

A CARBOXYMETHYLCELLULASE AND A XYLANASE FROM SCLEROTIUM ROLFSSII

by

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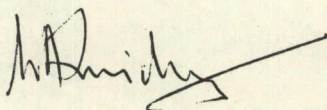


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## DECLARATION

The author received assistance with some of the studies reported in this thesis. Prof. N. C. K. Phillips demonstrated the validity of the various derivations of Schutz's law from considerations of product inhibition. Prof. A. M. Stephen and Dr. S. C. Churms analysed the xylooligosaccharide products of enzyme-catalysed xylan degradation by chromatography on paper and on a column of Bio-Gel P-2. Mr. R. Rinsma freeze-dried large volumes of culture filtrate.

All other original investigations reported in this thesis were the author's own work.

A handwritten signature in dark ink, appearing to read 'M. Smith', with a long horizontal stroke extending to the right.

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## CHAPTER 1

INTRODUCTION

Lignocellulosic materials constitute a large proportion of agricultural, industrial and municipal wastes in most developed countries. These wastes consist mainly of cellulose (40 to 60%) with lesser but significant amounts of hemicellulose (15 to 30%) and lignin (10 to 30%). As such they offer a renewable source of raw materials from which many useful chemicals may be derived (1). For example, the conversion of cellulose to D-glucose (the process of saccharification) can be the forerunner of many applications in the food industry, fermentation technology and fuel production. Apart from its obvious value in foods, glucose may be used as a starting material for nearly all fermentation products made at present, such as organic acids, amino acids, vitamins, antibiotics and a number of bacterial and fungal polysaccharides such as xanthan, curdlan and scleroglucan.

These polysaccharides find application in the food, cosmetic and pharmaceutical industries, as viscosity additives and also as drilling muds (e.g. xanthan) in the mining industry. In addition, bioconversion of cellulose to alcohol via glucose offers an attractive means of alleviating future fuel shortages, as motor vehicles can run on a mixture of ethyl alcohol and petrol, or even alcohol alone. A case in point is Brazil, where fermentation technology is particularly well developed, and where ethyl alcohol production has led to a reduction in

current petroleum imports by almost 20% with a further reduction of 5% anticipated by 1985 (2).

Hemicelluloses are also of particular interest industrially because of their high content in most agricultural wastes. They constitute a source of xylose from which xylitol and furfural can be derived. Since xylitol is of the same order of sweetness as sucrose or fructose (3) it could be used as a diabetic sweetener in foods. Furfural can be synthesised directly from xylose, and, since at present furfural is produced from raw bagasse, the direct conversion from xylose would constitute a simpler and cleaner process. Furfural finds use in the manufacture of furfural-phenol plastics, varnishes, pesticides and as a useful solvent for cellulosic derivatives.

Research studies aimed at the saccharification of wastes and the production of single cell protein (SCP) from these materials has received worldwide attention over the past 6 to 8 years. This interest has further intensified in countries lacking fossil fuel resources and has led to a large investment of money in projects concerned with the production of active cellulolytic and hemicellulolytic enzymes for use in saccharification. A review dealing with lignocellulosic waste bioconversion, particularly in a South African context, has been written by the author in collaboration with Dr. R.F.H. Dekker (1).

The current interest in bioconversion was a major reason for selecting cellulases and xylanases as a topic of study. Previous studies (4-6) have confirmed that Sclerotium rolfsii produces these

enzymes and, more recently, a mutant strain of S. rolfsii CPC 142, (7,8) was shown to produce enzymes especially effective in degrading cellulosic wastes. The paucity of detailed information on the cellulases and xylanases of S. rolfsii together with the potential usefulness of S. rolfsii in waste degradation are reasons for selecting this organism for closer investigation.

In order that the reader might fully appreciate the aims of this thesis and the approach taken by the author in solving some of the problems that arose in the course of study, some necessary background information on the enzymes and substrates is provided in the following sections.

### 1.1 Cellulose and cellulases

Cellulose occurs as a major structural component of the plant cell wall and it can also be produced by other organisms, such as certain bacteria (e.g. Acetobacter xylinum) (9), some fungi, where it occurs as a constituent of the mycelial cell wall (10), and by certain tunicates (e.g. Phallusia mammilata) (11).

Structurally, cellulose is a polysaccharide of high molecular weight composed of  $\beta(1\rightarrow4)$ -linked D-glucopyranosyl units of which cellobiose is the repeat unit. The degree of polymerization (D.P.) of these chains can be as high as 14 000 glucose residues (12). The glucose units adopt a chair conformation with the hydroxyl groups occupying the stable equatorial positions and the unbranched molecule

assumes a flat, ribbon-shaped structure. Neighbouring molecules associate closely along their length via hydrogen bonding, resulting in a fibrous crystalline material insoluble in water. At intervals, the strict association of chains becomes disrupted as a result of either mechanical stresses (12) or possibly by chain-end dislocations within the microfibrils (13). The result is an interspersion of paracrystalline (amorphous) and crystalline (highly-ordered) regions, the former being more susceptible to both chemical and enzymic degradation than the latter (12).

Enzymes capable of degrading cellulose (cellulases) are produced by a variety of bacteria and fungi, as well as by plants. Isolated plant cellulases lack the ability to degrade crystalline cellulose, although they readily attack non-crystalline (amorphous) cellulose preparations. The same is true of cellulases isolated from the majority of cellulolytic micro-organisms. In a few instances however, cellulases able to degrade crystalline cellulose have been isolated from strains of bacteria (14,15) and fungi (16-19) making it possible to study crystalline cellulose degradation in vitro.

An early theory of Reese (20) explained enzyme catalysed (crystalline) cellulose degradation in terms of the synergistic action of two types of cellulase designated  $C_1$  and  $C_x$ . The  $C_1$  enzyme was supposed to cause disaggregation of the hydrogen bonded cellulose polymers, opening up the substrate and thereby facilitating hydrolytic attack by  $C_x$  enzyme(s). Because insufficient evidence has accumulated in support of Reese's theory, it is no longer widely accepted.

Nevertheless, some of Reese's ideas continue to influence current ideas on cellulose degradation.

Although the exact mechanism by which cellulose is degraded enzymically is not yet known, recent research has provided some insight into the process. Many workers consider that at least three classes of enzymes are involved, namely endocellulases of different specificity, exocellulases (cellobiohydrolases) and  $\beta$ -D-glucosidases (cellobiases) (1,21,22). Together, these enzymes constitute the cellulosic complex and act synergistically to degrade native cellulose to D-glucose.

Endocellulase activity is usually assayed using soluble cellulosic derivatives, such as carboxymethyl- and hydroxyethylcellulose as substrates. These derivatives are attacked at random along the polymer chain, yielding some glucose, but mainly cellodextrins with a degree of polymerization of 2 to 6.

The exocellulases will hydrolyse soluble (i.e. substituted) celluloses only to a limited extent, but amorphous or non-crystalline celluloses are degraded more extensively (23,24). In an exo mode of attack, successive cellobiose units are cleaved from the cellulose polymer (21,22,25).

Cellobiase aids the overall conversion by hydrolysing cellobiose and cellooligosaccharides of D.P. 3 to 6 to glucose, thus relieving the inhibitory effect of cellobiose on exocellulase activity (26).

Enzymic attack on native cellulose is thought to be initiated by the action of the endocellulase components on less crystalline or amorphous regions, thereby creating chain ends for further attack by the exocellulases (27). Such a mechanism would result in a limited degree of hydrolysis yielding a polymeric fragment of high crystallinity. The crystallinity of the remaining fraction would present a barrier to further enzymic hydrolysis were it not for the presence of cellulases specific for highly ordered cellulose, for example the Avicelases. These enzymes have been found to be capable of degrading highly crystalline celluloses such as Avicel (27-30) and thereby overcome the inherent structural resistance to hydrolysis of native cellulose.

A model may be envisaged for cellulose breakdown in which cellulases able to degrade CMC (carboxymethylcellulose), i.e. the CMC-ases, attack the more accessible areas of the cellulose molecule, leaving the more ordered regions to the action of the Avicelases. Such concerted action leads to the production of cellodextrins of low D.P. which are readily attacked by cellobiase. It may be noted that the more modern view of cellulose degradation makes two major departures from Reese's original proposals. These are, (i) that endocellulases initiate attack on crystalline cellulose, and, (ii) that cellobiohydrolase (considered today to be the  $C_1$  of Reese's theory) acts as a hydrolytic enzyme.

Several questions about crystalline cellulose degradation still remain unanswered. For example, do the components of the cellulasic

complex act sequentially or do they act simultaneously as a single catalytic entity? A further question relates to the binding of cellulases to crystalline cellulose. The binding is often irreversible (at constant ionic strength) and yet these apparently immobile enzymes are nevertheless able to catalyse extensive degradation of the cellulose to which they are bound. How is this possible? These and other questions are addressed in Chapter 5 and in a recent publication by Lindner, Dennison, Quicke and Phillips (31).

#### 1.1.1 Modes of action of endocellulases

Cellulases have been the subject of several reviews (1,21,22,32,33). Little advance in the understanding of cellulase action on crystalline cellulose appears to have been made since the latest of these reviews was written. However, several recent publications have appeared that bring to light novel aspects of endocellulase action and these publications are reviewed below.

The endocellulase-catalysed degradation of a series of cellooligosaccharides was investigated by Hurst, Sullivan and Shepherd (34) using an enzyme preparation from Aspergillus niger. The enzyme displayed a preference for hydrolysing internal glycosidic bonds but also released substantial amounts (30% of the products formed) of glucose from the non-reducing end of cellotetraose.

A purified endocellulase from Clostridium thermocellum (35) degraded cellooligosaccharides of D.P.  $\geq 4$  without any evidence of

transglycosylation and the authors concluded that hydrolytic cleavage of internal bonds could account for their observations. Similarly an endocellulase isolated from Thermoascus aurantiacus (36) showed a preference for the internal bonds of cellooligosaccharide substrates, whilst exhibiting no transglycosylating activity. The T. aurantiacus enzyme, unlike the enzyme from C. thermocellum, could degrade both cellotriose and cellotriitol.

Halliwell and Vincent (37) investigated the mode of action of an endocellulase from Trichoderma koningii and concluded that the central bond of cellooligosaccharide substrates of D.P.  $\geq 4$  was always cleaved regardless of chain length. The central bond of an oligosaccharide composed of an odd number of monomers was defined as the bond on either side of the central monomer. Once again there was no evidence of transglycosylation.

Not all endocellulases catalyse simple hydrolysis of oligosaccharide substrates and several reports exist of enzyme-catalysed transglycosylations (38-40). Okada and Nisizawa (38) observed that two endocellulases (designated IIA and IIB) from Trichoderma viride catalysed the synthesis of cellotriose from p-nitrophenyl  $\beta$ -D-cellobioside ( $\beta$ -PNPC) and concluded that a terminal glucosyl residue of  $\beta$ -PNPC was transferred to cellobiose, (itself derived from  $\beta$ -PNPC). A further observation was that both enzymes could catalyse transglycosylation reactions with cellobiose as sole substrate, yielding either cellotetraose (in the case of cellulase IIB) or a mixture of cellotriose and glucose (in the case of cellulase IIA).

The observation of transglycosylation between cellobiose molecules is unusual, as most endocellulases are unable to attack cellobiose. Indeed, in the absence of  $\beta$ -glucosidase, cellobiose and glucose are the final products of cellulose degradation.

The degradation of both CMC and cellooligosaccharides by three purified T. viride endocellulases was investigated by Shoemaker and Brown (39). Two of the three enzymes (endoglucanases II and IV) acted in a similar way but the third enzyme, endoglucanase III attacked both CMC and cellooligosaccharides in a unique manner. Thus a plot of reducing sugar (on the abscissa) vs. specific fluidity for CMC hydrolysis by endoglucanase III exhibited a comparatively shallow slope, indicating that, unlike the other two enzymes, endoglucanase III showed a preference for glycosidic bonds near a CMC terminus. The attack of endoglucanase III on cellotetraitol and cellopentaitol was likewise unique. Endoglucanases II and IV catalysed the formation of reduced and non-reduced forms of cellobiose and cellotriose from both substrates. In contrast, the products of endoglucanase III action on cellotetraitol were cellobiitol and cellohexaitol. In addition only a single product, cellotriitol, was detected following attack by the same enzyme on cellopentaitol. The absence of cellobiose in the products of endoglucanase III action led the authors to conclude that endoglucanase III catalyses transfer of a cellobiosyl unit from one substrate molecule to another. Further support for this mechanism comes from the observed formation of cellohexaitol during degradation of cello-tetraitol.

The studies cited above provide direct evidence of differences in the modes of action of different endocellulases on cellulosic substrates. This conclusion is in accord with independent observations (41) indicating diverse roles for these enzymes in the degradation of crystalline cellulose. At present, however, there exists no obvious correlation between an enzyme's mode of attack and the possible role it might play in degrading crystalline cellulose.

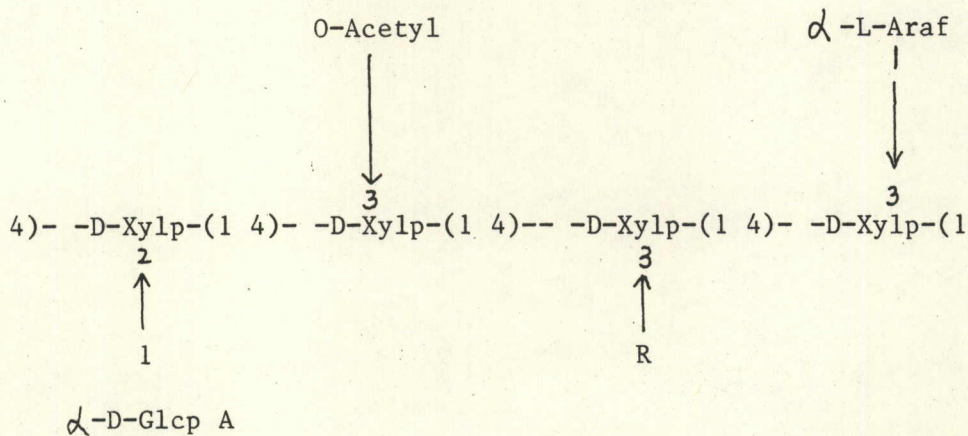
In this section some of the complexities of enzyme catalysed cellulose degradation have been outlined and it has been stressed that the mechanism of such degradation is not fully understood. In addition it was seen that considerable attention has been focused recently on endocellulase catalysed cellooligosaccharide degradation. Major differences exist between endocellulases in their mode of action on oligosaccharide substrates, however the significance of this in cellulose degradation remains obscure.

## 1.2 Xylan and xylanases

D-Xylans constitute a major part of that group of plant polysaccharides called hemicellulose and are a heterogeneous class of polymers possessing a number average degree of polymerisation of between 50 and 200 (42). They exist as structural components in the cell walls of both marine and land plants, where, in the latter, they make up between 7 and 30% of the plant dry mass (1,42). There are marked differences in the structure of xylans from land and marine plants and the generalisations given below will apply only to the

former group. The D-xylans from land plants have a main backbone chain of (1→4) linked  $\beta$ -D-xylopyranosyl residues but differ, according to source, in the composition of their side chain substituents. A single side chain may be composed of as many as three monosaccharide units, but commonly consists of one sugar residue only.

L-Arabinofuranoside, is a major appendage of xylans from monocotyledons. Angiosperm and Gymnosperm xylans are characterised by side-chains of single residues of 4-O-methyl-D-glucuronic acid, and have a degree of substitution (D.S.) of approximately 0,1 and 0,6 respectively. L-arabinose is bound to polymeric xylose by 1→3' linkages. Both arabinose, which occurs exclusively in the furanose form in xylan, and glucuronic acid invariably adopt the  $\alpha$ -configuration about C<sub>1</sub>. A general structure of D-xylan (43,44) is reproduced below:



where R may be any of the following:

1.  $\beta$ -D-Xylp - (1→2) - L - Araf - (1→
2.  $\alpha$ -D-Xylp - (1→3) - L - Araf - (1→

3. L-Galp-(1→4) - D - Xylp - (1→2)- L - Araf- (1→
4.  $\beta$ -D-Xylp- (1→
5.  $\beta$ -D-Galp-(1→5) - L - Araf- (1→
6.  $\beta$ -D-Galp-(1→4) - D - Xylp- (1→
7. D-Xylp- (1→2) - L - Araf- (1→2) - L - Araf - (1→
8. D-GlcA - (1→4) - D - Xylp - (1→4) - Galp - (1→

Araf = arabinofuranoside, Galp = galactopyranoside, GlcA = galactopyranoside uronide, Xylp = xylopyranoside. Further details of xylan structure are to be found in the reviews of Aspinall (42), Wilkie and Woo (44) and Timell (45,46).

It has been estimated that major agricultural wastes in South Africa annually comprise some 2,5 million tonnes of xylan (1), a large enough mass to be considered economically viable for conversion on an industrial scale. Xylanolytic enzymes provide one means whereby xylans may be degraded to simple sugars, including xylose. Enzymic conversion is probably more desirable than hydrolysis employing mineral acids as the former method does not require the use of resistant hardware or elevated temperatures, and, in general, creates fewer waste disposal problems.

The major group of xylanolytic enzymes are the D-xylanases (EC 3.2.1.8.), also known as endoxylanases or (1→4)- $\beta$ -D-xylan xylanohydrolases. These enzymes are elaborated by a number of organisms including bacteria, fungi, insects, and both marine and land plants. The most widely studied xylanases are those produced by fungi and bacteria and therefore any generalisations will apply largely to these

enzymes.

Xylanases are regarded in the main as constitutive enzymes although, in some cases, substrates such as cellulose, pectin, starch and glucomannan have been reported to induce xylanase synthesis (47). Xylan itself, although not an inducer, may exert a repressive effect on xylanase synthesis (47). It seems natural to suppose that xylan degradation occurs extracellularly since the size of the xylan polymer might be thought to severely limit its rate of entry into the cell. Indeed, during growth of most xylanolytic organisms, xylanase is found extracellularly in the growth medium, but surprisingly, there have been reports of intracellular xylanases as well (48). It is unclear whether the latter are active intracellularly or whether they merely await export from the cells.

The major difference between bacterial and fungal xylanases lies in their optimum pH for activity, the bacterial enzymes being most active around neutrality (pH 6 to 7) whereas the fungal enzymes have optima at acid pH values (3,5 to 5,5). In other respects, the physicochemical properties of xylanases from both fungi and bacteria exhibit no major differences. Optimal activity is, in both cases, around 50°C, isoelectric points fall anywhere between pH 3,5 and 9,5, and molecular masses range from  $1,0 \times 10^4$  to  $5,0 \times 10^4$ . The D-xylanases catalyse hydrolysis of the internal (1→4)-β-D-xylopyranosyl bonds of D-xylan, releasing xylose and xylose derivatives as products. In addition, some but not all, endoxylanases have the ability to hydrolyse the bond between polymeric xylose and arabinose

and this has led to an additional classification of these enzymes as either arabinose or non-arabinose releasing (47).

