTERSSON, G. (1972b). Electronmicroscopic observations on the degradation of cellulose fibres by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. *Journal of Applied Bacteriology* 35, 215-219.

BERG, B. and PETTERSSON, G. (1977). Location and formation of cellulases in *Trichoderma viride*. *Journal of Applied Bacteriology* 42, 65-75.

BERGHEM, L.E.R. and PETTERSSON, L.G. (1973). The Mechanisms of Enzymatic Cellulose Degradation. Purification of a cellulolytic enzyme from *Trichoderma viride* active on highly ordered cellulose. *European Journal of Biochemistry* 37, 21-30.

BERGHEM, L.E.R., PETTERSSON, L.G. and AXIO-FREDRIKSSON, U-B. (1975). The Mechanism of Enzymatic Cellulose Degradation. Characterization and enzymatic properties of a β-1,4-glucan cellobiohydrolase from *Trichoderma viride*. *European Journal of Biochemistry* 53, 55-62.

BERGHEM, L.E.R., PETTERSSON, L.G. and AXIO-FREDRIKSSON, U-B. (1976). The Mechanism of Enzymatic Cellulose Degradation. Purification and properties of two different 1,4-β-glucan glucanohydrolases from *Trichoderma viride*. *European Journal of Biochemistry* 61, 621-630.

BRINK, R.H.Jr., DUBACH, P. and LYNCH, D.L. (1960). Measurements of carbohydrates in soil hydrolysates with anthrone. *Soil Science* 89, 157-166.

BURNS, R.G. (1978). Enzymes in soil : some theoretical and practical considerations. In *Soil Enzymes*, pp. 295-339. Edited by R.G. Burns. London and New York : Academic Press.

BURNS, R.G. (1979). Interactions of microorganisms, their substrates and their products with soil surfaces. In *Adhesion of Microorganisms to Surfaces*, pp. 109-138. Edited by D.C. Ellwood, J. Melling and P. Rutter. London : Academic Press.

BURNS, R.G. (1980). Microbial adhesion to soil surfaces : consequences for growth and enzyme activities. In *Microbial Adhesion to Surfaces*, pp. 249-262. Edited by R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent. Chichester : Ellis Horwood.

BURNS, R.G. (1982a). Carbon mineralization by mixed cultures. In *Microbial Interactions and Communities*, pp. 473-541. Edited by A.T. Bull and J.H. Slater. London : Academic Press.

BURNS, R.G. (1982b). Enzyme activity in soil : location and a possible role in microbial ecology. *Soil Biology and Biochemistry* 14, 423-427.

BURNS, R.G. (1983). Extracellular enzyme-substrate interactions in soil. In *Microbes in their Natural Environments*. Symposium 34, Society for General Microbiology, pp. 249-298. Edited by J.H. Slater, R. Whittenbury, and J.W.T. Wimpenny. Cambridge : University Press.

BURNS, R.G., EL-SAYED, M.H. and McLAREN, A.D. (1972). Extraction of an urease-active organo-complex from soil. *Soil Biology and Biochemistry* 4, 107-108.

CANEVASCINI, G., COUDRAY, M.R., REY, J.P., SOUTHGATE, R.J.G. and MEIER, H. (1979). Induction and catabolite repression of cellulase synthesis in the thermophilic fungus *Sporotrichum thermophile*. *Journal of General Microbiology* 110, 291-303.

CANEVASCINI, G. and MEYER, H-P. (1979). β-Glucosidase in the cellulolytic fungus *Sporotrichum thermophile* Apinis. *Experimental Mycology* 3, 203-214.

CHAHAL, D.S. and WANG, D.I.C. (1978). *Chaetomium cellulolyticum*, growth and behaviour on cellulose and protein production. *Mycologia* 70, 160-170.

CHANG, W. and THAYER, D.W. (1977). The cellulase system of a *Cytophaga* species. *Canadian Journal of Microbiology* 23, 1285-1292.

CHESHIRE, M.V., MUNDIE, C.M. and SHEPHERD, H. (1973). The origin of soil polysaccharide : Transformation of sugars during the decomposition in soil of plant material labelled with carbon-14. *Journal of Soil Science* 24, 54-68.

CHILD, J.J., EVELEIGH, D.E. and SIEBEN, A.S. (1973). Determination of cellulase activity using hydroxyethylcellulose as a substrate. *Canadian Journal of Biochemistry* 51, 39-43.

CHRISTENSEN, P.J. (1977). The history, biology and taxonomy of the *Cytophaga* group. *Canadian Journal of Microbiology* 23, 1599-1653.

COOPER, K.E. (1972). The theory of antibiotic diffusion zones. In *Analytical Microbiology*, Vol II, pp. 13-42. Edited by F. Kavanagh. New York and London : Academic Press.

COWLING, E.B. and KIRK, T.K. (1976). Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes. *Biotechnology and Bioengineering Symposium* 6, 95-123.

CRAWFORD, D.L. and SUTHERLAND, J.B. (1979). The role of actinomycetes in the decomposition of lignocellulose. *Developments in Industrial Microbiology* 20, 143-151.