Further hydrolysis to simple sugars requires participation of one or more specific exoglycosidases that attack low molecular weight glycosides as well as xylan side chains, in an exo mode, releasing one monosaccharide at a time from the non-reducing end of the molecule. Glycosidases (e.g. the exoxylopyranosidases) do not attack the xylan backbone. To date there appears to be no firm evidence supporting the existence of an exoxylanase that would attack the xylan polymer from the non-reducing end to release xylose or xylobiose as the sole product. An exocellulase has recently been discovered that acts as an exoxylanase as well (49), but since the enzyme in question showed a greater affinity for cellulose than for xylan it is more properly classed as a cellulase.

The products arising from xylanase catalysed hydrolysis of native xylan exhibit considerable structural diversity reflecting differences in the mode of enzyme attack as well as structural differences in the various xylan substrates. Some of the main generalisations regarding product structure are summarised below.

1. It is common for both substituted and unsubstituted xylo-oligosaccharides to be released often by the same enzyme.
2. Substituted oligosaccharides are normally composed of a minimum of three monosaccharide units, two of which are xylose.

3. Arabinose is found attached at both the reducing and non-reducing ends of the oligosaccharide, as well as to the xylose residue next to the reducing end.
4. 4-O-methyl-D-glucuronic acid is normally found attached only at the non-reducing end of the molecule.
5. Although some xylanases can release free arabinose from their substrate, in this way resembling an exo-L-arabinofuranosidase, none appears able to attack the 1→2' linkage between xylose and glucuronic acid (or its 4-O-methyl derivative).

#### 1.2.1 A detailed review of selected recent xylanase literature

A more detailed examination of some recent xylanase studies is made below. Since the xylanases have been reviewed as recently as 1978 (50), commentary will be confined to literature appearing during or after this date. The examples below have been chosen either for their novel aspects or because they lend themselves to a critical appraisal. In either case the idea is to convey an impression of the diversity in both structure and action of xylanases.

A xylanase has been found associated with cell walls of the yeast, Cryptococcus albidus var. aerius (51). This enzyme proved to be somewhat exceptional since, unlike most xylanases from fungi and bacteria, it was unstable in free solution. Activity of the bound enzyme was completely destroyed following mild acid treatment, and, since the same

treatment did not appear to otherwise affect cell viability, the authors concluded that the xylanase was bound extracellularly. Again, unlike most other xylanases, the activity (measured in situ) of the enzyme decreased with increasing chain length of a series of linear xylooligosaccharides, although xylobiose was not attacked.

A particularly comprehensive study has been undertaken of the mode of action of a xylanase from the fungus Trametes hirsuta (52). The major neutral products liberated following enzyme attack on a glucuronoxylan substrate were xylotetraose and xylohexaose, while the main acidic products were aldotetrao- and aldohexaouronic acids. Further investigation, using linear xylooligosaccharides as substrates, revealed however that both xylotetraose and xylohexaose were further degradable, the former to xylobiose and the latter to a mixture of xylobiose and xylotetraose. In the light of the latter observations, it is not clear why an oligosaccharide of D.P. < 4 was not found to be a major product of the glucuronoxylan degradation.

John, Schmidt and Schmidt (53) separated five distinct arabinose releasing xylanases from A. niger, employing chromatography on hydroxylapatite as a key step in the purification scheme. All the enzymes possessed similar molecular masses (approx.  $5.0 \times 10^4$ ) and temperature optima but differed as to their respective pH optima and modes of action. Most distinctive was the xylanase designated IID that exhibited a pH optimum of 8, an unusually high value for a fungal xylanase. Individual differences were also noted in the degradation products resulting from the action of the xylanases on xylo-

oligosaccharide substrates. However, independent examination of the data by the author showed that these differences were quantitative rather than qualitative in nature, and became less marked after longer reaction times. It is therefore possible that early differences in product array reflect disparate hydrolysis rates rather than real dissimilarities in mode of action.

Uchino and Nakane (54) isolated an extracellular xylanase from Bacillus sp. 11-1S. The enzyme was capable of degrading CMC as well as xylan, however the  $K_m$  with CMC as substrate was half the corresponding value on xylan and the enzyme should perhaps be more correctly classed as a cellulase. The main products of larchwood xylan degradation were xylobiose and a compound thought to be composed of arabinose and xylose in the molar ratio of 1:3. The products of CMC hydrolysis included cellobiose and a number of larger oligosaccharides indicating endo-action of the enzyme on this substrate.

Several interesting chemical characteristics have emerged from a study of a xylanase elaborated by the Basidiomycete Schizophyllum commune (55). Analysis of the pure enzyme revealed a high proportion of the amino acids glycine, serine, threonine and aspartic acid which together accounted for some 50% of the protein mass. Of further interest was the discovery that carbohydrate was non-covalently bound to the enzyme but its removal did not entirely destroy enzyme activity. The authors conclude that the carbohydrate might play no part in the catalytic process at all. Several authors (50) have reported on a carbohydrate component of xylanases although in most cases the

association between carbohydrate and protein was covalent. The role of covalently bound carbohydrate in the catalytic process is unclear at present.

A particularly high molecular mass ( $9,0 \times 10^4$ ) has been assigned to a xylanase isolated from the Basidiomycete, Termitomyces clypeatus (56). The enzyme was homogeneous on SDS polyacrylamide gels but the authors were unable to exclude the possible existence of subunits of equal molecular weight. In this context it should be mentioned that a quaternary structure for xylanase has been suggested before (57) although such cases would appear to constitute a distinct minority. The present author considers that extensive association between carbohydrate and protein could account for the high molecular mass of the Termitomyces xylanase. The molar ratio of carbohydrate, estimated as glucose, to enzyme protein can be as high as 111:1 (55), which, based on an average molecular mass of 162 per anhydromonosaccharide unit, would increase the enzyme's molecular mass by  $1,8 \times 10^4$ .

This section has emphasised the heterogeneity of xylans from land plants and has indicated that xylans are subject to attack by more than one kind of enzyme. Attention has been drawn to the great diversity of xylanases both in their physicochemical properties and their modes of action. A review of the more recent literature has shown that fresh insights into these aspects of xylanases are still actively pursued.

### 1.3 Aims of this thesis

This thesis is a report on an investigation into the cellulases and xylanases of the plant pathogen S. rolfsii that seeks to broaden the existing knowledge of the polysaccharide degrading enzymes of this fungus. Previous studies of the cellulases and xylanases of S. rolfsii (4-6) were mainly concerned with the possible role of these enzymes in pathogenesis and no attempt was made to rigorously purify and characterise individual enzymes. In these previous investigations, the cellulase system (5) was the more intensively studied and was resolved into three partially purified endocellulase fractions. In addition, the physicochemical properties of each fraction were briefly examined. No attempt was made in the earlier studies to purify hemicellulase components, although an examination was made of the products released by the enzymes from native hemicellulosic substrates.

By contrast, in the work reported in this thesis a cellulase and a xylanase from S. rolfsii have been brought to a high state of purity and their respective properties and modes of action have received detailed study. The results of this thesis are presented in the following Chapters:

- Ch. 3. studies on the growth and enzyme production of S. rolfsii,
- Ch. 4. the isolation and characterisation of a CMCase,
- Ch. 5. Schutz's law and the action of cellulases,
- Ch. 6. the isolation and characterisation of a xylanase.

Growth and enzyme production (Chapter 3) were examined mainly with a view to optimising yields of cellulases and xylanases in liquid culture and this Chapter is largely self-explanatory.

Several aspects of the isolation and characterisation of both the cellulase and the xylanase (Chapters 4 and 6) however, require advance explanation. For example, since the effective degradation of both cellulose and xylan requires the synergistic action of several different enzymes, might it not be purposeless to study individual enzyme components in isolation? In answer one need merely point to the advances made in the understanding of cellulase action through the study of isolated cellulases. Even though enzyme catalysed cellulose degradation is not yet fully understood, the study of isolated cellulases has indicated at least possible roles for individual components of the cellulasic complex.

In identifying the properties of individual glycanases, it is essential that both the enzyme and the substrate be as pure as possible. Although this is sound advice in most enzyme studies, it is especially relevant in the present context. The assay procedures for glycanases usually involve an estimation of total non-specific reducing sugar liberated by enzyme action. This method of assay is particularly unsuitable for use with impure enzyme preparations. In the first place, glycanases often occur as families of related species whose members may differ in their mode of attack on a common substrate. Owing to the non-specific nature of the assay however, these differences are not distinguished and only the net activity of the

family of enzymes will be measured. In the second place, and this applies mainly to hemicelluloses, the substrates themselves are often contaminated by extraneous polysaccharides. Given this situation, one could not even be sure that a single family of enzymes was responsible for the measured activity, since an unspecified contribution to the activity might be made by unrelated enzymes capable of degrading the substrate impurities. Even in a case where the hemicellulose substrate was known to be pure, ambiguity could still arise through the non-specific measurement of activity. Thus hemicelluloses are more often than not heteropolymers, and consequently subject to attack by more than one group of enzymes. To take a specific example, D-xylans are subject to attack by both D-xylanases (which degrade internal bonds of the polymer backbone) and a group of specific exoglycosidases which degrade the polymer sidechains. The usual assay procedure would not distinguish between these two types of activity and, in these circumstances, the measured degradation of xylan by a crude enzyme preparation could not unequivocally be ascribed to xylanase action alone. It is for the abovementioned reasons that, wherever possible, care has been taken in the studies reported in this thesis to use highly purified enzymes and substrates when examining the properties of glycanases.

Chapter 4 includes an examination of the kinetics of endocellulase action. Unsuspected difficulties materialised during the course of routine endocellulase activity determinations, where the author encountered instances of non-linearity in both the enzyme progress and dilution curves. Examination of the literature revealed this to be a

fairly widespread occurrence with cellulases and consequently a study was undertaken to determine possible causes of the phenomena. One finding of the kinetic studies should be mentioned here, since it is of fundamental relevance to all quantitative work with glycanases. This finding is that no meaningful measurement of enzyme concentration can be made from activity determinations on mixtures of simultaneously acting enzymes that are undistinguished by the assay system. Both the xylanases and cellulases of S. rolfsii occur as families of related enzymes whose individual members could not be distinguished by the assay system. It was consequently not possible, even in principle, to determine meaningfully the degree of purification or yield of either cellulases or xylanases from activity measurements. Assessment of enzyme purity at each stage of the purification process was therefore largely effected using the techniques of SDS or non-SDS gel electrophoresis and gel electrofocusing. Much of the material in Chapter 4 has already been published by the author (58,59).

Chapter 5 deals with a re-examination of Schutz's law and its relevance to cellulase action. It is shown that Schutz's law is of more than historic interest, and that its manifestation by cellulases could reveal a novel enzymic mechanism.

## CHAPTER 2

MATERIALS AND METHODS2.1 Reagents and buffers

Unless otherwise stated, all reagents were of analytical reagent grade. In most cases buffers were made by preparing a solution of the weak acid in distilled water and titrating to the desired pH with base before diluting to the required concentration. Phosphate buffer was normally made by mixing appropriate quantities of the mono- and dibasic sodium salts and diluting to volume.

2.2 Sclerotium rolfsii

Under stressful conditions, such as drought, S. rolfsii has the ability to produce hard, compact masses of mycelium called sclerotia, that are dark brown in colour and vary in size from a pin head to a match head. Under suitable conditions sclerotia become the centres of renewed fungal growth.

Sclerotia were obtained as a gift from Prof. F.J. Rijkenberg of the Microbiology Department at the University of Natal. To initiate fungal growth, sclerotia were surface-sterilised for 30 seconds in a solution of sodium hypochlorite (Jik household detergent diluted 1 in 10 with water) and washed in sterilised distilled water before being transferred to Petri dishes containing potato dextrose agar (PDA). Two

or three sclerotia were placed in each dish. All transfer operations were performed under laminar flow and inoculated Petri dishes were left on the Laminar flow bench throughout the growth period.

### 2.3 Growth of *S. rolfsii* in liquid culture

Growth on PDA was allowed to proceed at room temperature until the plate was evenly covered with mycelium (7 to 10 days). At the end of this period one or more blocks were cut from the agar and transferred to vessels containing liquid growth medium. Sclerotia were not transferred to the liquid medium.

#### 2.3.1 Liquid growth medium

The liquid growth medium employed was originally developed by Mandels and Reese (11) and later modified by Reese and Maguire (60). One litre of the medium contained the following components: (numbers refer to mass in g).

$(\text{NH}_4)_2 \text{SO}_4$	1,4
$\text{KH}_2 \text{PO}_4$	2,0
Urea	0,3
$\text{CaCl}_2$	0,3
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0,3
Proteose peptone	0,75
Tween 80	2,2
Avicel PH 101	10,0
$\text{FeSO}_4$	$5 \times 10^{-3}$

CoCl <sub>2</sub>	2 x 10 <sup>-3</sup>
MnSO <sub>4</sub>	2 x 10 <sup>-3</sup>
ZnCl <sub>2</sub>	1,66 x 10 <sup>-3</sup>

Several of the components listed above are unusual additives to fungal growth media and require some explanation. The explanations offered apply specifically to Trichoderma (61), however, they may also be relevant to other fungi. Tween 80 was found to double cellulase yield although the mechanism of enhancement is apparently not known. Addition of proteose peptone decreased the lag in both growth and enzyme production in the case of spore inocula. Finally, addition of cobalt, although not required to promote growth, stimulated cellulase production.

The growth medium was autoclaved at 120°C for between 0,25 and 1 hour before use in small scale growth experiments and for 1 hour in the case of large scale growth experiments. Glass wool air filters employed for aeration during large scale growth were autoclaved in situ along with the growth medium.

### 2.3.2 Studies of growth and enzyme production on a small scale

Enzyme production on different carbon sources. A square of agar (1 cm<sup>2</sup>) was transferred to Erlenmeyer flasks (normally of 250 cm<sup>3</sup> capacity) containing autoclaved growth medium incorporating the carbon source as a 10 g dm<sup>-3</sup> suspension (or solution). The volume of growth medium was 60 cm<sup>3</sup> in the case of growth on microcrystalline cellulose,

filter paper and CMC, and  $100 \text{ cm}^3$  for growth on bagasse and hemi-cellulose. Flasks were shaken at room temperature at  $3,5 \text{ s}^{-1}$  in a Labotec 202 reciprocal shaker.

At 24 to 48 hour intervals a sample (between  $5,0$  and  $10,0 \text{ cm}^3$ ) was removed aseptically from each flask under laminar flow and centrifuged ( $267 \text{ s}^{-1}$ , 15 minutes) in a Beckman J-21 centrifuge using the JA-21 rotor,) to remove mycelial mass. The supernatant was adjusted to a pH of ca.  $6,0$  by dropwise addition of  $100 \text{ g dm}^{-3}$  sodium hydroxide, with constant stirring, and stored frozen. Aliquots were removed from the thawed solutions and assayed for enzyme activity.

Growth and enzyme production on Avicel. Discs of  $0,83 \text{ cm}$  diameter were cut from the PDA with a cork borer and were transferred in pairs to separate  $250 \text{ cm}^3$  Erlenmeyer flasks containing an autoclaved  $10 \text{ g dm}^{-3}$  suspension of Avicel PH 101 in liquid growth medium ( $50 \text{ cm}^3$ ). Flasks were incubated in a New Brunswick controlled environment shaker at  $27$  to  $30^\circ\text{C}$  and agitated at  $2,5 \text{ s}^{-1}$ .

At appropriate times duplicate flasks were taken from the incubator and thereafter treated individually. Mycelial mass was separated from the culture filtrate by centrifugation ( $83 \text{ s}^{-1}$ , 15 minutes,  $5^\circ\text{C}$ ) in a Beckman J-21 centrifuge using the JA-10 rotor and both the supernatant and the mycelial pellet retained. A sample of the supernatant ( $10 \text{ cm}^3$ ) was removed, adjusted to a pH of ca.  $6,0$  with sodium hydroxide, as described above, and frozen; residual supernatant was discarded. The mycelial pellet was resuspended in  $9,0 \text{ g dm}^{-3}$

sodium chloride ( $50 \text{ cm}^3$ ) and centrifuged as before. After discarding the supernatant, the mycelial pellet was removed with a spatula and stored frozen.

### 2.3.3 Studies of growth and enzyme production on a large scale

Between 20 and 30  $\text{cm}^2$  of mycelial mat, still attached to the underlying agar, was transferred to 20  $\text{dm}^3$  glass carboys containing an autoclaved 10  $\text{g dm}^{-3}$  suspension of Avicel PH 101 (FMC) in liquid growth medium (14  $\text{dm}^3$ ). Inoculated carboys were incubated at room temperature for 10 to 12 days. Throughout the growth period the carboy contents were continuously agitated by means of a magnetic stirrer and sparged with air filtered through sterilised glass wool.

In order to obtain substantial yields of extracellular enzymes, comparatively large volumes (30 to 50  $\text{dm}^3$ ) of culture filtrate were collected. For practical reasons it was more convenient to grow the fungus in several batches rather than in a single large volume. However, it seemed probable that separate batches of culture filtrate would exhibit small differences in enzyme composition. Therefore, following growth, separate batches of culture filtrate were mixed to obtain a homogeneous starting material.

Preparation of acetone powders. It was desirable to remove, as early on as possible, unwanted culture filtrate components (e.g. inorganic salts) that might interfere with subsequent enzyme purification. Two ways of producing a partially purified homogeneous starting material

for enzyme purification were examined on a pilot scale (section 4.1.2) and the method ultimately chosen for routine application is described below.

Growth was terminated by removing mycelial mass by centrifugation ( $83,3 \text{ s}^{-1}$ , 15 minutes,  $5^{\circ}\text{C}$ ) in a Beckman J-21 centrifuge using the JA-10 rotor. The supernatant was filtered through a nylon stocking, adjusted to a pH of between 5,5 and 6,0 with sodium hydroxide as before and, after addition of merthiolate ( $0,05 \text{ g dm}^{-3}$ ), was stored frozen.

Frozen culture filtrates were thawed, filtered through glass wool and freeze-dried. The freeze-dried preparation (25 g) was reconstituted in  $5 \times 10^{-3} \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, ( $100 \text{ cm}^3$ ) and after centrifugation, ( $25 \text{ to } 30 \text{ s}^{-1}$ , 15 minutes,  $5^{\circ}\text{C}$ ) in an International PR-2 centrifuge using a rotor of 20 cm radius to remove undissolved solids, protein was precipitated at  $0^{\circ}\text{C}$  by the addition of 2,5 volumes of pre-chilled acetone. The precipitated protein was dried in vacuo and stored below  $0^{\circ}\text{C}$ .

## 2.4 Enzyme assays

### 2.4.1 Enzyme activity on polysaccharide substrates

Polysaccharide degrading activity was routinely assayed by measuring the rate of reducing sugar production in buffered mixtures of enzyme and polysaccharide substrate. The concentrations of both the enzyme and the substrate used in the assays was varied in response to

different experimental demands. Thus, where only limited supplies of enzyme were available, the volume of the enzyme solution in the assay was decreased and, if necessary, the reaction time increased to ensure production of a measurable amount of reducing sugar. The need for choosing different substrate concentrations for assay purposes was dictated by the nature of the substrate used. For example, because of its carboxymethyl substituents, not all the glucosidic bonds of CMC are available to the enzyme. It was therefore necessary in the case of a highly substituted CMC, to use comparatively high substrate concentrations to overcome the possible effects of substrate limitation. The D.P. of polymeric substrates also influenced the choice of substrate concentration. Low viscosity CMC (D.P. ca. 200) is relatively free-flowing at a concentration  $20 \text{ g dm}^{-3}$ , whereas at a similar concentration, high viscosity CMC (D.P. ca. 1000) is highly viscous. Since highly viscous solutions are difficult to transfer rapidly and quantitatively, the concentration of high viscosity CMC was kept at comparatively low levels in enzyme assays.

However, the substrate solutions employed in this thesis for measuring enzyme activity in column effluents were generally the same. To avoid repetition, reference is made merely to substrate "stock solution" in the legends to the pertinent figures. The preparation of these stock solutions is described below.

CMC stock solution. Low viscosity CMC (Sigma D.S. 0,7) (0,2 g) was measured into a test tube and dissolved in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, ( $10 \text{ cm}^3$ ). The buffer was initially added in small

aliquots (ca.  $0,3 \text{ cm}^3$ ) and the CMC ground into a transparent paste using a glass rod. The remainder of the buffer was added in larger aliquots and the solution well stirred after each addition. The final pH was 4,9.

Xylan stock solution. Xylan (Sigma) (0,05 g) was measured into a test tube and dissolved in  $0,5 \text{ mol dm}^{-3}$  sodium hydroxide ( $1,0 \text{ cm}^3$ ). Complete dissolution of the xylan required that the sodium hydroxide initially be added in small aliquots (ca.  $0,1 \text{ cm}^3$ ) and that the resulting xylan paste be continually ground against the wall of the test tube with a glass rod. (A thorough grinding of the paste appeared to be more critical in effecting solubilisation of the xylan than of the CMC described previously). The xylan solution was diluted with distilled water ( $8,0 \text{ cm}^3$ ) and partially neutralised by adding  $0,5 \text{ mol dm}^{-3}$  acetic acid ( $1,0 \text{ cm}^3$ ). The final pH was adjusted to 4,5 with either concentrated hydrochloric acid or glacial acetic acid.

Testing the specificity of the purified CMCase F2. Enzyme ( $6,0 \times 10^{-6} \text{ g}$ ) was incubated with  $10 \text{ g dm}^{-3}$  substrate in  $0,05 \text{ mol dm}^{-3}$  ammonium acetate buffer, pH 4,5, ( $0,52 \text{ cm}^3$ ) at  $50^\circ\text{C}$  for 80 minutes prior to measuring the release of reducing sugars. The substrates (Sigma) were: arabinoxylan, arabinogalactan, galactomannan and pectin (methyl ester). Activity on phosphoric acid swollen cellulose (a gift from R.K. Berry) was assayed similarly, except the reaction time was 22 hours. Activity on Avicel was monitored after an overnight incubation at  $50^\circ\text{C}$  and pH 4,5 employing  $20 \text{ g dm}^{-3}$  Avicel PH 101 (FMC) and enzyme ( $3,0 \times 10^{-6} \text{ g}$ ) in the same buffer. Sodium azide ( $0,2 \text{ g dm}^{-3}$ ) was added to all reaction

mixtures.

Testing the specificity of the purified xylanase Ul. Enzyme ( $1,0 \times 10^{-6}$  g) was incubated with  $5,0 \text{ g dm}^{-3}$  substrate in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, ( $0,51 \text{ cm}^3$ ) at  $40^\circ\text{C}$  for 3 hours before measuring the release of reducing sugars. The substrates (Sigma) were: CMC (D.S. 0,7), arabinogalactan and galactomannan. Sodium azide ( $0,2 \text{ g dm}^{-3}$ ) was added to all reaction mixtures.

Defining a unit of polysaccharide degrading activity. The conventional way of expressing enzyme activity is in terms of enzyme units. One unit of enzyme activity is the amount of enzyme catalysing the transformation of  $1,0 \times 10^{-6}$  mol of substrate per minute under defined assay conditions (62). This definition is not always suitable in the case of cellulases and xylanases. One reason is that the molecular weight of the substrate is not always known. In addition, these enzymes are usually assayed by measuring the rate of product formation rather than substrate transformation. In theory, the latter difficulty could be overcome by applying a knowledge of the stoichiometry of the reaction to the calculation of substrate utilisation from the observed product formation. However, during cellulase or xylanase catalysed degradation, a variety of different products is often formed. The individual isolation and assay of these products proves too time consuming for routine enzyme assay. Instead an average, non-stoichiometric measurement of product concentration is made in the reducing sugar assay. This non-stoichiometric measurement cannot be used to calculate the corresponding substrate transformation with

accuracy.

Probably owing to the abovementioned difficulties, there seems to be at present no generally accepted definition of a unit of cellulase or xylanase activity. However, in order to express enzyme activity in a concise way, it is useful to define an arbitrary enzyme unit. Accordingly, in this thesis, an enzyme unit is defined as the amount of enzyme that will catalyse the production of  $1,0 \times 10^{-6}$  mol of reducing sugar per minute under the stated assay conditions. Reducing sugar is measured using either glucose or xylose as a reference standard in the case of cellulases and xylanases respectively. It is stressed that the molar extinction coefficients of the reaction products are not necessarily identical to those of the standards and, therefore, only relative product concentration is being estimated. Further, for reasons already given above, uniform assay conditions were not used in this thesis. It is, consequently, not always possible to compare directly the enzyme activities reported for different experiments.

#### 2.4.2 Enzyme activity on aryl- $\beta$ -glycosides

As in the case of polysaccharide degrading activity, different assay conditions were used to suit different purposes. Both *p*-nitrophenyl- $\beta$ -D-glucoside (PNG) and *o*-nitrophenyl- $\beta$ -D-xyloside (ONX) were employed as substrates. A typical assay (63) was performed as follows: a suitable amount of enzyme was incubated with  $4,0 \times 10^{-3}$  mol dm<sup>-3</sup> PNG in 0,1 mol dm<sup>-3</sup> sodium acetate buffer (0,9 cm<sup>3</sup>) at 50°C for 10 minutes. The reaction was quenched by adding 0,25 mol dm<sup>-3</sup> sodium carbonate (3,0

cm<sup>3</sup>) before reading absorbance at 401 nm.

Testing the specificity of F2 and U1. The action of F2 and U1 on both PNG and ONX was tested using the same assay conditions, already described, for testing the specificities of the respective enzymes on polysaccharide substrates. The extent of reaction was monitored by the absorbance at 401 nm.

#### 2.4.3 Enzyme activity on oligosaccharide substrates

Cellooligosaccharides. In a typical assay oligosaccharide or PNG (in transglycosylation experiments) ( $2,0 \times 10^{-3}$  g) was mixed with enzyme (ca.  $3,0 \times 10^{-6}$  g) in  $0,05 \text{ mol dm}^{-3}$  ammonium acetate buffer, pH 4,5 ( $0,11 \text{ cm}^3$ ), and the mixture incubated at 50 to 52°C for 2 hours. The course of oligosaccharide degradation was monitored in the same buffer and at the same temperature in a total of  $1,5 \text{ cm}^3$  containing enzyme ( $1,5 \times 10^{-5}$  g) and substrate ( $1,5 \times 10^{-2}$  g). Samples ( $0,2 \text{ cm}^3$ ) were withdrawn at intervals and quenched by boiling for 5 minutes.

Xylooligosaccharides. Oligosaccharide was mixed with enzyme (ca.  $4,0 \times 10^{-7}$  g) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5 ( $0,06 \text{ cm}^3$ ) containing  $0,2 \text{ g dm}^{-3}$  sodium azide, and the mixture incubated at 50°C for 16 hours. The approximate masses ( $\text{g} \times 10^{-3}$ ) of xylooligosaccharides in the reaction mixture, were: xylobiose (4,0), xylotriose (10), xylo-tetraose (1,0), xylopentaose (0,3) and xylohexaose (0,3). Similar assay conditions applied when examining the products of xylanase action on ONX except that ammonium acetate buffer was employed. The final ONX

concentration in the reaction mixture was  $8,5 \times 10^{-3} \text{ g cm}^{-3}$ .

## 2.5 Assay for reducing sugar

For the assay of reducing sugars, a modified form of the Nelson-Somogyi procedure (64) was always used unless stated otherwise. The method closely resembled the method of Marais, de Wit and Quicke (65).

### Reagents

- A: sodium carbonate (25 g), sodium potassium tartrate (25 g) sodium bicarbonate (20 g) and sodium sulphate (200 g) were dissolved in water, made up to  $1,0 \text{ dm}^3$ , filtered and stored.
- B: copper sulphate pentahydrate (30 g) was dissolved in  $200 \text{ cm}^3$  of water containing  $0,4 \text{ cm}^3$  of concentrated sulphuric acid.
- C: 1 part of B was added to 25 parts of A (prepared fresh daily).
- D: ammonium molybdate (25 g) was dissolved in water and made up to  $450 \text{ cm}^3$ , concentrated sulphuric acid ( $21 \text{ cm}^3$ ) was added and the resulting solution thoroughly mixed.
- E: sodium arsenate heptahydrate (3 g) was dissolved in distilled water ( $25 \text{ cm}^3$ ).

F: D and E were mixed together, made up to 500 cm<sup>3</sup> with water and incubated either for 24 hours at 37°C or for 30 minutes at 55°C. This reagent was stored in a dark bottle.

Procedure. Sugar solution (1,0 cm<sup>3</sup>) was mixed with reagent C (1,0 cm<sup>3</sup>) in a Folin-Wu tube of 25 cm<sup>3</sup> capacity and heated in a boiling water bath for 20 minutes. After heating, the tube was removed and cooled in tap water. Reagent F (1,0 cm<sup>3</sup>) was added, followed by vigorous shaking, to ensure good mixing as well as to hasten evolution of carbon dioxide. After standing at room temperature for 10 minutes, the contents were made to volume (either 12.5 or 25 cm<sup>3</sup>) and their absorbance read at 520 nm. Standard curves relating absorbance to sugar content over the range 0,0 to 2,5 x 10<sup>-4</sup> g were constructed for xylose and glucose.

## 2.6 Estimation of carbohydrate

The method employed to determine total carbohydrate was that of DuBois, Gilles, Hamilton, Rebers and Smith (66).

### Reagents

Phenol reagent: Phenol (55,0 g) was made up in distilled water to 500 cm<sup>3</sup> and the mixture was distilled at 99°C to obtain the azeotrope. Azeotrope (250 g) was mixed with distilled water (410 cm<sup>3</sup>), yielding the desired phenol concentration, namely 50 g dm<sup>-3</sup>. This reagent was stored in a dark bottle.

Procedure. Carbohydrate solution ( $1,0 \text{ cm}^3$ ) was mixed with phenol reagent ( $0,5 \text{ cm}^3$ ). Concentrated sulphuric acid ( $2,5 \text{ cm}^3$ ) was added in a single operation and the resulting mixture vigorously shaken. Solutions were left at room temperature for 30 minutes prior to the determination of absorbance. Pentose content was estimated from the absorbance at 480 nm and hexose from the absorbance at 490 nm from standard curves constructed using xylose ( $0,0$  to  $1,0 \times 10^{-4} \text{ g}$ ) and glucose ( $0,0$  to  $8,0 \times 10^{-5} \text{ g}$ ) respectively.

## 2.7 Estimation of protein

Protein was determined according to the method of Hartree (67).

### Reagents

- A: potassium sodium tartrate (2,0 g) and sodium carbonate (100 g) were dissolved in  $500 \text{ cm}^3$  of  $1 \text{ mol dm}^{-3}$  sodium hydroxide and made to  $1,0 \text{ dm}^3$  with distilled water.
- B: potassium sodium tartrate (2,0 g) and copper sulphate pentahydrate (1,0 g) were dissolved in  $90 \text{ cm}^3$  of distilled water to which  $10 \text{ cm}^3$  of  $1 \text{ mol dm}^{-3}$  sodium hydroxide was added.
- C: commercial Folin-Ciocalteu reagent was diluted with 15 volumes of water immediately before use.

Procedure. Protein solution ( $1,0 \text{ cm}^3$ ) was mixed with reagent A ( $0,9 \text{ cm}^3$ ) and heated at  $50^\circ\text{C}$  for 10 minutes. The solution was cooled in tap water prior to addition of reagent B ( $0,1 \text{ cm}^3$ ). After standing at room temperature for 10 minutes, reagent C ( $3,0 \text{ cm}^3$ ) was added with vigorous stirring and the resulting mixture was again heated at  $50^\circ\text{C}$  for 10 minutes. After cooling in tap water the absorbance was read at 650 nm.

## 2.8 Column chromatography

### 2.8.1 Avicel PH 102

Several authors (68-71) have successfully fractionated cellulases on columns of crystalline cellulose, in each case applying the protein to the column at relatively high ionic strength and eluting with buffers of decreasing ionic strength or with water. Under these conditions at least two cellulase fractions were invariably obtained, one that eluted under the starting conditions and another that became desorbed at low ionic strength. Work with rumen cellulases (72), however, suggests that it is not merely a lowering of the ionic strength that promotes desorption of bound cellulases. Instead, there seems to be an interval of values of the ionic strength over which adsorption is maximal, desorption occurring at values on either side of this interval. Neither the precise nature of the cellulose/cellulase interaction nor its biological significance seems to be known with certainty.

Avicel appears to have a comparatively large capacity for binding crude cellulase (71) and chromatography on Avicel was therefore considered suitable as a preliminary enzyme fractionation step. In addition, since at optimal ionic strength there appeared to be no gradual desorption of adsorbed cellulases from the cellulose, use could be made of a column with large diameter to height ratio. Problems arising from cellulose bed compression were consequently avoided and relatively high flow rates were obtained.

Preparation and operation of the Avicel column. Dry cellulose powder (Avicel PH 102) was suspended in 2 to 3 volumes of  $0,02 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, and allowed to settle before decanting the supernatant. The procedure was repeated until the supernatant, which at first was tinged, became colourless.

The suspension was left overnight at ca.  $4^{\circ}\text{C}$  and packed in a column (4,0 x 9,0 cm). The column was operated at a temperature of ca.  $4^{\circ}\text{C}$  and was fitted at the top with a glass fibre disc to prevent disturbance of the bed surface. The Avicel was further equilibrated by passing through it 1 to 2 bed volumes of  $0,02 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6. Buffer was removed from the Avicel surface before applying the sample.

The sample was prepared by suspending acetone powder (10 g) in  $0,02 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, ( $40 \text{ cm}^3$ ) and centrifuging (in a bench top centrifuge) to remove undissolved solids. The supernatant was applied to the column and allowed to drain into the

Avicel bed under gravity. Protein was eluted from the column with 0,02 mol dm<sup>-3</sup> sodium phosphate buffer (1 bed volume) followed by 2 to 3 bed volumes of the same buffer after 40 fold dilution. Fractions (200 drops, equivalent to ca. 12 cm<sup>3</sup>) were collected at a flow rate of 1,0 cm<sup>3</sup> per minute.

#### 2.8.2 DEAE-Sephadex A-50

The anion exchange resin, DEAE-Sephadex, has proved useful in the resolution of both cellulases and hemicellulases from a variety of microorganisms (23,73,74). This resin, particularly type A-50, exhibits a comparatively high (available) capacity for protein, and consequently this type was chosen for further resolution of fractions resulting from prior chromatography on cellulose. Although the use of a cationic buffer is recommended for use with anion-exchange resins, several authors (23,73,74) have achieved success using anionic buffers (notably acetate and formate), encouraging the author to do likewise. In practice there exists a wide choice of possible anionic buffers, but as the samples to be applied to the ion-exchanger were already dissolved in phosphate buffer following chromatography on cellulose, the DEAE-Sephadex was prepared in the phosphate form. In this way the necessity of changing the sample buffer was avoided.

Preparation and operation of the DEAE-Sephadex column. DEAE-Sephadex A-50 was prepared in the phosphate form according to the manufacturer's general instructions for changing the counter ion. After fully swelling the resin in 0,01 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6,6, it

was packed in a column (24 x 2,5 cm) and further equilibrated by passage of 2 bed volumes of the same buffer through the resin bed. The column was operated at ca. 4°C.

Protein sample dissolved in 0,01 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6,6, was applied to the top of the column and pumped into the resin bed. Elution was effected with, in sequence, 1,5 bed volumes of (i) 0,01 mol dm<sup>-3</sup> sodium phosphate buffer pH 6,6, (ii) 0,05 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6,6, and (iii) ca. 4 bed volumes of 0,05 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6,6, containing 0,5 mol dm<sup>-3</sup> sodium chloride. Fractions (ca. 12 cm<sup>3</sup>) were collected at a flow rate of 1,0 cm<sup>3</sup> per minute.

### 2.8.3 Molecular exclusion chromatography

In molecular exclusion chromatography solutes are fractionated according to molecular size on columns of gel beads, most commonly dextran or polyacrylamide. Separation is effected under mild conditions of both pH and ionic strength and the method is therefore ideally suited to enzyme purification. An added advantage is that the molecular weights of solutes can be deduced from a knowledge of their elution volumes on suitably calibrated columns.

Since many polysaccharide degrading enzymes are glycoproteins, wherever possible, polyacrylamide gels were used in preference to dextran gels. This precaution was taken to minimise complications that might have arisen from the interaction between the dextran beads and

the carbohydrate moiety of the glycoprotein (75).

Molecular exclusion chromatography was used to fractionate both proteins and mixtures of oligosaccharides.

Chromatography of proteins on Bio-Gel. Both Bio-Gel P-100 and P-150 were used to fractionate proteins. The general method of preparation and operation of the columns was the same in either case and followed the guidelines of the Bio-Rad handbook (75).

Resins were equilibrated with  $0,05 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, at room temperature. Protein sample, dissolved in the equilibration buffer ( $2,0$  to  $5,0 \text{ cm}^3$ ) was applied at the top of the column, and eluted with the same buffer at a flow rate of ca.  $10 \text{ cm}^3$  per hour. The fraction size was usually  $4,5 \text{ cm}^3$ . Column dimensions were  $100 \times 2,5 \text{ cm}$  for the P-100 and  $145 \times 2,5 \text{ cm}$  for the P-150 resin.