DANIELS, J. (1961). *Chaetomium piluliferum* sp. nov. The perfect state of *Botryotrichum piluliferum*. *Transactions of the British Mycological Society* 44, 79-86.

DESHPANDE, V., ERIKSSON, K-E. and PETTERSSON, B. (1978). Production, purification and partial characterization of 1,4-β-glucosidase enzymes from *Sporotrichum pulverulentum*. *European Journal of Biochemistry* 90, 191-198.

DINGLE, J., REID, W.W. and SOLOMONS, G.L. (1953). The Enzymic Degradation of Pectin and other Polysaccharides. II-Application of the 'cup-plate' assay to the estimation of enzymes. *Journal of the Science of Food and Agriculture* 4, 149-155.

DOONAN, S., VERNON, C.A. and BANKS, B.E.C. (1970). Mechanisms of enzyme action. *Progress in Biophysics and Molecular Biology* 20, 247-327.

DROZDOWICZ, A. (1971). The behaviour of cellulase in soil. *Revues of Microbiology* 2, 17-23.

EMERT, G.H., GUM, E.K.Jr., LANG, J.A., LIU, T.H. and BROWN, R.D.Jr. (1973). Cellulases. *Advances in Chemistry Series* 136, 79-100.

ENARI, T-M. and MARKKANEN, P. (1977). Production of cellulolytic enzymes by fungi. *Advances in Biochemical Engineering* 5, 3-22.

ERIKSSON, K-E. (1969). New methods for the investigation of cellulases. *Advances in Chemistry Series* 95, 83-104.

ERIKSSON, K-E. (1978). Biotechnology Review. Enzyme mechanisms involved in cellulose hydrolysis by the rot fungus *Sporotrichum pulverulentum*. *Biotechnology and Bioengineering* 20, 317-332.

ERIKSSON, K-E. and HAMP, S.G. (1978). Regulation of endo-1,4-β-glucanase production in *Sporotrichum pulverulentum*. *European Journal of Biochemistry* 90, 183-190.

ERIKSSON, K-E, and PETTERSSON, B. (1968). Extracellular Enzyme-system Utilized by the Rot Fungus *Sterum sanguinolentum* for the Breakdown of Cellulose. II-Purification of cellulase. *Archives of Biochemistry and and Biophysics* 124, 142-153.

ERIKSSON, K-E, and PETTERSSON, B. (1975a). Extracellular Enzyme-system Utilized by the Fungus *Sporotrichum pulverulentum (Chrysosporium lignorium)* for the Breakdown of Cellulose. 1. Separation, purification and physico-chemical characterization of 5 endo-β-1,4-glucanases. *European Journal of Biochemistry* 51, 193-206.

ERIKSSON, K-E. and PETTERSSON, B. (1975b). Extracellular Enzyme-system Utilized by the Fungus *Sporotrichum pulverulentum (Chrysosporium lignorium)* for the Breakdown of Cellulose. 3. Purification and physico-chemical characterization of an exo-1,4-β-glucanase. *European Journal of Biochemistry* 51, 213-218.

FAN, L.T., LEE, Y-H. and BEARDMORE, D.H. (1980a). Major chemical and physical features of cellulosic materials as substrates for enzymatic hydrolysis. *Advances in Biochemical Engineering* 14, 101-117.

FAN, L.T., LEE, Y-H. and BEARDMORE, D.H. (1980b). Mechanisms of the enzymatic hydrolysis of cellulose : effects of major structural features of cellulose on enzymatic hydrolysis. *Biotechnology and Bioengineering* 22, 177-199.

FERCHAK, J.D., HÄGERDAL, B. and PYE, E.K. (1980). Saccharification of Cellulose by the Cellulolytic Enzyme System of *Thermomonospora* sp. II Hydrolysis of cellulosic substrates. *Biotechnology and Bioengineering* 22, 1527-1542.

FILIP, Z. (1973). Clay minerals as a factor influencing the biochemical activity of soil microorganisms. *Folia Microbiologica* 18, 56-74.

GALAS, E., PYE, R., PYE, K. and SIWINSKA, K. (1981). Universal method for expressing activity of cellulolytic enzymes. *European Journal of Applied Microbiology and Biotechnology* 11, 229-233.

GHOSE, T.K. and BISARIA, V.S. (1979). Studies on the mechanism of enzymic hydrolysis of cellulosic substances. *Biotechnology and Bioengineering* 21, 131-146.

GIBSON, W.P. (1977). The loss of pesticides from soil and soil components. Ph.D. thesis, University of Kent at Canterbury.

GOKSØEYR, J., EIDSA, G., ERIKSEN, J. and OSMUNDSVAG, K. (1975). A comparison of cellulases from different microorganisms. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 217-230. Edited by M. Bailey, T-M. Enari and M.Linko. Sitra, Helsinki.

GONG, C-S. and TSAO, G.T. (1979). Cellulase and biosynthesis regulation. *Annual Reports of Fermentation Processes* 3, 111-140.

GRAY, T.R.G. (1976). Survival of vegetative microbes in soil. In *The Survival of Vegetative Microbes*. Symposium 26, Society for General Microbiology, pp. 327-364. Edited by T.R.G. Gray and J.R. Postgate. Cambridge : University Press.