Chromatography of xylooligosaccharides on Sephadex G-15. Chromatography on Sephadex G-15 was employed to isolate xylooligosaccharides from either acid or enzymic digests of xylan. The resin was equilibrated in distilled water at room temperature and packed into a column ( $124 \times 1,6 \text{ cm}$ ). Oligosaccharides, dissolved in either distilled water ( $1,0$  to  $2,0 \text{ cm}^3$ ) or, in some cases, in a similar volume of  $0,05 \text{ mol dm}^{-3}$  acetate buffer, pH 4,5, were loaded at the top of the column and eluted with water at room temperature. Fractions ( $1,8 \text{ cm}^3$ ) were collected at a flow rate of  $12 \text{ cm}^3$  per hour.

#### 2.8.4 Chromatography of cellooligosaccharides on a mixture of carbon and celite

Cellooligosaccharides of degree of polymerisation 2 to 6 were generated from Avicel PH 101 by the method of Miller (76) and subsequently chromatographed on a carbon-celite column.

Preparation and operation of the column. The column (91 x 5,5 cm) was prepared from a 1:1 mixture of Darco G-60 and celite 545 according to Miller (76) and operated at room temperature. After loading the sample and eluting glucose with water (2,5 dm<sup>3</sup>), oligosaccharides were eluted using a water/ethanol gradient. The subsequent elution of oligosaccharides of D.P.  $\geq 2$  will be described in some detail since it differs from the published procedure.

Elution was commenced at a flow rate of 33 drops per minute (ca. 2,0 cm<sup>3</sup> per minute) employing a linear gradient of 40% (v/v) ethanol (in the donor vessel) and water. The fraction size was 220 drops (ca. 13 cm<sup>3</sup>). At fraction 203, after approximately 4,0 dm<sup>3</sup> had flowed through the column, the contents of the donor vessel were replaced by 50% (v/v) ethanol (2,0 dm<sup>3</sup>) and 30% (v/v) ethanol (1,0 dm<sup>3</sup>) was added to the recipient vessel.

As the alcohol content of the eluant increased, so the drop size decreased and it became necessary, periodically, to increase the number of drops per fraction so as to maintain a constant fraction volume. Details of these adjustments are given in the legend to Fig. 21.

## 2.9 Electrofocusing

In recent years electrofocusing has become a powerful technique for resolving the components of heterogeneous protein mixtures. The method involves the generation of an electrical field in a solution containing the protein sample together with an appropriate mixture of ampholytes. The ampholytes are normally polyamino-polycarboxylic acid compounds which, under the influence of an applied electric field, establish a stable linear pH gradient. Each protein species bearing a net electric charge in the solution will likewise migrate in the field to a region of the gradient in which the pH will be equal in value to the pI of the protein. At this point the protein bears no net charge and in consequence stops moving. In this manner proteins exhibiting different pI values become focused at different regions in the pH gradient and are thus separated from one another.

### 2.9.1 Preparative column electrofocusing

Preparative electrofocusing was performed at a constant power of 15 W in the LKB 8101 column (capacity: 110 cm<sup>3</sup>) employing a manually constructed sucrose density gradient. Both the construction of the density gradient and the incorporation of the protein sample were as recommended by the manufacturers. The temperature was maintained between 5 and 10°C and the focusing allowed to proceed for 16 to 20 hours. At the end of this period the column was emptied by pumping water in at the top at a flow rate of 2,0 cm<sup>3</sup> per minute and effluent was collected in fractions (2,4 cm<sup>3</sup>). The pH of each fraction was

determined at the running temperature using a glass electrode.

### 2.9.2 Preparative flat-bed electrofocusing

The LKB 2117 Multiphor was employed for preparative flat-bed electrofocusing. Construction of the Sephadex G-50 flat-bed and incorporation of the protein sample were according to the manufacturers recommendations. Samples were focused at a constant power of 6 W for 18 hours and the temperature was maintained at 7°C. On completion of the focusing step, the gel was sliced into 1 cm wide segments and each segment suspended separately in 0,12 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6.5 (1,0 cm<sup>3</sup>). Protein present in the gel suspension was recovered by filtering and washing the gel with more of the same buffer (2,0 cm<sup>3</sup>).

### 2.9.3 Analytical electrofocusing

Analytical electrofocusing was performed in polyacrylamide gels using the LKB 2117 Multiphor. Gel slabs were either bought in pre-cast form from LKB or alternatively were prepared in the laboratory. Gels prepared in the laboratory were cast in a rectangular glass mould consisting of three glass plates of identical length and breadth. Two of the plates were 0,3 mm thick and constituted the two broad, outer walls of the mould. These two plates were separated from each other by, in order, a silicone rubber gasket and a thinner (1 mm) plate, the thin plate acting as a gel support once the gel was removed from the mould. The side of this plate facing away from the gasket rested flat

against a thick plate. Gel solution was polymerised in the space bounded by a thick plate, the thin plate and the rubber gasket. It is tacitly assumed (73) that on opening the mould following polymerisation the gel will adhere to the thin plate alone, however, more often than not the gel either stuck to the thick plate or, in some cases, to neither plate. The problem was solved by silanising the thin plate in Silane Al74 before assembling the mould.

Silanisation of gel support plates. Silanising solution was made by dissolving Silane Al74 (Pharmacia) ( $8,0 \text{ cm}^3$ ) in distilled water ( $2,0 \text{ dm}^3$ ), previously adjusted to pH 3,5 with glacial acetic acid. The final solution was slightly turbid. Glass plates were soaked in silanising solution for 1 hour at room temperature, after which they were rinsed in distilled water and air-dried.

Preparation of the gels and electrofocusing. Further details of the preparation of the gel and the loading of protein samples are given by Winter, Ek and Andersson (77). Electrofocusing was normally carried out for 1,5 hours over the pH range 3,5 to 9,5 or for 2,5 hours when a narrower pH range was selected. Power was maintained at  $0,09 \text{ W cm}^{-2}$  of gel surface and the temperature at between 3 and  $7^\circ\text{C}$ . Glycerol was omitted from the gel solution in cases where enzymes were to be eluted after focusing.

## 2.10 Polyacrylamide gel electrophoresis (PAGE)

Originally developed by Ornstein (78) and Davis (79), disc gel

electrophoresis is designed to concentrate proteins into thin starting zones prior to their resolution by the co-operative effects of electrophoresis and molecular sieving. Starting with narrow protein zones has the advantage of partially offsetting the subsequent zone broadening brought about by thermal diffusion and leads ultimately to sharper definition in the protein bands following separation.

Gels for disc electrophoresis are cast in two segments which differ as to both pH and average pore size and are designated respectively the spacer gel, and the running gel. Proteins are initially layered on the spacer gel which is a lightly cross-linked structure with large average pore size and which, in consequence, does not impede the passage of ions through it. In contrast the running gel, in which the actual separation occurs, has a comparatively small average pore size and thus restricts the velocity of large ions moving through the matrix. This retardation increases with the molecular size of the ion and the final separation achieved will depend on the size as well as on the charge of the constituent proteins.

Conditions are chosen such that proteins, once they enter the spacer gel, constitute an ionic species with mobilities intermediate between a leading ion present in the spacer gel and a trailing ion present in the reservoir buffer above this gel segment. In the presence of an electric field the leading ions endeavour to move ahead of the trailing ions, leaving behind their common boundary a region of lower conductance and therefore of greater field strength. This field discontinuity serves to accelerate the trailing ions and a steady

current is rapidly established under conditions in which both leading and trailing ions obey the relationship:

$$\frac{dx}{dt} \propto E\mu = \text{a constant}$$

where  $\frac{dx}{dt}$  is the velocity,  $E$  is field strength (the voltage gradient) and  $\mu$  is the mobility (78). Thus a situation is reached where, despite their different mobilities, both leading and trailing ions are travelling at the same velocity down the gel. Because of their intermediate mobilities protein ions ahead of the leading ion-trailing ion boundary are slowed relative to the leading ions and fall back. On the other hand, protein ions behind the boundary are accelerated in the higher field to velocities exceeding the boundary itself. As a result, the proteins are confined to an extremely thin zone between the leading and the trailing ions.

At the interface between the spacer gel and the running gel the boundary experiences an abrupt change in pH, the magnitude of which is so designed as to increase the mobility of the trailing ion to a value exceeding that of the proteins. Consequently, on reaching the running gel, the boundary overtakes the proteins leaving these to separate down the length of this gel in a uniform electric field.

The PAGE system used here resembled closely system I of Gabriel (80). It was, however, observed that narrow protein zones could be obtained with or without a stacking gel, and consequently a stacking gel was not always employed. Since the method employed for preparative electrophoresis was based on the analytical system, the latter will be

described first.

### 2.10.1 Analytical electrophoresis

#### Reagents

Stock solutions :-

- A: tris base (36,3 g), TEMED (N,N,N',N'-tetramethylenediamine) (1,0 cm<sup>3</sup>) and 1 mol dm<sup>-3</sup> hydrochloric acid (48,0 cm<sup>3</sup>) were made up to a final volume of 100 cm<sup>3</sup> with distilled water. The pH was 8,9.
- B: acrylamide (45 g) and N, N'-methylenebisacrylamide (1,2 g) were dissolved and made up to 100 cm<sup>3</sup> with distilled water.
- C: acrylamide (10 g) and N, N'-methylenebisacrylamide (2,5 g) were made up to 100 cm<sup>3</sup> with distilled water.
- D: tris base (1,45 g) and 1 mol dm<sup>-3</sup> hydrochloric acid (6,4 cm<sup>3</sup>) were made up to 100 cm<sup>3</sup> with distilled water. The pH was 6,9.
- E: ammonium persulphate (0,014 g) dissolved in distilled water (10 cm<sup>3</sup>) was made immediately before use.

Working solutions:-

Running gel: 1 part A  
1,3 parts B  
3,2 parts distilled water  
2,5 parts E

Spacer gel : 1,5 parts C  
1 part D  
0,5 parts distilled water  
1 part E  
0,02 cm<sup>3</sup> TEMED

Reservoir buffer: tris base (1,2 g) and glycine (5,76 g) were dissolved and made up to 1,0 dm<sup>3</sup> with distilled water. The final pH was 8,3.

Application buffer: glycerol (5,0 cm<sup>3</sup>), D (5,0 cm<sup>3</sup>), mercaptoethanol (0,036 to 0,05 cm<sup>3</sup>) and 0,6 cm<sup>3</sup> of a 5,0 g dm<sup>-3</sup> aqueous solution of bromophenol blue were mixed.

Procedure. Cylindrical glass tubes (10 x 0,5 cm), thoroughly cleansed with chromic acid, were clamped in a vertical position, and one end of each tube was sealed with a rubber cap or alternatively with Parafilm. Running gel solution was introduced carefully with a Pasteur pipette to a height of 7 cm, the surface was gently overlaid with distilled water (ca. 0,02 cm<sup>3</sup>) and the solution allowed to polymerise. (The time required for the gel to form was estimated by observing the rate of polymerisation of an aliquot of running gel solutions retained in the

Pasteur pipette). Occasionally polymerisation was unduly delayed in which case it was found expedient to de-aerate the running gel solution prior to the addition of ammonium persulphate. After the gel had set, the water layer was removed by absorption into a strip of tissue paper, stacking gel solution was introduced to a height of 1,0 cm and subsequently treated in a fashion analogous to that of the running gel solution. Following polymerisation of the stacking gel solution, the cap (or Parafilm) was removed and replaced by a drop of reservoir buffer which remained suspended from the end of the gel and displaced any air pockets which might have formed. Tubes were clamped in the electrophoresis tank, submerging the running gel end in the lower electrode buffer. The sample ( $0,1 \text{ cm}^3$ , containing  $1,0 \times 10^{-4} \text{ g}$  protein) was mixed with application buffer ( $0,025 \text{ cm}^3$ ) and applied to the top of the stacking gel. After filling the space above the (dense) sample with (less dense) electrode buffer, the upper chamber was filled and electrophoresis commenced at a constant current of 3 mA per tube and a temperature of 5 to  $10^\circ\text{C}$  until the bromophenol blue marker had reached the end of the running gel. Gels were stained by overnight immersion in a 23% (v/v) methanol solution containing Coomassie Blue ( $1,3 \text{ g dm}^{-3}$ ) and 4,6% glacial acetic acid. Gels were destained by soaking in methanol/acetic acid/water (10:15:175).

### 2.10.2 Preparative electrophoresis

Procedure. The LKB Uniphor 7900 was employed for preparative electrophoresis. Preparation of both the protein samples and the gels were as described for analytical electrophoresis, except that stacking

gel was omitted. The Uniphor was assembled and operated according to the manufacturer's instructions.

The gel was polymerised as a column (10 x 2,5 cm) and electrophoresis effected at an initial power of 20 W. Voltage was normally set so as not to exceed 1,5 Kv. The temperature of the gel was maintained at between 5 and 10°C. Fractions (4,0 cm<sup>3</sup>) were collected at room temperature at a flow rate of 12 to 15 cm<sup>3</sup> per hour.

### 2.10.3 SDS gel electrophoresis

Electrophoresis in SDS polyacrylamide gels, a technique originated by Shapiro, Vinuela and Maizel (81), was one way in which the changing composition of protein fractions was monitored during enzyme purification. SDS (sodium dodecyl or lauryl sulphate) is an ionic detergent which complexes with protein in a fixed proportion by mass, approximately 1,4 g of SDS per g of protein in the majority of cases. Moreover, following complex formation, the natural charge on the protein becomes masked, being replaced by a net negative charge whose magnitude is determined solely by the mass of SDS bound. As a result individual SDS-protein complexes exhibit a constant charge to mass ratio and, in the absence of retarding forces, possess almost identical mobilities. When subjected to electrophoresis in a gel, however, the complexes experience a steric resistance to their movement through the gel matrix, larger components being more severely retarded than smaller ones and in this way separation is achieved. Because protein mobility in SDS gels is a function solely of molecular size, and further, since

SDS-protein complexes all assume a similar rod shape, the method can be used to estimate protein molecular weights. In practice absolute mobilities are not normally measured, instead mobility relative to some small molecule (e.g. bromophenol blue) is determined. This is probably done to minimize the effects of slight differences in experimental conditions (e.g. gel composition) on separately treated samples.

### Reagents

#### Stock solutions :-

- A: sodium dihydrogen phosphate monohydrate (7,81 g), disodium hydrogen phosphate dihydrate (25,6 g) and sodium dodecyl sulphate (2,0 g) were made up to a final volume of  $1,0 \text{ dm}^3$  with distilled water.
- B: acrylamide (11,1 g) and N,N'-methylenebisacrylamide (0,3 g) were dissolved and made up to  $50 \text{ cm}^3$  with distilled water.
- C: sodium dihydrogen phosphate monohydrate (1,08 g) and disodium hydrogen phosphate dihydrate (2,17 g) were made up to  $200 \text{ cm}^3$  with distilled water.
- D: sodium dodecyl sulphate (1,0 g) and mercaptoethanol ( $0,9 \text{ cm}^3$ ) were made up to  $10 \text{ cm}^3$  with solution C previously diluted 10 fold.
- E: ammonium persulphate ( $0,015 \text{ g cm}^{-3}$ ) was made up in distilled water.

## Working Solutions:-

Gel solution: 15,0 cm<sup>3</sup> A  
10,13 cm<sup>3</sup> B  
1,5 cm<sup>3</sup> E  
3,33 cm<sup>3</sup> distilled water  
0,045 cm<sup>3</sup> TEMED

Application buffer: glycerol (5,0 cm<sup>3</sup>), C (5,0 cm<sup>3</sup>) and 5,0 g dm<sup>-3</sup> bromophenol blue (0,6 cm<sup>3</sup>) were mixed.

Reservoir buffer: 1 part A was mixed with 1 part distilled water.

Procedure. The procedure used closely resembled the procedure described in the BDH handbook (82). Sample (0,1 cm<sup>3</sup>, containing between  $1,5 \times 10^{-5}$  and  $1,0 \times 10^{-4}$  g protein) was mixed with solution D (0,01 cm<sup>3</sup>) and boiled for 2 minutes. After cooling, an aliquot of the treated sample (0,1 cm<sup>3</sup>) was mixed with application buffer (0,025 cm<sup>3</sup>) and mercaptoethanol (0,005 cm<sup>3</sup>) before being applied to the top of the gel (7 x 0,5 cm). Electrophoresis was performed at room temperature at a constant current of 8 mA per tube. In other respects the procedure was similar to that used for disc gel electrophoresis, with the exception that no stacking gel was present. Molecular weight markers (BDH), subjected to electrophoresis concurrently in a separate gel, were treated as recommended by the manufacturers (82). Staining of SDS-containing gels for protein was performed as for non-SDS gels (section 2.10.1).

Estimation of molecular mass. Protein molecular mass was estimated from a plot of protein mobility (relative to bromophenol blue) vs. log molecular mass (82) for the series of marker proteins with molecular weights spanning the range  $1,43 \times 10^4$  to  $7,15 \times 10^4$ .

### 2.11 Purification of Sigma xylan

Following sulphuric acid hydrolysis of Sigma xylan (see section 2.12), paper chromatographic analysis of hydrolysates revealed the presence of glucose, indicating the presence of a glucan contaminant in the original xylan preparation (see section 6.3.1). The glucan was removed by barium chloride precipitation using a procedure based on that reported by Taiz and Honigman (83).

Procedure. Crude xylan (10 g) was dispersed in distilled water ( $70 \text{ cm}^3$ ) and the suspension cooled in an ice bath. The xylan was dissolved by adding potassium hydroxide ( $30 \text{ cm}^3$  of a solution containing  $333,3 \text{ g dm}^{-3}$ ) accompanied by vigorous stirring. The volume of the resulting solution was measured and an aqueous solution containing  $360 \text{ g dm}^{-3}$  barium chloride (0,2 volumes) added dropwise in order to precipitate glucan. (During both the dissolution and precipitation steps the xylan suspension was kept on ice and periodically sparged with nitrogen). After centrifugation in a benchtop centrifuge, the precipitate was discarded and the supernatant brought to a pH of between 5 and 6 by addition of glacial acetic acid. The neutralised solution was left to stand for 1 to 2 hours before

centrifugation ( $233,3 \text{ s}^{-1}$ , 20 minutes,  $5^{\circ}\text{C}$ ) in a Beckman J-21 centrifuge using the JA-20 rotor. The resulting precipitate, comprising the purified xylan, was dialysed against distilled water and freeze-dried.

## 2.12 Acid hydrolysis of polysaccharides

### 2.12.1 Sulphuric acid hydrolysis

Polysaccharide (0,06 g) was suspended in water ( $5,0 \text{ cm}^3$ ), 72% sulphuric acid ( $0,2 \text{ cm}^3$ ) was added, and the suspension heated in a boiling water bath for 3 hours in a sealed tube.

### 2.12.2 Trifluoroacetic acid hydrolysis

Trifluoroacetic acid hydrolysis of purified xylan (Sigma) was performed according to the method of Havlicek and Samuelson (84), except that the hydrolysis period was shortened to 8 days.

## 2.13 Paper chromatography

### 2.13.1 Preparative paper chromatography

Preparative paper chromatography was employed to obtain pure individual xylooligosaccharides from acid or enzyme digests of xylan.

Procedure. Oligosaccharides (ca. 0,02 g) dissolved in distilled water

(ca.  $1,0 \text{ cm}^3$ ) or in some cases dilute ammonium acetate buffer pH 4,5, ( $0,05 \text{ mol dm}^{-3}$ ), were applied as a 17 cm long streak to Whatman 17 paper. Chromatograms were equilibrated overnight and developed in ethyl acetate/pyridine/water (10:4:3) by the descending method at room temperature. Resolution of smaller oligosaccharides (up to a D.P. of 4) normally required a 4 hour development and larger oligosaccharides a 6 to 8 hour development.

After drying the papers in air, two strips were cut from the chromatogram, parallel to the direction of development and 1,0 cm in from each end of the application streak. Oligosaccharides were visualised on these strips with a suitable reagent and subsequently used to determine the positions of the corresponding sugars on the unstained chromatogram. Areas of paper containing sugars were cut from the unstained chromatogram and extracted twice in water ( $2 \times 50 \text{ cm}^3$ ) for ca. 2 hours. Sugar solutions were concentrated either by freeze-drying or rotary evaporation below  $40^\circ\text{C}$ .

### 2.13.2 Analytical paper chromatography

Procedure. Whatman No. 1 paper was employed for analytical chromatography of carbohydrates. Monosaccharides ( $5,0 \times 10^{-5}$  to  $1,0 \times 10^{-4} \text{ g}$ ) and oligosaccharides ( $1,5 \times 10^{-4}$  to  $2,0 \times 10^{-4} \text{ g}$ ) were spotted on at the origin and papers were developed in ethyl acetate/pyridine/water either in the ratio 10:4:3 (for mono- and oligosaccharides) or 8:2:1 (for monosaccharides). Papers were equilibrated for 2 to 3 hours with the developing solvent and developed at room temperature. Development

times were 16 to 18 hours for monosaccharides and disaccharides and 24 hours for oligosaccharides.

Identification of oligosaccharides by paper chromatography. It appeared at first as though paper chromatography offered little aid in identifying oligosaccharides. Neither cello- nor xylooligosaccharides were available commercially as chromatographic standards. In addition, differences were often noted between published  $R_f$  values and those, obtained experimentally by the author, for known monosaccharides. There seemed, therefore, no reason to expect agreement between published and experimental  $R_f$  values for oligosaccharides either. Consequently comparative  $R_f$  data in the absence of standards was judged to be of little diagnostic value.

However, there is a method that can be used to infer the D.P. of members of a known homologous series of oligosaccharides and identify them (85). In the case of homopolymeric linear oligosaccharides the monomer is the only standard needed. Accurately reproducible  $R_f$  values are not necessary. The method involves chromatographing members of the homologous series, including the monomer, measuring the  $R_f$  of each and constructing a plot of (suspected) D.P. vs.  $\log(1 - R_f/R_f)$ . A straight line relationship confirms the suspected D.P. A method similar to that just outlined but using  $R_x$  in place of  $R_f$  (52) was used in this thesis to identify xylooligosaccharides.

The general procedure was similar to that already described for analytical paper chromatography except that temperature was kept

constant at 22°C and the equilibration time was extended to 16 hours. In order to ensure that oligosaccharides of D.P.  $\geq 4$  migrated a measurable distance, the developing solvent was allowed to run off the end of the paper. Since, during extended development times, xylose was eluted from the paper, the  $R_x$  values of the larger oligosaccharides had to be determined indirectly.

Two paper chromatograms were prepared and developed under identical conditions. Relatively fast-moving oligosaccharides, including the monomer, were chromatographed on one paper which was removed before the solvent front reached the end of the paper. The  $R_x$  values of these sugars was measured directly. The slower-moving oligosaccharides were chromatographed, alongside a faster-moving oligosaccharide (with known  $R_x$ ) on the second paper and the solvent allowed to run off the end of the paper. (The mobility of the faster-moving oligosaccharide had to be such that this oligosaccharide was not eluted from the paper during the longer development time). The distance moved by the slower oligosaccharides was calculated as a fraction (Q) of the distance moved by the faster oligosaccharide. Since the  $R_x$  value of the faster oligosaccharide was known from direct measurements on the first paper, the  $R_x$  value of the slow-moving oligosaccharides could be calculated according to the formula:

$$R_x \text{ (of slow oligosaccharide)} = Q \times R_x \text{ (of faster moving oligosaccharide)}.$$

Visualisation of reducing sugars on paper chromatograms. Reducing sugars were visualised with alkaline silver nitrate reagent (86). Air-dried papers were dipped in a solution containing 30% (w/v) aqueous silver nitrate (1,5 cm<sup>3</sup>) in acetone (100 cm<sup>3</sup>). After drying in air, papers were sprayed with alcoholic sodium hydroxide made by dissolving saturated aqueous sodium hydroxide (2,0 cm<sup>3</sup>) in ethanol (25 cm<sup>3</sup>). When visualisation was complete (5 minutes), chromatograms were dipped in 5 to 10% (w/v) aqueous sodium thiosulphate, washed in tap water and dried.

#### 2.14 Thin layer chromatography (TLC)

An analytical thin layer chromatographic method was developed by the author (59) that achieved a comparatively rapid separation of both xylo- and cellooligosaccharides. An added advantage of the method was that oxidised and reduced cellooligosaccharides present in admixture, could be completely resolved in a single dimension.

Procedure. Carbohydrate ( $2,0 \times 10^{-5}$  to  $8,0 \times 10^{-5}$  g) was spotted on at the origin of a 0,05 cm thick Kieselgel 60 (Merck) layer. The developing solvent, ethyl acetate/acetic acid/water (3:3:1) was allowed to ascend the plate twice to a height of ca. 12 cm.

Visualisation of sugars on TLC plates. Reducing sugars were detected by spraying with aniline phthalate spray reagent (Merck). Both reducing and non-reducing sugars were visualised by spraying with 20% (v/v) sulphuric acid in ethanol. After spraying with either reagent,

plates were heated at 100 to 150°C for 5 to 10 minutes.

### 2.15 Determination of the degree of polymerisation of xylooligosaccharides by sodium borohydride reduction

There are, in principle, several ways to establish the degree of polymerisation of linear oligosaccharides. These ways include: (i) a stoichiometric determination of reducing end groups, e.g. by hypiodate oxidation or the Kiliani reaction (87), (ii) periodate oxidation (88), and, (iii) methylation analysis (88). The first two methods require an accurate knowledge of the mass of starting material. This is not always a simple requirement, particularly in those cases where only relatively small amounts of carbohydrate are available or where the water of crystallisation is not accurately known. The third method does not suffer from this disadvantage since it relies on the determination of relative quantities of methylated products. However, the identification of methyl derivatives (by paper or gas chromatography) could prove difficult for the non-specialist.

In order to obviate the difficulties mentioned above, a simple method was developed for the determination of the degree of polymerisation of linear xylooligosaccharides. At the time of writing, however, it was discovered that a similar procedure had, in fact, been published some years before (89). The method possesses the following advantages: (i) there is no requirement for specialised instrumentation, (ii) a relatively simple non-stoichiometric end group assay (e.g. Nelson-Somogyi) can be employed, (iii) an accurate knowledge of

the mass of starting material is not required, and (iv) as little as  $1,0 \times 10^{-4}$  g of carbohydrate suffices for a single determination.

In outline, the procedure involves the hydrolysis, in parallel, of two samples of the unknown oligosaccharide, one of which has been previously reduced with sodium borohydride, the other serving as a control. Following hydrolysis, the neutralised hydrolysates are assayed for monosaccharide content by the Nelson-Somogyi method. Reaction with borohydride renders the reducing terminal of the oligosaccharide unreactive to the Nelson-Somogyi reagents. Consequently, following hydrolysis, the reduced oligosaccharide sample yields, (on a molar basis), one less reducing monosaccharide than the control. From the measured ratio,  $\lambda$ , of monosaccharide in the reduced sample to that in the control the degree of polymerisation of the oligosaccharide can be calculated according to the formula:

$$\text{degree of polymerisation} = \frac{1}{1 - \lambda}$$

Procedure. Oligosaccharide ( $1,0 \times 10^{-4}$  to  $2,0 \times 10^{-4}$  g) was incubated with sodium borohydride ( $5,0 \times 10^{-4}$  g) in water ( $0,8 \text{ cm}^3$ ) in a rimless test-tube at  $26^\circ\text{C}$  for 1 hour.  $5,0 \text{ mol dm}^{-3}$  sulfuric acid ( $0,2 \text{ cm}^3$ ) was added, the tube was evacuated, sealed in a flame and placed in a boiling water bath for 1,5 hours. The contents of the tube were neutralised with  $4,0 \text{ mol dm}^{-3}$  sodium hydroxide ( $0,5 \text{ cm}^3$ ), transferred quantitatively to a  $2,0 \text{ cm}^3$  volumetric flask and made to volume. A sample ( $0,9 \text{ cm}^3$ ) was withdrawn from the volumetric flask, and mixed with  $0,1 \text{ cm}^3$  of  $0,5 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, in a Folin

Wu tube before determining the reducing sugar content. A control, in which sodium borohydride was omitted, was run in parallel with each sample.

## CHAPTER 3

STUDIES ON MYCELIAL GROWTH AND POLYSACCHARIDASE PRODUCTIONBY SCLEROTIUM ROLFSSII3.1 Carboxymethylcellulase and xylanase production in liquid culture

Both the quantity and the quality of polysaccharide degrading enzymes elaborated by an organism depend on the nature of the carbon source supporting the organism's growth. It is known for example that cellulases can be induced either by their substrate or, in some cases, by various small molecules such as sophorose and cellobiose which may be added to the growth medium (90,91). In addition, where cellulose is the inducer, the physical structure of the substrate can effect the specificities of the induced cellulases (92). Hemicellulases, in contrast, generally appear to be constitutive enzymes although recently a xylanase from the yeast Cryptococcus albidus has been shown to be induced by both xylan and xylooligosaccharides (93). The carbon source is however not without effect, even on constitutive hemicellulase production, since synthesis of these enzymes can be subject to substrate repression (47).

In view of the above considerations it became necessary to determine which carbon sources best supported enzyme synthesis before initiating large scale enzyme production.

### 3.1.1 The Carbon Sources

Both cellulosic and hemicellulosic substrates were used as carbon sources for S. rolfsii. Details of individual substrates are presented below.

Cellulosic carbon sources. Microcrystalline cellulose (MXC) belongs to a category of celluloses known as hydrocelluloses. Hydrocelluloses are defined as "a group of macromolecular substances formed by the hydrolysis of cellulose with an acid, any member of the group being a hydrocellulose" (94). The effect of controlled acid hydrolysis is to preferentially degrade and solubilise the amorphous regions of the cellulose leaving the crystalline regions unchanged. This treatment results in a hydrocellulose product with a reduced degree of polymerisation but an enhanced degree of crystallinity. MXC is such a product and as it consists almost entirely of crystalline material, it is representative of a highly ordered cellulosic substrate.

CMC is a water soluble polymer derived from cellulose by introducing carboxymethyl substituents at carbons 2, 3 and 6 of the anhydroglucose units constituting the polymer (95). It is these substituents which are responsible for conferring solubility on the otherwise insoluble cellulose. Several types of CMC are commercially available, manifesting a wide range of characteristics due to variations in the degrees of polymerisation and substitution. For purposes of growing the organism in liquid culture, a CMC of low viscosity type (Sigma, D.S. 0,7 and D.P. 200) was employed in order to

minimise any limitations on growth imposed by diffusion of nutrients and gases through a viscous medium. CMC is an example of a non-crystalline, soluble cellulose substrate.

Filter paper (Whatman No.1) represents a substrate intermediate in crystallinity between MXC and CMC, in that filter paper contains both ordered crystalline regions and regions of disorder (96).

Mixed and hemicellulosic carbon sources. Growth substrates in this category included sugar-cane bagasse and a hemicellulose preparation from Eragrostis teff. The former is a substrate of mixed composition containing on a dry weight basis approximately 45 % cellulose and 30% xylan (1). The Eragrostis hemicellulose fraction was prepared by Dr. R.F.H. Dekker and consisted largely of xylan.

### 3.1.2 Production of CMCase and hemicellulase during growth on cellulosic carbon sources

CMCase production. CMCase was synthesised during growth on all three cellulosic substrates. Where either MXC or filter paper served as the carbon source, no significant differences were observed in either the rates of production or in the peak activities of this enzyme (Figs. 1 and 2). Maximum CMCase activity was reached after approximately five days, attaining a peak of 0,06 to 0,075 units per  $\text{cm}^3$  of culture filtrate. By comparison, CMCase production seems to have been both reduced and slowed where CMC itself was the carbon source, reaching a maximum value of only 0,05 units per  $\text{cm}^3$  of culture filtrate after a

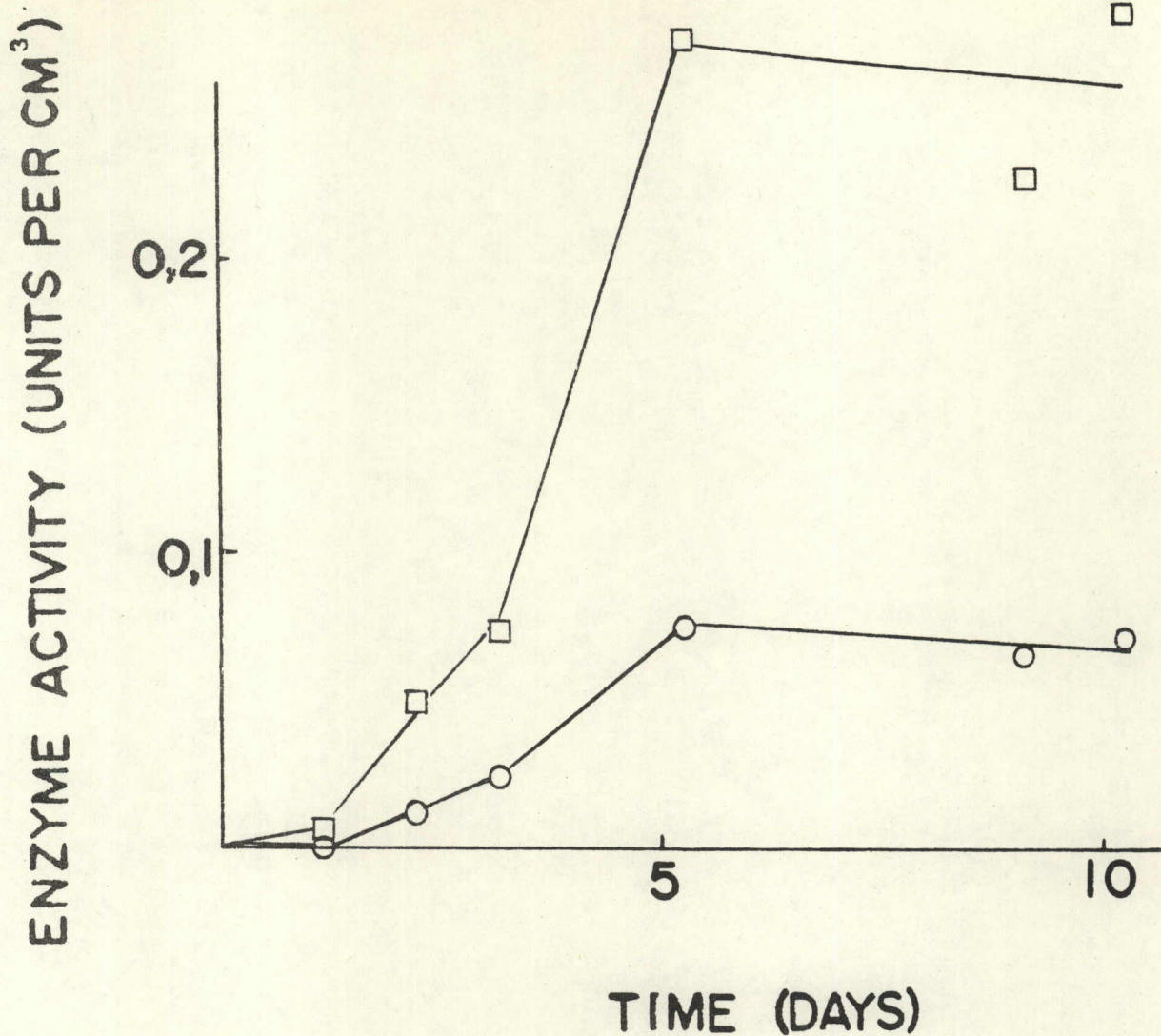


Figure 1. Enzyme production by *S. rolfsii* growing on microcrystalline cellulose. At the times indicated samples of the culture filtrate were removed and assayed for CMCase (○) and hemicellulase (□) activities. The concentrations of the substrate solutions were  $5,0 \text{ g dm}^{-3}$  (Sigma CMC, D.S. 0,1) and  $10,0 \text{ g dm}^{-3}$  (*Eragrostis teff* hemicellulose) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5 (CMC) and pH 5,7 (hemicellulose). In both cases enzyme activity was measured by the release of reducing sugar after incubating enzyme ( $0,25 \text{ cm}^3$ ) with substrate solution ( $0,5 \text{ cm}^3$ ) at  $36^\circ\text{C}$  for 1 hour. Each determination was corrected for a blank value obtained after incubating enzyme in the absence of substrate but otherwise under the same experimental conditions.