GRAY, T.R.G. and WILLIAMS, S.T. (1971). *Soil Microorganisms*, pp. 72-74. London and New York : Longman.

GREEN, R.D. and FORDHAM, S.J. (1973). *Soil Survey Record No.14*, Soils of Kent 1. Rothamstead Experimental Station, Harpenden, Herts.

GREENLAND, D.J. (1956a). The Adsorption of Sugars by Montmorillonite. I. X-ray studies. *Journal of Soil Science* 7, 319-328.

GREENLAND, D.J. (1956b). The Adsorption of Sugars by Montmorillonite. II. Chemical studies. *Journal of Soil Science* 7, 329-334.

GRIFFIN, D.M. (1972). *The Ecology of Soil Fungi.* London : Chapman and Hall.

GRITZALI, M. and BROWN, R.D. Jr. (1979). The cellulase system of *Trichoderma* : Relationships between purified extracellular enzymes from induced or cellulose-grown cells. *Advances in Chemistry Series* 181, 237-260.

GULL, K. (1975). Mycelial branch patterns of *Thamnidium elegans*. *Transactions of the British Mycological Society* 64, 321-324.

HÄGERDAL, B.G.R., FERCHAK, J.D. and PYE, E.K. (1978). Cellulolytic enzyme systems of *Thermoactinomyces* sp. grown on microcrystalline cellulose. *Applied and Environmental Microbiology* 36, 606-612.

HÄGERDAL, B.G.R., FERCHAK, J.D. and PYE, E.K. (1980). Saccharification of Cellulose by the Cellulolytic Enzyme System of *Thermomonospora* sp. 1. Stability of cellulolytic activities with respect to time, temperature and pH. *Biotechnology and Bioengineering* 22, 1515-1526.

HÅKANSSON, U., FÄGERSTAM, L., PETERSSON, G. and ANDERSSON, L. (1978). Purification and characterization of a low molecular weight 1,4-β-glucan glucanohydrolase from the cellulolytic fungus *Trichoderma viride* QM 9414. *Biochimica et Biophysica acta* 524, 385-392.

HALLIWELL, G. and GRIFFIN, M. (1973). The nature and mode of action of the cellulolytic component $C_1$ of *Trichoderma koningii* on native cellulose. *Biochemical Journal* 135, 587-594.

HAN, Y.W. (1982). Nutritional requirements and growth of a *Cellulomonas* species on cellulosic substrates. *Journal of Fermentation Technology* 60, 99-104.

HANCOCK, J.G. and BENHAM, G.S. (1980). Fungal decay of buried cotton stems. *Soil Biology and Biochemistry* 12, 35-41.

HANKIN, L. and ANAGNOSTAKIS, S.L. (1977). Solid media containing carboxymethyl cellulose to detect $C_x$ cellulase activity of microorganisms. *Journal of General Microbiology* 98, 109-115.

HANKIN, L., POINCELOT, R.P. and ANAGNOSTAKIS, S.L. (1976). Microorganisms from composting leaves : Ability to produce extracellular degradative enzymes. *Microbial Ecology* 2, 296-308.

HARTENSTEIN, R. (1982). Soil macroinvertebrates, aldehyde oxidase, catalase, cellulase and peroxidase. *Soil Biology and Biochemistry* 14, 387-391.

HERR, D. (1979). Secretion of cellulase and β-glucosidase by *Trichoderma viride* ITCC-1433 in submerged culture on different substrates. *Biotechnology and Bioengineering* 21, 1361-1371.

v. HOFSTEN, B. (1975). Topological effects in enzymatic and microbial degradation of highly ordered polysaccharides. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 281-295. Edited by M. Bailey, T.M. Enari and M. Linko. Sitra, Helsinki.

HONG, J., LADISCH, M.R., GONG, C-S., WANKAT, P.C. and TSAO, G.T. (1981). Combined product and substrate inhibition equation for cellobiase. *Biotechnology and Bioengineering* 23, 2779-2788.

HOPE, C.F.A., ALEXANDER, J.M. and BURNS, R.G. (1980). β-D-Glucosidase activity in soil. *Society of General Microbiology Quarterly* 8, 41.

HSU, T-A., GONG, C-S., and TSAO, G.T. (1980). Kinetic studies of cellodextrins hydrolyses by exocellulase from *Trichoderma reesei*. *Biotechnology and Bioengineering* 22, 2305-2320.

HUANG, A.A. (1975a). Enzymic hydrolysis of cellulose to sugar. *Biotechnology and Bioengineering Symposium* 5, 245-252.

HUANG, A.A. (1975b). Kinetic studies on insoluble cellulose-cellulase systems. *Biotechnology and Bioengineering* 17, 1421-1433.

HULME, M.A. and STRANKS, D.W. (1971). Regulation of cellulase production by *Myrothecium verrucaria* grown on non-cellulosic substrates. *Journal of General Microbiology* 69, 145-155.

IBISTER, J.D., SHIPPEN, R.S. and CAPLAN, J. (1980). A new method for measuring cellulose and starch degradation in soils. *Bulletin of Environmental Contamination and Toxicology* 24, 570-574.