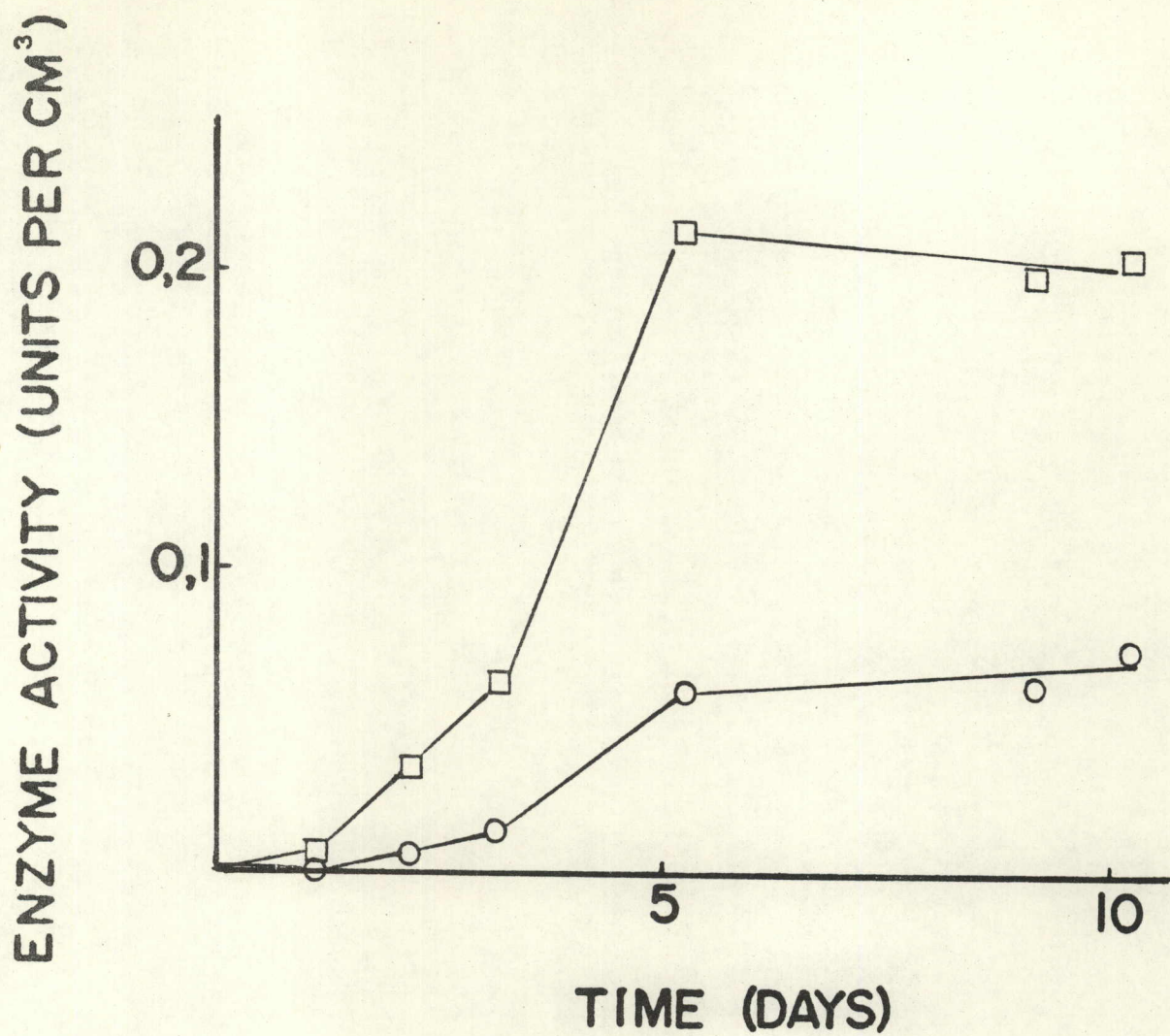


Figure 2. Enzyme production by *S. rolfsii* growing on filter paper. CMCCase (○), hemicellulase (□). Other experimental details were as in Figure 1.

period of nine days (Fig. 3).

Hemicellulase production. Hemicellulase production followed a pattern similar to CMCase production (Figs. 1 to 3). Greatest hemicellulase activity (0,28 units per  $\text{cm}^3$  of culture filtrate) was observed when MXC served as carbon source (Fig. 1). By comparison, peak activity was somewhat reduced (0,22 units per  $\text{cm}^3$  of culture filtrate) when either filter paper or CMC constituted the carbon source (Figs. 2 and 3). As in the case of CMCase production, peak activity was recorded after ca. five days growth on either MXC or filter paper, whereas during growth on CMC, the time to peak activity was extended to nine days.

### 3.1.3 Production of CMCase and hemicellulase during growth on hemicellulosic and mixed carbon sources

CMCase Production. CMCase was synthesised by S. rolfsii when either bagasse or hemicellulose served as carbon sources (Figs. 4 and 5). However, maximal CMCase production was attained more rapidly (after six days) during growth on bagasse than on hemicellulose (maximum production at eleven days). In addition, higher peak levels of CMCase activity were observed during growth on bagasse (0,04 units per  $\text{cm}^3$  of culture filtrate) than during growth on hemicellulose (0,03 units per  $\text{cm}^3$  of culture filtrate).

Hemicellulase production. Comparison of the hemicellulase production curves during bagasse- or hemicellulose-supported growth reveals that, as in the case of CMCase, peak production was achieved sooner in the

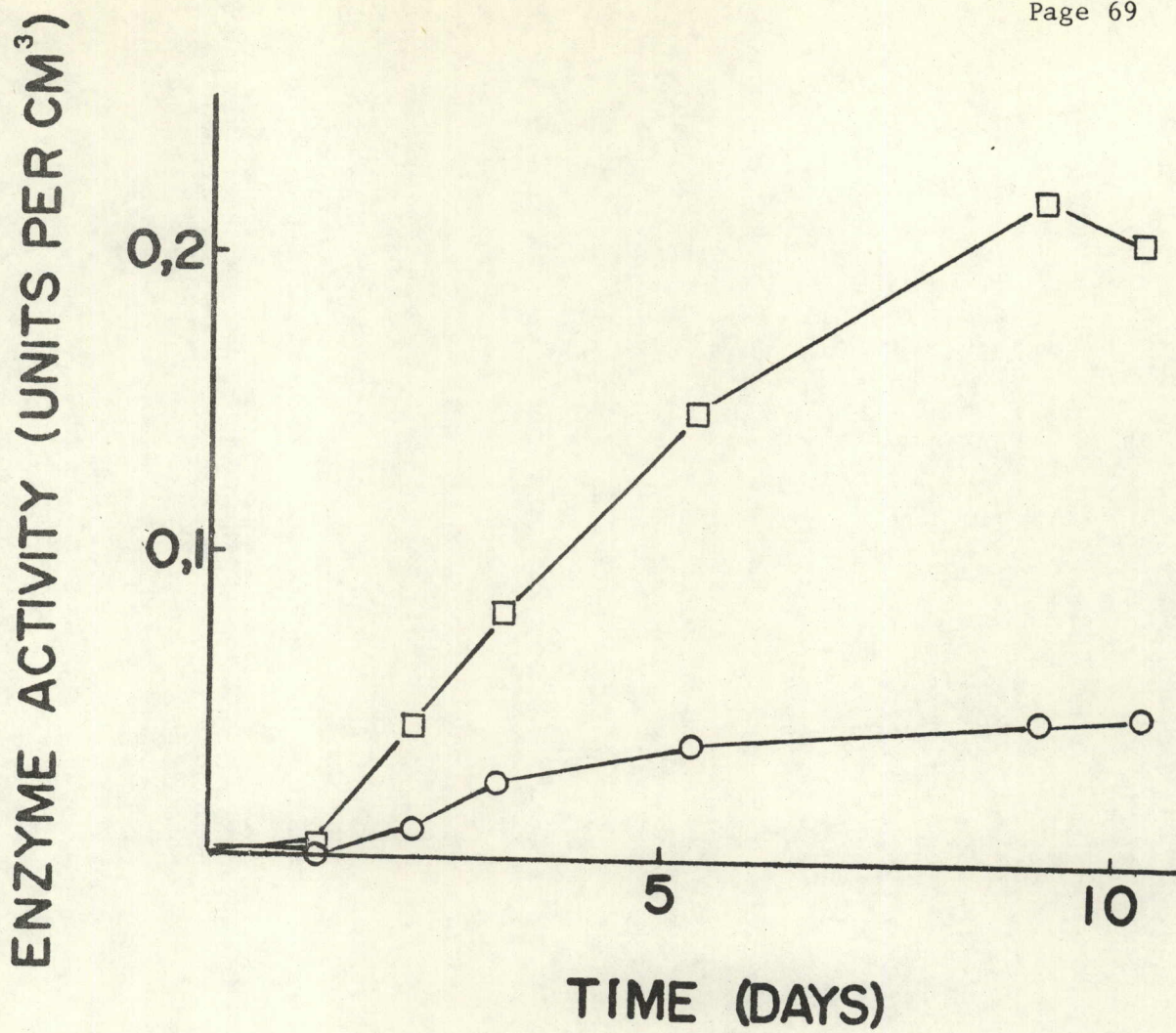


Figure 3. Enzyme production by *S. rolfsii* growing on CMC. CMCase (hemicellulase (□)). Other experimental details were as for Figure 1.

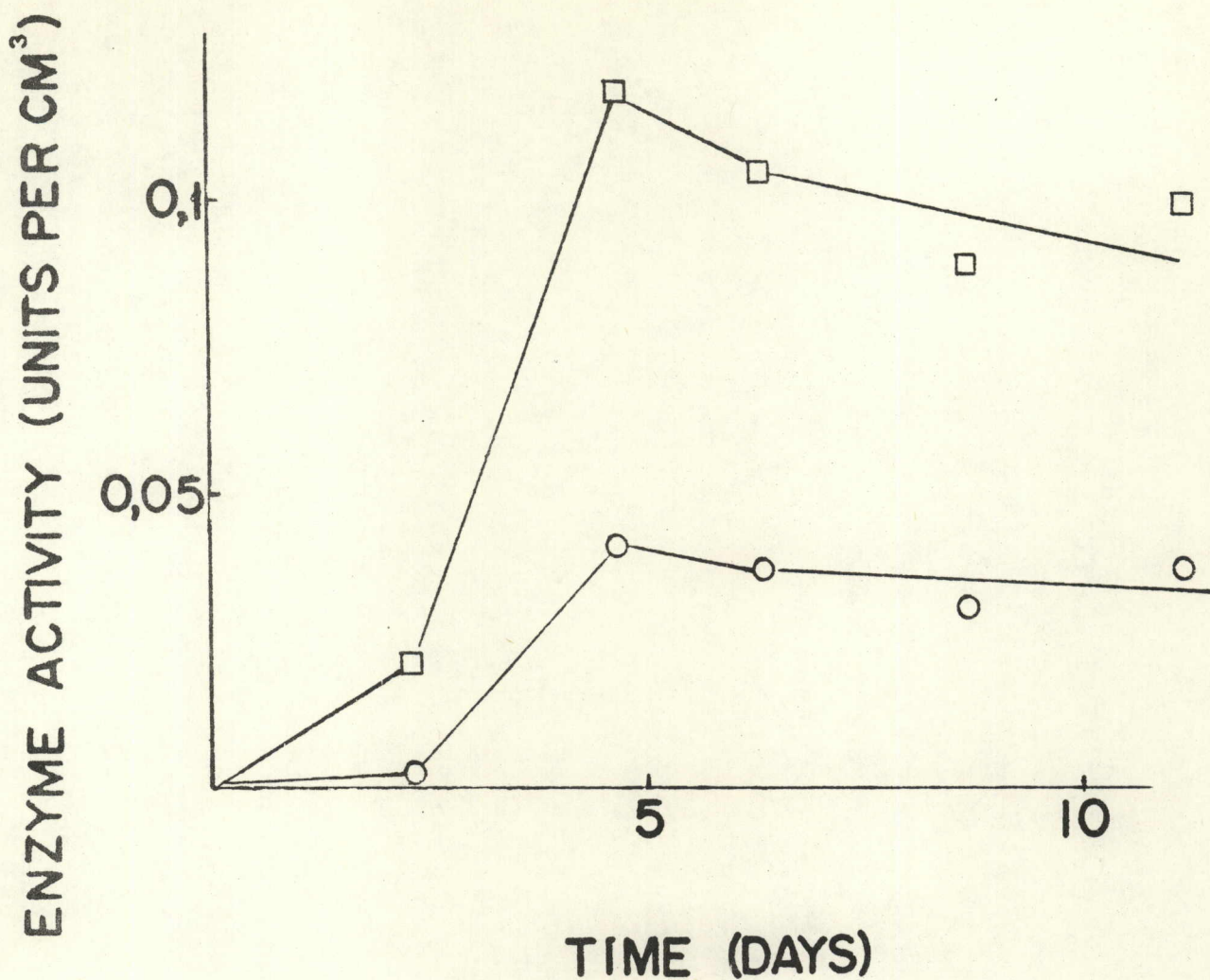


Figure 4. Enzyme production by *S. rolfsii* growing on bagasse. CMCase (○), hemicellulase (□). Details of the enzyme assays were as for Figure 1 except that, from the 190 hour sample on, the CMC stock solution was in  $0,005 \text{ mol dm}^{-3}$  sodium acetate buffer pH 4,5.

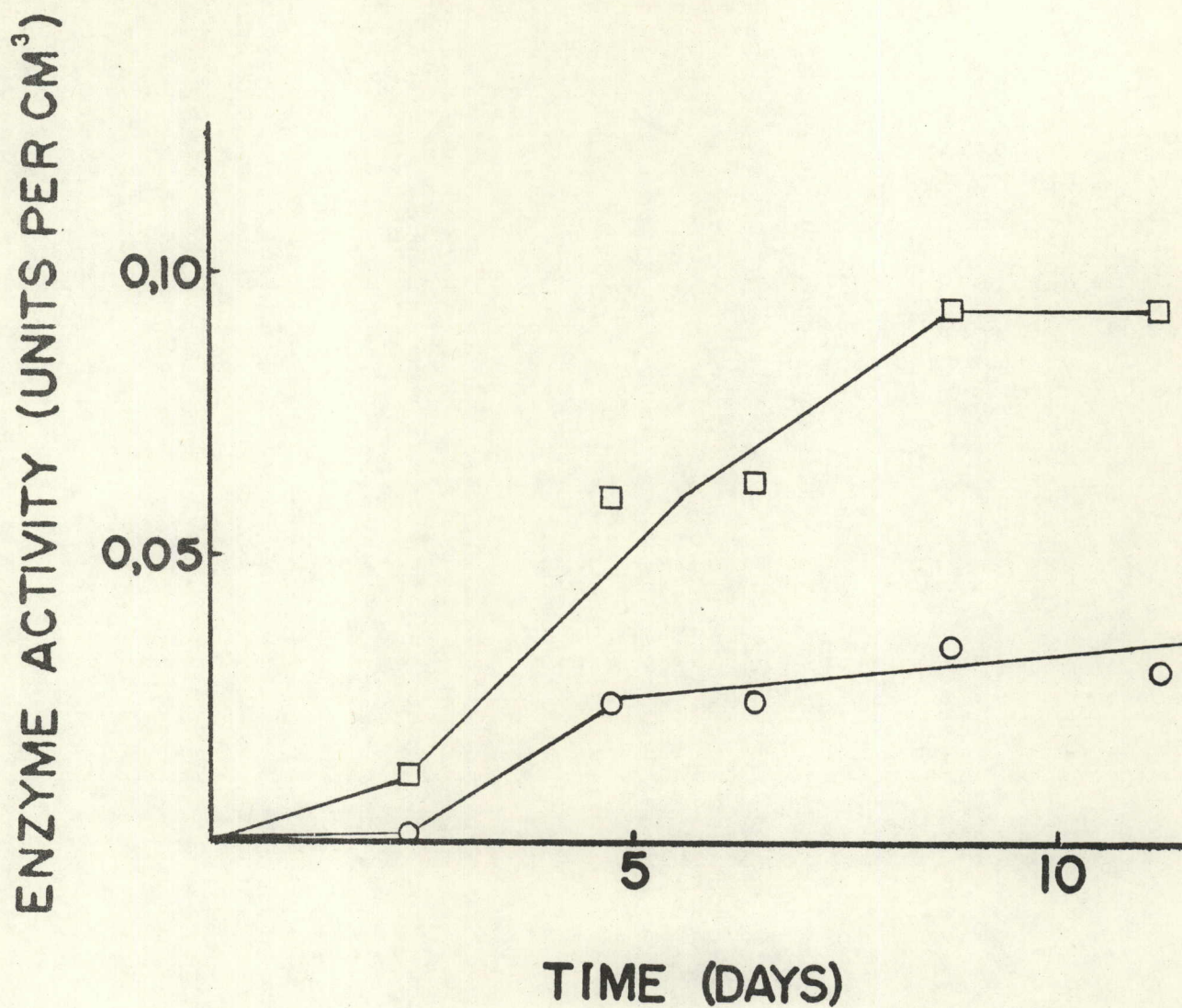


Figure 5. Enzyme production by *S. rolfsii* growing on hemicellulose CMCase (○), hemicellulase (□). Other details were as for Figure 4.

former than in the latter case, (Figs. 4 and 5). Maximal activity was reached after six days growth on bagasse and after eleven days on hemicellulose. Respective maxima (peak activities) were 0,12 units and 0,09 units per cm<sup>3</sup> of culture filtrate.

#### 3.1.4 Discussion of enzyme production on different carbon sources

Both CMCase and hemicellulase were synthesised by S. rolfsii during growth on either cellulose or hemicellulose. It appears therefore that the synthesis of at least some members of each group of enzymes did not require induction by substrate. However, induction per se cannot be ruled other out on the basis of the data presented here. For example, hemicellulase might have been induced by cellulose or its hydrolysis products. Similarly, CMCase synthesis could have been induced by products of hemicellulose breakdown.

Comparison of the hemicellulase activity profiles during fungal growth on hemicellulose and bagasse suggests that hemicellulase synthesis was repressed when hemicellulose was the sole growth source. The most suitable growth source for purposes of enzyme production appeared to be MXC. High levels of both CMCase and hemicellulase were synthesised during growth on MXC and substrate repression effects were absent. In addition MXC appeared to induce the synthesis of cellulases with the highest activity on crystalline cellulose substrates.

### 3.2 Further studies of growth and enzyme production on microcrystalline cellulose

With microcrystalline cellulose selected as the most suitable carbon source, the problem remained of determining at what stage of the growth period maximal enzyme production was attained. It was considered impractical to monitor enzyme activity continuously during large scale growth. The large vessels employed for this purpose were necessarily unwieldy and it was feared that, whilst removing samples for analysis, microbial contamination of the vessel contents might occur. Although this problem might have been overcome with special apparatus, some other measure of enzyme production was sought which would not entail opening the vessel. One possible indicator of enzyme production appeared to be the extent of mycelial growth and a detailed study was accordingly initiated to establish a possible correlation between these two parameters. For this experiment Avicel PH 101 (FMC) was chosen as a source of microcrystalline cellulose.