IOWENSTEIN, H. and INGILD, A. (1976). A micromethod for determination of proteolytic enzymes in the pH range 2.8 to 4.8. *Analytical Biochemistry* 71, 204-208.

ISHAQUE, M. and KLUEPFEL, D. (1980). Cellulase complex of a mesophilic *Streptomyces* strain. *Canadian Journal of Microbiology* 26, 183-189.

ISMANDE, J. (1970). Regulation of penicillinase synthesis : Evidence for a unified model. *Journal of Bacteriology* 101, 173-180.

IWASAKI, T., TOKUYASU, K. and FUNATU, M. (1963). Determination of cellulase activity employing glycol cellulose as a substrate. *Journal of Fermentation Technology* 41, 340-345.

JACKSON, R.W. and DeMOSS, J.A. (1965). Effects of toluene on *Escherichia coli*. *Journal of Bacteriology* 90, 1420-1425.

JENKINSON, D.S. and LADD, J.N. (1981). Microbial biomass in soil : Measurement and turnover. In *Soil Biochemistry*, Vol. 5, pp. 415-471. Edited by E.A. Paul and J.N. Ladd. New York : Marcel Dekker.

JENKINSON, D.S. and POWLSON, D.S. (1976). The Effects of Biocidal Treatments on Metabolism in Soil-V. A method for measuring soil biomass. *Soil Biology and Biochemistry* 8, 209-213.

JERMYN, M.A. (1955). Fungal Cellulases IV. Production and purification of an extracellular β-glucosidase of *Stachybotrys atra*. *Australian Journal of Biological Science* 8, 541-562.

KALNINS, A. (1930). Aerobic soil bacteria that decompose cellulose. *Acta Universitatis Latviensis, Lauksaimnieciebas Fak.* Ser 1, 221-299.

KANAMOTO, J., SAKAMOTO, R., ARAI, M. and MURAO, S. (1979). Enzymatic properties of two carboxymethyl cellulose hydrolysing enzymes from *Aspergillus aculeatus*. *Journal of Fermentation Technology* 57, 163-168.

KANDA, T., NAKAKUBA, S., WAKABAYASHI, K. and NISIZAWA, K. (1979). The mode of enzymatic degradation of cellulose based on the properties of cellulase components. *Advances in Chemistry Series* 181, 211-236.

KAPLAN, D.L. and HARTENSTEIN, R. (1979). Problems with toluene and the determination of extracellular enzyme activity in soils. *Soil Biology and Biochemistry* 11, 335-338.

KATZ, D., GODSTEIN, D. and ROSENBERGER, R.F. (1972). Model for branch initiation in *Asperigillus nidulans* based on measurements of growth parameters. *Journal of Bacteriology* 109, 1097-1100.

KAUFMANN, A., FEGAN, J., DOLEAC, P., GAINER, C., WITTICH, D. and GLANN, A. (1976). Identification and characterization of a cellulolytic isolate. *Journal of General Microbiology* 94, 405-408.

KAVANAGH, F. (1960). A commentary on microbial assaying. *Advances in Applied Microbiology* 2, 65-94.

KAVANAGH, F. (1975). Antibiotic assays, principles and precautions. In *Methods in Enzymology*, Vol. 43, pp. 55-69. Edited by J.H. Hash. New York : Academic Press.

KIM, C. (1974). In ARO report 74-2. Proceedings of the 1974 army numerical analysis conference, p. 507. *Cited by* Lee *et al.* (1980).

KING, K.W. and VESSAL, M.I. (1969). Enzymes of the cellulase complex *Advances in Chemistry Series* 95, 7-25.

KUBICEK, C.P. (1981). Release of carboxymethyl-cellulase and β-D-glucosidase from cell walls of *Trichoderma reesei*. *European Journal of Applied Microbiology and Biotechnology* 13, 226-231.

KUMAR, A. (1980). Ph.D. Thesis. Birmingham University.

LADISH, M.R., ANDERSON, A.W. and TSAO, G.T. (1979). Measurement of cellulolytic activity by low pressure liquid chromatography. *Journal of Liquid Chromatography* 2, 745-760.

LAMOT, E. and VOETS, J.P. (1976). Cellulolytic activity of aerobic soil actinomycetes. *Zeitschrift fur Allgemeine Mikrobiologie* 16, 345-351.

LATTER, P.M. and HOWSON, G. (1977). The use of cotton strips to indicate cellulose decomposition in the field. *Pedobiologia* 17, 145-155.

LAUBE, V.M. and MARTIN, S.M. (1981). Conversion of cellulose to methane and carbon dioxide by triculture of *Acetovibrio cellulolyticus*, *Desulfovibrio* sp. and *Methanosarcina bakeri*. *Applied and Environmental Microbiology* 42, 413-420.

LEATHERWOOD, J.M. (1969). Cellulase complex in *Ruminococcus* and a new mechanism for cellulose degradation. *Advances in Chemistry Series* 95, 53-59.

LEE, Y-H. and FAN, L.T. (1980). Properties and mode of action of cellulase. *Advances in Biochemical Engineering* 17, 101-129.

LEE, Y-H, FAN, L.T. and FAN, L-S. (1980). Kinetics of hydrolysis of insoluble cellulose by cellulase. *Advances in Biochemical Engineering* 17, 131-168.

LETHBRIDGE, G. (1978). Soil enzyme - soil amendment interactions. Ph.D. thesis, University of Kent at Canterbury.

LODHA, B.C. (1974). Decomposition of digested litter. In *Biology of Plant Litter Decomposition*, Vol. 1, pp. 213-241. Edited by C.H. Dickenson and G.J.F. Pugh. London and New York : Academic Press.

LOW, P.F. (1962). Effect of quasi-crystalline water on rate processes involved in plant nutrition. *Soil Science* 93, 6-15. *Cited from* Stotzky and Rem, 1967.

LYNCH, D.L. and COTNOIR, L.J.Jr. (1956). The influence of clay minerals on the breakdown of certain organic substrates. *Soil Science Society of America Proceedings* 20, 367-370.

LYNCH, D.L., WRIGHT, L.M. and COTNOIR, L.J.Jr. (1956). The adsorption of carbohydrates and related compounds on clay minerals. *Soil Science Society of America Proceedings* 20, 6-9.

LYNCH, J.M. (1982). Limits to microbial growth in soil. *Journal of General Microbiology* 128, 405-410.

LYNCH, J.M., SLATER, J.H., BENNET, J.A. and HARPER, S.H.T. (1981). Cellulase activities of some aerobic microorganisms isolated from soil. *Journal of General Microbiology* 127, 231-236.

McLAREN, A.D. (1963). Enzyme Reactions in Structurally Restricted Systems IV. The digestion of insoluble substrates by hydrolytic enzymes. *Enzymologia* 26, 237-246.

McLAREN, A.D. and SKUJINS, J. (1968). The physical environment of microorganisms in soil. In *The Ecology of Soil Bacteria*, pp. 3-24. Edited by T.R.G. Gray and D. Parkinson. Liverpool : University Press.

MANDELS, M. (1975). Microbial sources of cellulase. *Biotechnology and Bioengineering Symposium* 5, 81-105.

MANDELS, M. (1982). Cellulases. *Annual Reports on Fermentation Processes* 5, pp. 35-78.

MANDELS, M., ANDREOTTI, R. and ROCHE, C. (1976). Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium* 6, 21-33.

MANDELS, M., PARRISH, F.W. and REESE, E.T. (1962). Sophorose as an inducer of cellulase in *Trichoderma viride*. *Journal of Bacteriology* 83, 400-408.

MANDELS, M. and REESE, E.T. (1963). Inhibition of cellulases and β-glucosidase. In *Advances in Enzymatic Hydrolysis of Cellulose and Related Materials*, pp. 115-157. Edited by E.T. Reese. Oxford : Pergamon Press.

MARSHMAN, N.A. and MARSHALL, K.C. (1981). Bacterial growth on proteins in the presence of clay minerals. *Soil Biology and Biochemistry* 13, 127-134.

MARTIN, J.P.,FILIP, Z. and HAIDER, K. (1976). Effect of montmorillonite and humate on growth and metabolic activity of some actinomycetes. *Soil Biology and Biochemistry* 8, 409-413.

MARTIN, J.P. and HAIDER, K. (1979). Effect of concentration on decomposition of some $^{14}C$-labelled phenolic compounds, benzoic acid, glucose, cellulose, wheat straw and *Chlorella* protein in soil. *Soil Science Society of America Journal* 43, 917-920.

MILLER, G.L., BLUM, R., GLENNON, W.E. and BURTON, A.L. (1960a). Measurement of carboxymethyl cellulase activity. *Analytical Biochemistry* 1, 127-132.

MILLER, G.L., DEAN, J. and BLUM, R. (1960b). A study of methods for preparing oligosaccharides from cellulose. *Archives of Biochemistry and Biophysics* 91, 21-26.

MOLTON, P.M., MILLER, R.K., DONOVAN, J.M. and DEMMITT, T.F. (1979). Identification by GLC-MS of low molecular weight products from aqueous alkaline degradation of cellulose. *Carbohydrate Research* 75, 199-206.

MORGAN, H.W. and CORKE, C.T. (1976). Adsorption, desorption and activity of glucose oxidase on selected clay species. *Canadian Journal of Microbiology* 22, 684-693.

MUSOLAN, C. LUNGU, M. and VASU, S. (1978). Semiquantitative estimation of cellulase by radial diffusion in carboxymethyl cellulose-agarose gels. *Revue Roumaine de Biochimie* 15, 63-66.

NAKAYAMA, M., TOMITA, Y., SUZUKI, H. and NISIZAWA, K., (1976). Partial proteolysis of some cellulase components from *Trichoderma viride* and the substrate specificity of the modified products. *Journal of Biochemistry* 79, 955-966.

NISIZAWA, K. (1973). Mode of action of cellulases. *Journal of Fermentation Technology* 51, 267-304.

NISIZAWA, T., SUZUKI, H. and NISIZAWA, K. (1972). Catabolite repression of cellulase formation in *Trichoderma viride*. *Journal of Biochemistry* 71, 999-1007.