#### 3.2.1 Mycelial protein

There appeared to be no simple way of separating the mycelium from the solid cellulose present in the growth medium. Mycelial protein, determined by the Kjeldahl method (97), rather than mycelial mass was therefore taken as an index of fungal growth. Growth of S. rolfsii on Avicel displayed no appreciable lag phase as a measurable increase in mycelial protein was observed after twenty four hours incubation. Thereafter, growth proceeded exponentially for a further four days, at

which time a sudden acceleration in the rate occurred. The period of rapid growth (the tropophase) was short-lived giving way to a somewhat extended idiophase, during which the rate of mycelial protein production decreased steadily. Growth was accompanied by a rapid drop in pH of the growth medium, the rate of fall in pH being greatest just prior to the sudden acceleration in mycelial growth marking tropophase (Fig. 6).

### 3.2.2 Enzyme production

The enzyme production curves, apart from minor differences, appeared to reflect the general form of the growth curve (Figs 7 and 8). CMCase production exhibited the longest lag phase and continued to lag behind the growth curve throughout the experiment (Fig 7). In this respect CMCase production exhibited a degree of non-growth-associated behaviour.

### 3.2.3 Discussion of growth and enzyme production

Except for relatively small shifts in phase the enzyme production curves on Avicel appeared to follow the pattern of mycelial protein formation. It was therefore concluded that the extent of mycelial growth was in fact a reasonable indicator of enzyme concentration in the culture filtrate.

The S. rolfsii growth and enzyme production curves appear to be similar to those exhibited by several other microorganisms. However,

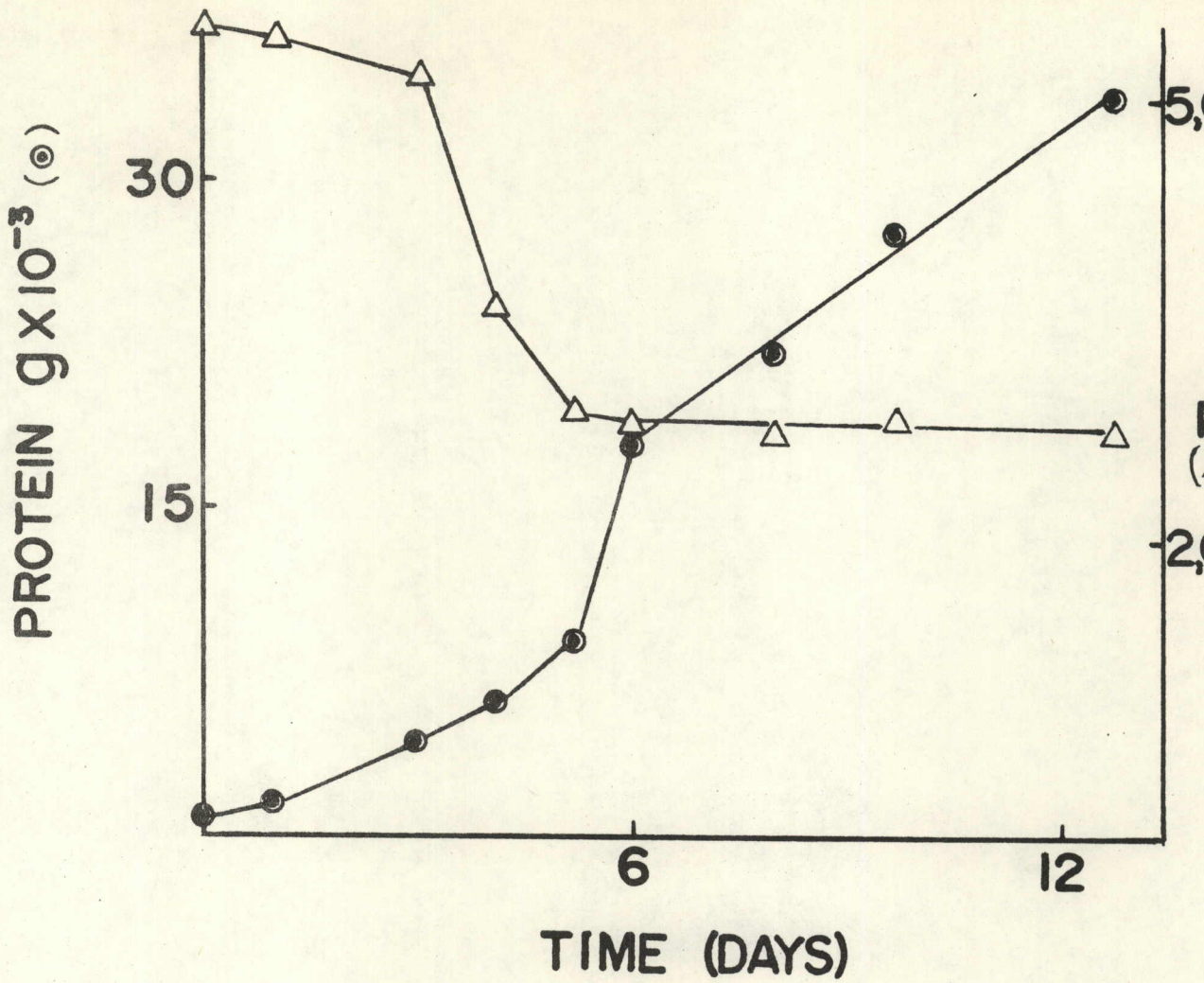


Figure 6. Mycelial protein and pH of the growth medium during growth of *S. rolfsii* on Avicel PH 101. At the times indicated, duplicate flasks were removed and the mycelial mass recovered by centrifugation. The pH of the medium was determined using a glass electrode and mycelial protein was measured by the Kjeldahl method for N (97). Individual points on the graph are means of the duplicate determinations.

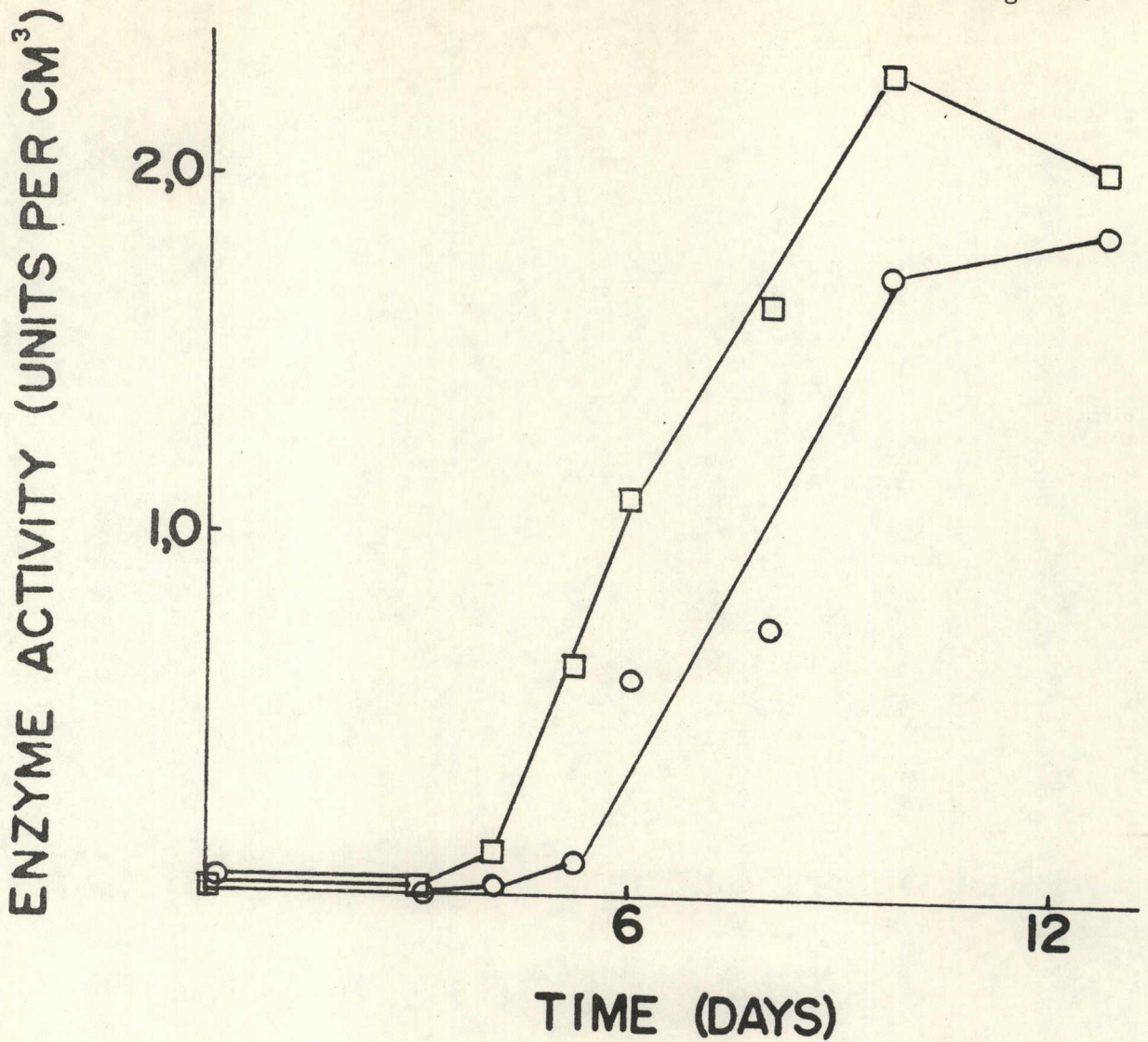


Figure 7. CMCase and xylanase production by *S. rolfsii* growing on Avicel PH 101. At the times indicated, duplicate flasks were taken and, after removal of mycelial mass by centrifugation, the supernatant was assayed for CMCase (●) and xylanase (□) activities. Individual points on the graph are means of the duplicate determinations. Enzyme activity was measured by the release of reducing sugar following incubation of enzyme (0,02 cm<sup>3</sup>) with substrate stock solution (0,2 cm<sup>3</sup>) at 50°C for 10 minutes.

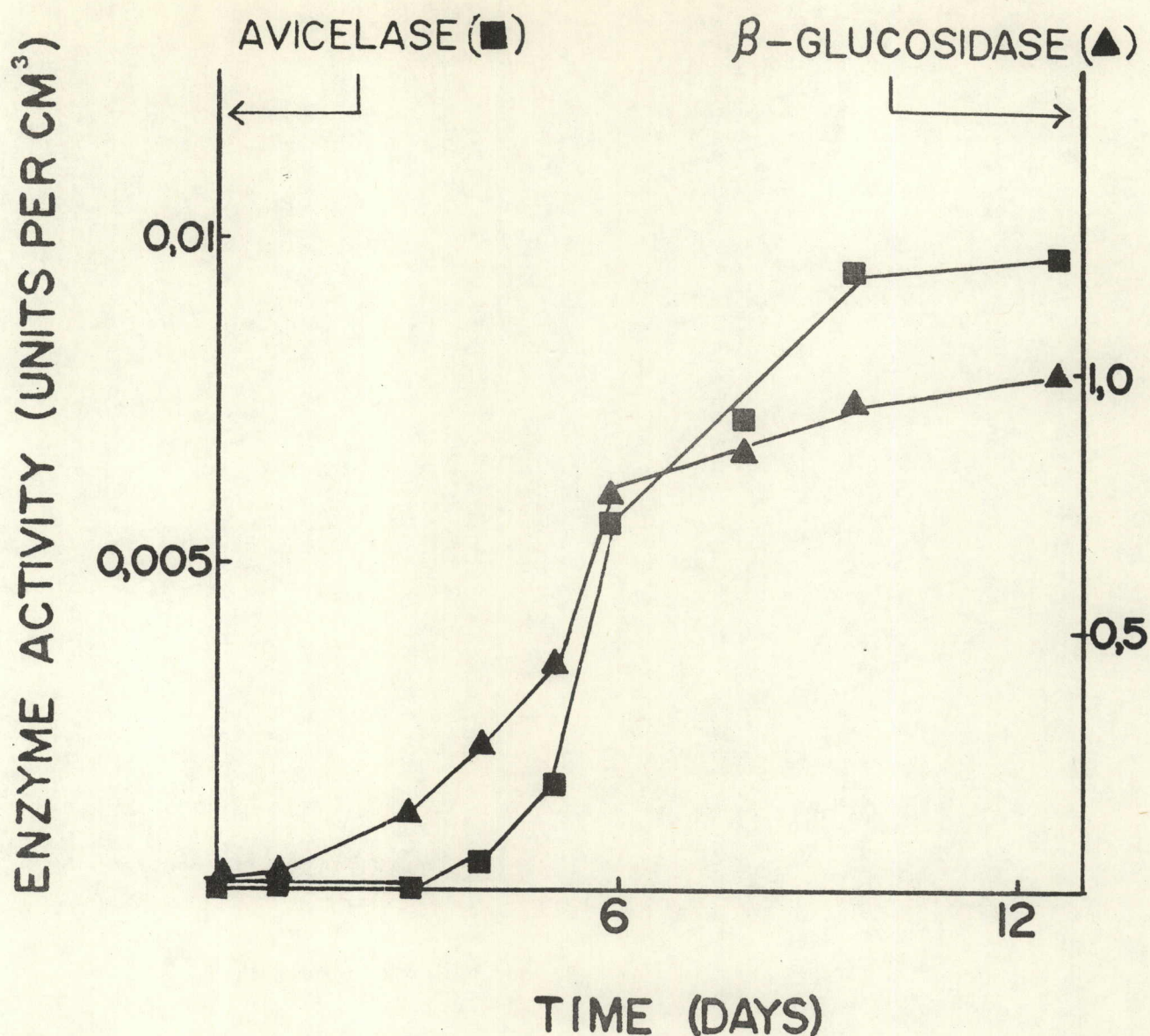


Figure 8. Avicelase and  $\beta$ -glucosidase production by *S. rolfsii* growing on Avicel PH 101. Avicelase activity was determined from the release of reducing sugar following incubation of enzyme ( $0,05 \text{ cm}^3$ ) with a  $10,0 \text{ g dm}^{-3}$  suspension of Avicel PH 101 in sodium acetate buffer pH 4,5 ( $0,5 \text{ cm}^3$ ) at  $54^\circ\text{C}$  for 16 hours. Reaction mixtures contained  $0,2 \text{ g dm}^{-3}$  sodium azide as preservative.  $\beta$ -glucosidase activity was assayed by measuring the increase in absorbance at  $401 \text{ nm}$  following incubation of enzyme ( $0,02 \text{ cm}^3$ ) with  $0,004 \text{ mol dm}^{-3}$  *p*-nitrophenyl- $\beta$ -D-glucopyranoside in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5 ( $0,2 \text{ cm}^3$ ) at  $50^\circ\text{C}$  for 10 minutes. The reaction was terminated by the addition of  $0,26 \text{ mol dm}^{-3}$  sodium carbonate ( $4,8 \text{ cm}^3$ ). Other experimental details were as for Figure 7. Both vertical axes represent enzyme activity (units per  $\text{cm}^3$ ).

examination of published data for other other microorganisms reveals no fixed relationship between growth and either cellulase or xylanase production. In the majority of cases CMCase production appears to occur during late tropophase, although several exceptions have been noted. This disparate behaviour appears to be as much a function of the organism as of the culture conditions. The effect of culture conditions is exemplified by the work of Miele and Linkins (98), who showed that the water mould Aschyla bisexualis, when grown on glucose, elaborated extracellular CMCase throughout the growth period whereas, when cellobiose was employed as carbon source, CMCase production was restricted to late idiophase. These authors showed in addition that, during growth on insoluble cellulose, most of the CMCase became adsorbed to the substrate and CMCase release into the growth solution was consequently delayed. A similar effect has been noted to occur in the case of CMCase production by Cellulomonas (99). Culture conditions, (particularly ambient pH) have also been shown to influence both the timing and extent of cellulase production in Trichoderma reesei (61).

In other cases intrinsic differences between organisms appear to be responsible for their disparate enzyme production patterns. For example, Van Hofsten and Berg (100) have shown that CMCase production in the bacterium Cellvibrio fulvus is growth-associated but the enzyme remains attached to the cell wall during active growth. The appearance of CMCase in the culture fluid during late idiophase appeared to be as a result of its release following death and consequent dissolution of the organism. Cellulase production in T. reesei also occurs late in

the fermentation. In fact, during batch culture a large portion of the filter paper degrading activity is produced during idiophase (90). In contrast to the situation in Cellvibrio, this late release of enzyme does not result from cell death (nor is it a result of slow desorption from the solid substrate), but appears to be the result of continued enzyme synthesis (90).

In contrast to the cellulases, relationships between growth and hemicellulase production appear seldom, if ever, to have been examined. The author has been unable to find a single example.

## CHAPTER 4

THE PURIFICATION AND CHARACTERISATION OF CARBOXYMETHYLCELLULASES FROM  
SCLEROTIUM ROLFSII

The main objectives of the studies reported in this Chapter were to purify one or more CMCase components from culture filtrates of S. rolfsii, to determine the physicochemical properties of the purified enzymes and to investigate their modes of action on various cellulosic substrates.

These aims were attained to the extent that a CMCase component, designated F2, from S. rolfsii culture filtrates was brought to a high state of purity and subsequently characterised. In addition another CMCase, P4, was also partially purified and characterised.

Since there is no prescribed method for separating cellulases from culture filtrates, the purification scheme adopted in the present investigation necessarily involved a degree of trial and error. However, the individual purification steps were not chosen arbitrarily and, to emphasise this, the reasons for making a particular choice are explained in each case.

A variety of experimental conditions are used by workers in the cellulase field when determining the kinetic properties of cellulases. It therefore proved difficult, and sometimes impossible, to compare meaningfully some of the kinetic data obtained for the S. rolfsii CMCase components with data published for other CMCase components (see section 4.7.1).

Valid comparative data could however be obtained by contrasting the properties of F2 and P4.

It is relatively difficult to study the mechanism of CMCase catalysed degradation using CMC as a substrate. CMC is a substituted cellulose moreover the substituents occur at random along the length of the cellulose chain making it difficult to assign a precise structure to the CMC molecule. In addition, identification of the products of enzyme action on CMC is complicated by the presence of substituents and appropriate chromatographic standards appear to be unavailable. In contrast the use of soluble linear cellooligosaccharides as substrates for mode of action studies obviates these difficulties.

Cellooligosaccharides were consequently the substrates of choice in the present investigation for examining the modes of action of the purified CMCases. Studies on the mechanism of cellooligosaccharide degradation by F2 progressed far enough to allow the proposal of a mechanism for transglycosylation by cellulases.

In the course of the studies reported in this Chapter it became clear that the assay of CMCase activity was subject to serious pitfalls, and an investigation of the assay system was therefore initiated (section 4.6). This investigation might be viewed as a digression from the main theme of this Chapter viz. to purify and characterise CMCases. However, in view of the importance of the CMCase assay to the whole question of the isolation and characterisation of these enzymes, the digression seems amply justified.