NOVAKOVA, J. (1972a). Effect of increasing concentrations of clay on the decomposition of glucose. I. Effect of bentonite. *Zentralblatt fur Bakteriologie Parisitkenkunde Infektionskrankheiten und Hygiene II.* Abt II. 127, 359-366. *Cited from* R.G. Burns, (1979).

NOVAKOVA, J. (1972b). Effect of increasing concentrations of clay on the decomposition of glucose. II. Effect of kaolinite. *Zentrablatt fur Bakteriologie Parisitkenkunde Infektionskrankheiten und Hygiene II.* Abt II. 127, 367-372. *Cited from* R.G. Burns, (1979).

OADES, J.M. and JENKINSON, D.S. (1979). Adenosine triphosphate content of the soil microbial biomass. *Soil Biology and Biochemistry* 11, 201-204.

OLNESS, A. and CLAPP, C.E. (1975). Influence of polysaccharide structure on dextran adsorption by montmorillonite. *Soil Biology and Biochemistry* 7, pp. 113-118.

PANCHOLY, S.K. and RICE, E.L. (1973). Soil enzymes in relation to old field succession : amylase, cellulase, invertase, dehydrogenase and urease. *Soil Science Society of America Proceedings* 37, 47-50.

PEITERSEN, N., MEDEIROS, J. and MANDELS, M. (1977). Adsorption of *Trichoderma* cellulase on cellulose. *Biotechnology and Bioengineering* 29, 1091-1094.

PETTERSSON, G. and PORATH, J. (1966). A cellulolytic enzyme from *Penicillium notatum.* In *Methods in Enzymology,* Vol. 8, pp. Edited by E.F. Neufeld and V. Ginsburg. New York and London : Academic Press.

PETTIT, N.M. (1978). Investigations of the characteristics and stability of accumulated soil enzymes. Ph.D. thesis, University of Kent at Canterbury.

POLLOCK, M.R. (1962). Exoenzymes. In *The Bacteria,* Vol. IV, pp. 121-178. Edited by I.C. Gunsalas and R.Y. Stanier. New York and London : Academic Press.

PYCRAFT, C.J.H. and HOWARTH, P. (1980). A new method for measuring enzymic degradation of cellulose fibres. *Paper Technology and Industry* 21, 283-285.

RAMASAMY, K. and VERACHTERT, H. (1980). Location of cellulase components in *Pseudomonas* sp. isolated from activated sludge. *Journal of General Microbiology* 117, 181-191.

RANBY, B. (1969). Recent progress on the structure and morphology of cellulose. *Advances in Chemistry Series* 95, 139-151.

RANDERATH, K. (1966). *Thin Layer Chromatography.* New York and London : Academic Press.

REESE, E.T. (1977). Degradation of polymeric carbohydrates by microbial enzymes. *Recent Advances in Phytochemistry* 11, 311-361.

REESE, E.T. (1982). Elution of cellulase from cellulose. *Process Biochemistry* 17, 2-7.

REESE, E.T., SIU, R.G.H. and LEVINSON, H.S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bateriology* 59, 485-497.

RIFAI, M.A. (1969). A revision of the genus *Trichoderma*. *Mycological Papers* No.116.

ROBERGE, M.R. (1970). Behaviour of urease added to unsterilized, steam-sterilized and gamma-radiation sterilized black spruce humus. *Canadian Journal of Microbiology* 16, 865-870.

ROBINSON, P.M. (1978). *Practical Fungal Physiology*. Chichester, New York, Brisbane and Toronto : John Wiley and Sons.

ROBINSON, P.M., PARK, D. and GRAHAM, T.A. (1968). Autotropism in fungal spores. *Journal of Experimental Botany* 19, 125-134.

ROE, J.H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological Chemistry* 212, 335-343.

ROSENBERG, S.L. (1980). Patterns of diffusibility of lignin and carbohydrate-degrading systems in wood-rotting fungi. *Mycologia* 72, 798-812.

ROSS, D.J. (1974). Glucose oxidase activity in soil and its possible interference in assays of cellulase activity. *Soil Biology and Biochemistry* 6, 303-306.

ROSS, D.J. and SPEIR, T.W. (1978). Studies on a Climosequence of Soils in Tussock Grasslands. 17. Litter decomposition : some glycoside hydrolase activities. *New Zealand Journal of Science* 21, 285-296.

ROSS, D.J. and SPEIR, T.W. (1979). Studies on a Climosequence in Soils in Tussock Grasslands. 23. Cellulase and hemicellulase activities of topsoils and tussock plant materials. *New Zealand Journal of Science* 22, 25-33.

ROWLAND, S.P. (1975). Selected aspects of the structure and accessibility of cellulose as they relate to hydrolysis. *Biotechnology and Bioengineering Symposium* 5, 183-191.

ROZYCKI, M. and BARTHA, R. (1981). Problems associated with the use of azide as an inhibitor of microbial activity in soil. *Applied and Environmental Microbiology* 41, 833-836.

SADANA, J.C., LACHKE, A.H. and SHEWALE, J.G. (1979). Biochemistry of cellulose degradation and cellulose utilization for feeds and for protein. *Journal of Scientific and Industrial Research* 38, 442-453.

SATO,K. (1981). Relations between soil microflora and $CO_2$ evolution upon decomposition of cellulose. *Plant and Soil* 61, 251-258.

SCHIMZ, K.L., RÜTTEN, B. and TRETTER, M. (1979). Cellobiase from *Cellulomonas* species cleaves cellobiose by phosphorolysis. *Hoppe-Seyler's Zeitschrift physiologische Chemie* 360, 1191.

SHEWALE, J.G. and SADANA, J.C. (1979). Enzymic hydrolysis of cellulosic materials by *Sclerotium rolfsii* culture filtrate for sugar production. *Canadian Journal of Microbiology* 25, 773-783.

SHIELDS, J.A. LOWE, W.E., PAUL, E.A. and PARKINSON, D. (1973). Turnover of microbial tissue in soil under field conditions. *Soil Biology and Biochemistry* 5, 753-764.

SHOEMAKER, S.P. and BROWN, R.D.Jr. (1978). Enzymic activities of endo-1, 4-β-D-glucanases purified from *Trichoderma viride*. *Biochimica et Biophysica Acta* 523, 133-146.

SIHTOLA, H. and NEIMO, L. (1975). Structure and properties of cellulose. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 9-21. Edited by M. Bailey, T.M. Enari and M. Linko. Sitra, Helsinki.

SKINNER, F.A., JONES, P.C.T. and MOLLISON, J.E. (1952). A comparison of a direct- and a plate-counting technique for quantitative estimation of soil microorganisms. *Journal of General Microbiology* 6, 261-271.

SKUJINS, J. (1976). Extracellular enzymes in soil. *Critical Reviews in Microbiology* 4, 383-421.

SLATER, J.H. and BULL, A.T. (1978). Interactions between microbial populations. In *Companion to Microbiology*, pp. 181-206. Edited by A.T. Bull and P.M. Meadow. London : Longmans Group Ltd.

SOMOGYI, M. (1952). Nelson sugar determination. *Journal of Biological Chemistry* 195, 19-23.

SPANO, L.A. (1976). Enzymatic hydrolysis of cellulosic wastes to fermentable sugars and the production of alcohol. *Clean Fuels, Biomass, Sewage, Urban Refuse and Agricultural Wastes, Symposium Papers*, pp. 324-348. U.S. Army Natick Research and Development Command, Massachsetts.

SPANO, L., TASSINARI, T., RYU, D.D.Y., ALLEN, A. and MANDELS, M. (1979). Enzymatic hydrolysis of cellulose to fermentable sugars for the production of ethanol. Presented at the 29th Canadian Chemical Engineering Conference, Sanaria, Ontario.

SPARLING, G.P. and CHESHIRE, M.V. (1979). Effects of soil drying and storage on subsequent microbial growth. *Soil Biology and Biochemistry* 11, 317-319.

SPEIR, T.W. and ROSS, D.J. (1975). Effects of storage on the activities of protease, urease, phosphatase and sulphatase in three soils under pasture. *New Zealand Journal of Science* 18, 231-237.

SPIRO, R.G. (1966). Analysis of sugars found in glycoproteins. In *Methods in Enzymology*, Vol. 8, pp. 3-26. Edited by E.F. Neufeld and V. Ginsberg. London and New York : Academic Press.

STAHL, E. and KALTENBACH, U. (1961). Thin-layer chromatography. VI Trace analysis of sugar mixtures on layers of Kieselguhr G. *Journal of Chromatography* 5, 351-355. *Cited from* RANDERATH, K. (1966).

STERNBERG, D. (1976). Production of cellulase by *Trichoderma*. *Biotechnology and Bioengineering Symposium* 6, 35-53.

STERNBERG, D., VIJAYAKUMAR, P. and REESE, E.T. (1977). β-Glucosidase : microbial production and effect on enzymatic hydrolysis of cellulose. *Canadian Journal of Microbiology* 23, 139-147.

STEWART, B.J. and LEATHERWOOD, J.M. (1976). Derepressed synthesis of cellulase by *Cellulomonas*. *Journal of Bacteriology* 128, 609-615.

STONE, J.E., SCALLAN, A.M., DONEFER, E. and AHLGEN, E. (1969). Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. *Advances in Chemistry Series* 95, 219-241.

STORER, G.B., GAWTHORNE, J.M., FRANCIS, G.L. and ILLMAN, R.J. (1979). The determination of cellulase activity by gas-liquid chromatography. *Analytical Biochemistry* 92, 270-275.

STOTZKY, G. (1974). Activity, ecology and population dynamics of microorganisms in soil. In *Microbial Ecology*, pp. 57-135. Edited by A. Laskin and H. Lechevalier. Cleveland, Ohio : CRC Press.

STOTZKY, G. and BURNS, R.G. (1982). The soil environment : clay-humus-microbe interactions. In *Experimental Microbial Ecology*, pp. 105-133. Edited by R.G. Burns and J.H. Slater. Oxford : Blackwell Scientific Press.

STOTZKY, G. and DeMUMBRUM, L.E. (1965). Effect of autoclaving on X-ray characteristics of clay minerals. *Soil Science Society of America Proceedings* 29, 225-227.

STOTZKY, G. and REM, L.T. (1967). Influence of Clay Minerals on Microorganisms. IV Montmorillonite and kaolinite on fungi. *Canadian Journal of Microbiology* 13, 1535-1550.

STREAMER, M., ERIKSSON, K-E. and PETTERSSON, B. (1975). Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum (Chrysosporium lignorum)* for the breakdown of cellulose. *European Journal of Biochemistry* 59, 607-613.

STUTZENBERGER, F.J. (1972). Cellulolytic acitivity of *Thermomonospora curvata* : optimal assay conditions, partial purification and product of the cellulase. *Applied Microbiology* 24, 83-90.

SUGA, K., v.DEDEM, G. and MOO-YOUNG, M. (1975). Degradation of polysaccharides by endo and exo enzymes - a theoretical analysis. *Biotechnology and Bioengineering* 17, 433-439.

SUZUKI, H., YAMANE, K. and NISIZAWA, K. (1969). Extracellular and cell-bound cellulase components of bacteria. *Advances in Chemistry Series* 95, 60-82.

SWIFT, M.J. (1977). The roles of fungi and animals in the immobilization and release of nutrient elements from decomposing branch wood. In *Soil Organisms as Components of Ecosystems*, pp. 193-202. Edited by U. Lohm and T. Persson. Stockholm : Ecological Bulletins No.25.

TOMITA, Y., SUZUKI, H. and NISIZAWA, K. (1974). Purification and properties of 'Avicelase' a cellulose component of the less-random type from *Trichoderma viride*. *Journal of Fermentation Technology* 52, 233-246.

TRIBE, H.T. (1960). Aspects of Cellulose Decomposition in Canadian Soils. I. Observations with the microscope. *Canadian Journal of Microbiology* 6, 309-316.

TRIBE, H.T. (1961). Microbiology of cellulose decomposition in soil. *Soil Science* 92, 61-77.

TRIBE, H.T. (1966). Interactions of soil fungi on cellulose film. *Transactions of the British Mycological Society* 49, 457-466.

TRINCI, A.P.J. (1969). A kinetic study of the growth of *Aspergillus nidulans* and other fungi. *Journal of General Microbiology* 57, 11-24.

VAHERI, M., LEISOLA, M. and KAUPPINEN, V. (1979). Transglycosylation products of the cellulase system of *Trichoderma reesei*. *Biotechnology Letters* 1, 41-46.

VIRKOLA, N-E. (1975). Available cellulosic materials. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 23-40. Edited by M. Bailey, T.M. Enari and M. Linko. Sitra, Helsinki.

WALKER, E. and WARREN, F.L. (1937). Decomposition of cellulose by *Cytophaga I*. *Biochemical Journal* 32, 31-43.

WAKESMAN, S.A. and STARKEY, R.L. (1924). Influence of organic matter upon the development of fungi, actinomycetes and bacteria in the soil. *Soil Science* 17, 373-378.

WELLS, G.B. and LESTER, R.L. (1979). Rapid separation of acetylated oligosaccharides by reverse-phase high-pressure liquid chromatography. *Analytical Biochemistry* 97, 184-190.

WHITE, A.R. and BROWN, R.M.Jr. (1981). Enzymatic hydrolysis of cellulose : Visual characterization of the process. *Proceedings of the National Academy of Sciences of the United States of America* 78, 1047-1051.

WILLIAMS, S.T. and GRAY, T.R.G. (1974). Decomposition of litter on the soil surface. In *Biology of Plant Litter Decomposition*, Vol. 2, pp. 611-632. Edited by C.H. Dickenson and G.J.F. Pugh. London and New York : Academic Press.

WOOD, T.M. (1971). The cellulase of *Fusarium solani*. *Biochemical Journal* 121, 353-362.

WOOD, T.M. and McCRAE, S.I. (1972). The purification and properties of the $C_1$ component of *Trichoderma koningii* cellulase. *Biochemical Journal* 128, 1183-1192.

WOOD, T.M. and McCRAE, S.I. (1975). The cellulase complex of *Trichoderma koningii*. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 231-254. Edited by M. Bailey, T.M. Enari and M. Linko. Sitra, Helsinki.

WOOD, T.M. and McCRAE, S.I. (1978). The cellulase of *Trichoderma koningii*. *Biochemical Journal* 171, 61-72.

WOOD, T.M. and McCRAE, S.I. (1979). Synergism between enzymes involved in the solubilization of native cellulose. In *Advances in Chemistry Series* 181, 181-209.

WOOD, T.M., McCRAE, S.I. and MACFARLANE, C.C. (1980). The isolation, purification and properties of the cellobiohydrolase component of *Penicillium funiculosum* cellulase. *Biochemical Journal* 189, 51-65.

ZANTUA, M.I. and BREMNER, J.M. (1976). Production and persistence of urease activity in soils. *Soil Biology and Biochemistry* 8, 369-374.

ZUNINO, H., BORIE, F., AGUILERA, S., MARTIN, J.P. and HAIDER, K. (1982). Decomposition of $^{14}C$-labelled glucose, plant and microbial products and phenols in volcanic ash-derived soils of Chile. *Soil Biology and Biochemistry* 14, 37-43.