#### 4.1 Preliminary enzyme fractionation

##### 4.1.1. Concentration of culture filtrates

Following large scale growth and subsequent removal of the mycelium, the problem remained of isolating extracellular enzymes from a large volume (ca. 40 dm<sup>3</sup>) of culture filtrate. Two methods of reducing the volume to more manageable proportions were investigated, namely rotary evaporation and freeze-drying. Rotary evaporation on the scale required proved impractical since, to avoid possible denaturation of the enzymes, culture filtrates were maintained below 35°C and, at these temperatures, evaporation was slow. In addition, some losses of enzyme activity were noted following rotary evaporation. In contrast, facilities for comparatively rapid large scale freeze-drying were available, and the results of pilot experiments showed no losses of enzyme activity resulted from freeze-drying. Freeze-drying consequently became the chosen method for concentrating culture filtrates.

##### 4.1.2. Protein precipitation

Freeze-drying possessed the disadvantage of simultaneously concentrating unwanted components of the culture filtrate, such as inorganic salts and the soluble products of fungal growth. These compounds represented a possible source of interference during subsequent protein fractionation and it seemed advantageous to remove them in the early stages of enzyme purification.

Two methods of separating protein from culture filtrate contaminants were investigated, namely precipitation by addition of either ammonium sulphate or acetone. The results indicated that either method could be used to yield precipitates retaining most of the original enzyme activity (Tables 1 and 2). In the case of certain of the enzymes, activity seems to have been enhanced following ammonium sulphate precipitation. Details of the precipitation procedures are given in the table legends.

Despite the enhanced activity observed following ammonium sulphate precipitation, acetone precipitation was preferred since acetone-precipitated protein required only to be dried before storage as an acetone powder. In contrast, ammonium sulphate precipitated protein would have required dialysis to remove ammonium sulphate before drying and storage.

#### 4.1.3. Removal of scleroglucan

During growth in liquid culture, S. rolfsii produced an extracellular polysaccharide called scleroglucan, that remained in solution after removing mycelial mass by centrifugation. Owing to the high viscosity of culture filtrates containing scleroglucan, it was desirable to remove this polysaccharide before enzyme fractionation.

Culture filtrates were stored frozen and it was noticed that, after thawing, scleroglucan had become less soluble and could be removed by filtration through glass wool. Because of its convenience

TABLE 1. PRECIPITATION OF CULTURE FILTRATE ENZYMES WITH AMMONIUM SULPHATE.<sup>a</sup>

AMMONIUM SULPHATE SATURATION (%)	CMCase (units cm <sup>-3</sup> culture filtrate)	CMCase YIELD (%)	HEMICELLULASE (largely xylanase) (units cm <sup>-3</sup> culture filtrate)	HEMICELLULASE YIELD (%)
0	0,35	100	0,35	100
0 to 55	0,24	68,6	0,26	74,3
56 to 65	0,22	62,9	0,21	60,0
66 to 80	0,13	37,1	0,04	11,4
81 to 100	0,018	5,0	0,018	5,0

<sup>a</sup> Culture filtrate was buffered at pH 5,7 with 0,05 mol dm<sup>-3</sup> sodium-potassium phosphate buffer and kept at 0°C during precipitation. The CMC solution used in the assay was the stock solution described in section 2.4.1. The hemicellulose substrate solution contained 10 g dm<sup>-3</sup> *Eragrostis tef* hemicellulose in 0,05 mol dm<sup>-3</sup> sodium-potassium phosphate buffer, pH 5,7. The assay temperature was 43°C and the reaction time was 1 hour.

TABLE 2. PRECIPITATION OF CULTURE FILTRATE ENZYMES WITH ACETONE.<sup>a</sup>

VOLUMES OF ACETONE	CMCase (units cm <sup>-3</sup> culture filtrate)	CMCase YIELD	HEMICELLULASE (largely xylanase) (units cm <sup>-3</sup> culture filtrate)	HEMICELLULASE YIELD (%)
0	0,23	100,0	0,17	100,0
0 to 1,5	0,10	43,4	0,06	35,3
1,5 to 2,5	0,12	52,2	0,09	52,9
2,5 to 4,0	0,03	13,0	0,01	5,9

<sup>a</sup> Culture filtrate was buffered at pH 6,0 with 0,005 mol dm<sup>-3</sup> sodium-potassium phosphate buffer and kept at 0°C during precipitation. Other details were as for Table 1.

this simple procedure was the one routinely adopted for removing scleroglucan. An alternative procedure noted, but not used routinely by the author, was to precipitate scleroglucan from unfrozen culture filtrates by addition of 0,7 volumes of acetone.

#### 4.2 Purification of CMCases

As an aid to the ready comprehension of section 4.2, a flow diagram is presented in Fig.9.

##### 4.2.1. Fractionation of the acetone powder on Avicel PH 102

Chromatography of the acetone powder on Avicel yielded two fractions, A1 and A2, the first, (A1), eluting under the starting conditions (0,02 mol dm<sup>-3</sup> sodium phosphate buffer, pH 6,6) and the second, (A2), under conditions of reduced ionic strength (the same buffer diluted 40 fold) (Fig. 10). Each fraction exhibited enzymic activity on cellulose, xylan and p-nitrophenyl- $\beta$ -D-glucoside suggesting the presence of several different enzymes. In addition A1 and A2 were heterogeneous by SDS gel electrophoresis indicating the need for further purification of both fractions.

##### 4.2.2. Chromatography of A1 on DEAE-Sephadex A-50

Examination of the elution profile obtained following ion-exchange chromatography of A1 on DEAE-Sephadex (Fig. 11), reveals that most of the protein and enzyme activity eluted at high ionic strength

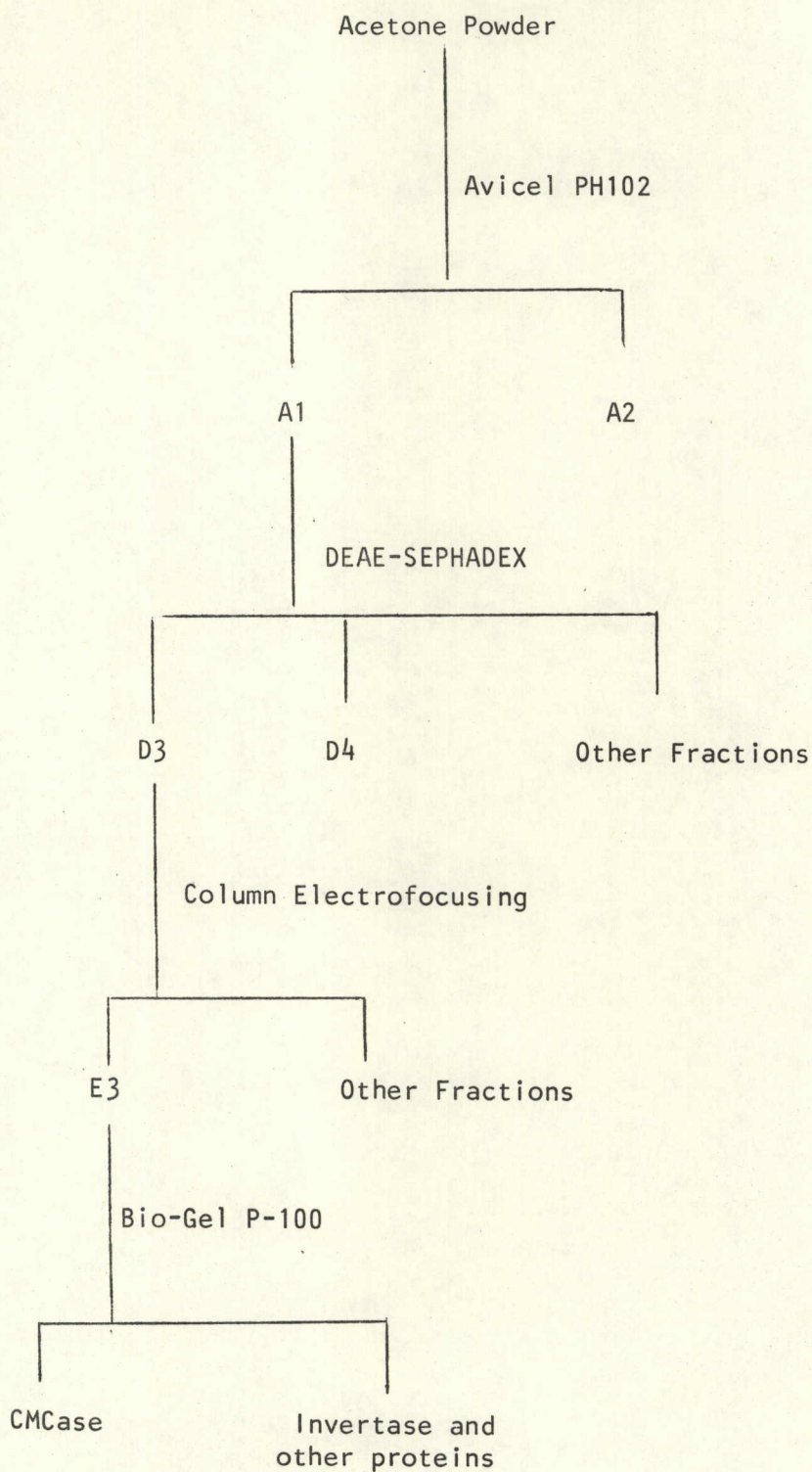


Figure 9. Initial purification scheme for a CMCase in fraction A1.

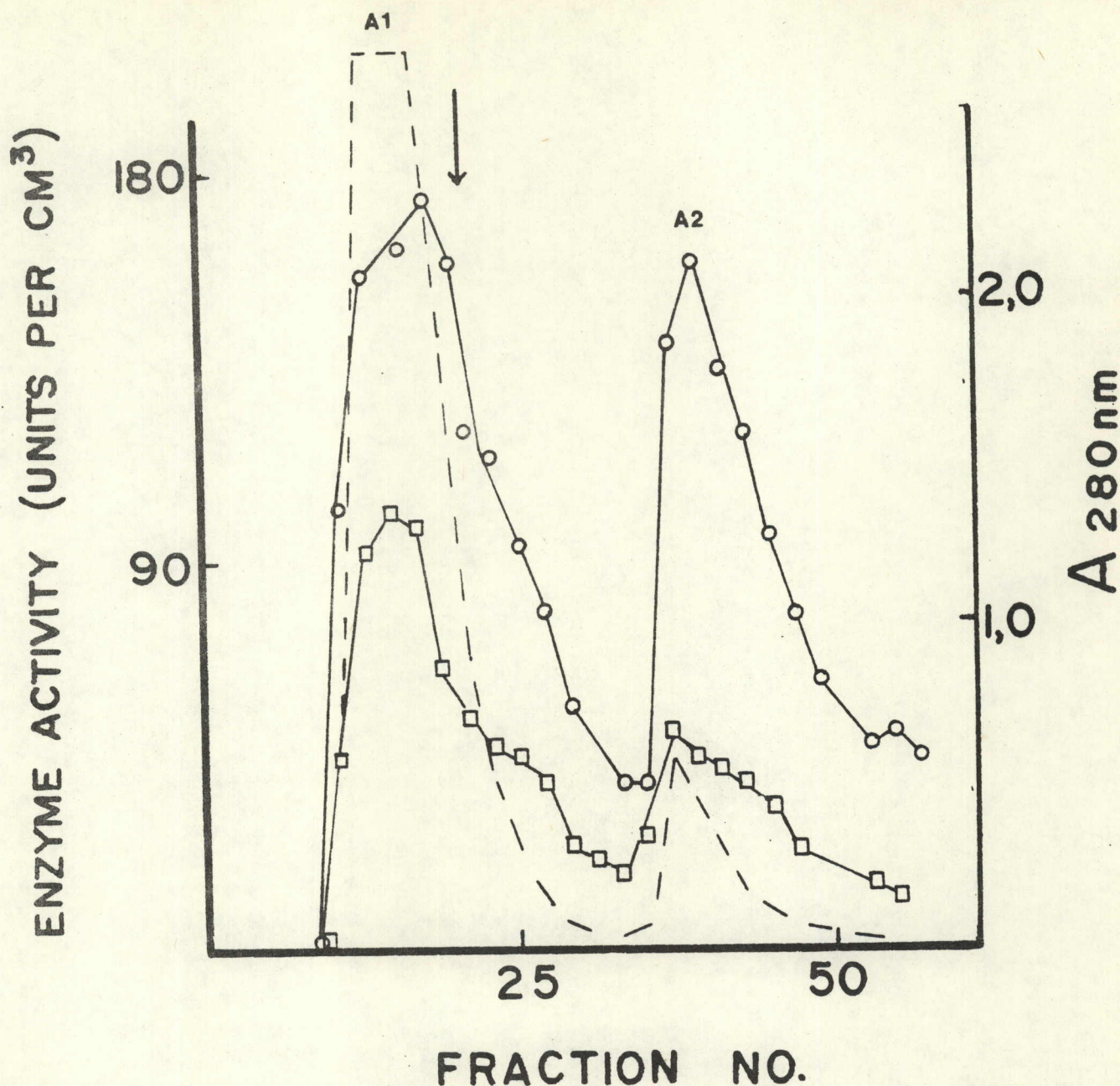


Figure 10. Chromatography of the acetone powder on Avicel PH 102. Soluble material from the acetone powder (10 g) was dissolved in  $0,02 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, and applied to a column (4,0 x 9,0 cm) of Avicel PH 102 equilibrated in the same buffer and maintained at ca.  $4^{\circ}\text{C}$ . The column was eluted with equilibrating buffer, followed by the same buffer diluted 40 fold with water. The arrow indicates the point at which the eluant was changed. Elution with water alone appeared to generate fine cellulose particles that passed through the column supporting disc and contaminated the fractions. The practice of eluting with water alone was therefore abandoned. However, studies on a separate batch of culture filtrate indicated that  $<12\%$  of the protein remained on the column after elution with 40 fold diluted sodium phosphate buffer. Fractions ( $12,0 \text{ cm}^3$ ) were collected at a flow rate of  $1,2 \text{ cm}^3$  per minute. Enzyme activity was assayed by the release of reducing sugar from CMC (○) and from xylan (□). The CMC substrate solution contained  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,1 \text{ mol dm}^{-3}$  sodium acetate pH 4,5. The xylan substrate solution was the stock solution described in section 2.4.1. Enzyme ( $0,1 \text{ cm}^3$ ) was incubated with substrate solution ( $0,2 \text{ cm}^3$ ) at  $45^{\circ}\text{C}$  for 1 hour before determining reducing sugar. Protein (---) was estimated by absorbance at 280 nm.

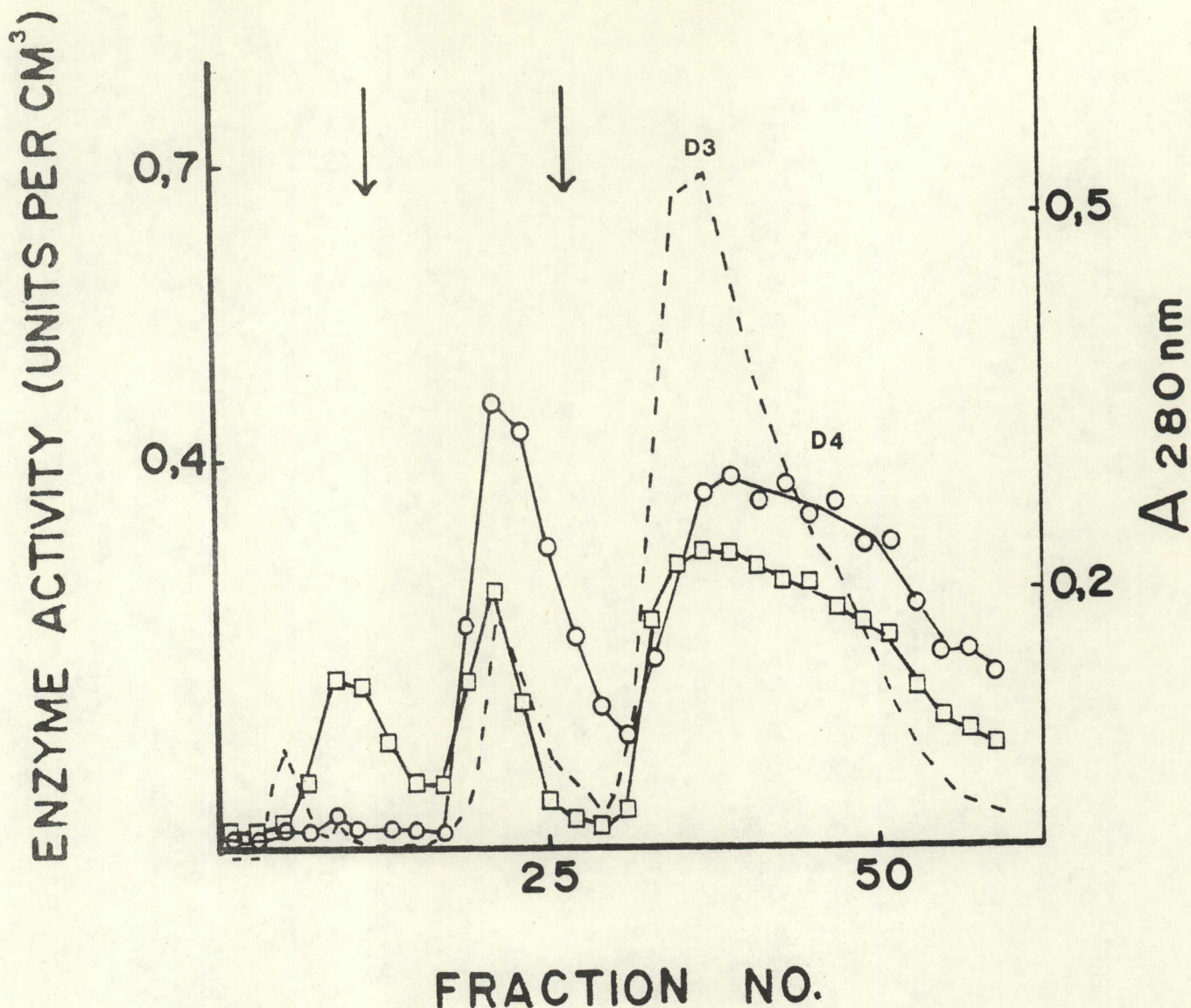


Figure 11. Chromatography of A1 on DEAE-Sephadex A-50. A sample of A1 was washed with  $10 \text{ mol dm}^{-3}$  sodium phosphate, pH 6,6 on an Amicon PM 10 ultrafilter before being applied to a column of DEAE-Sephadex ( $27 \times 2,5 \text{ cm}$ ) equilibrated with the same buffer and maintained at ca.  $4^\circ\text{C}$ . Elution was with equilibrating buffer,  $0,05 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6 and finally with the same buffer incorporating  $0,5 \text{ mol dm}^{-3}$  sodium chloride. Arrows indicate the points at which the eluting buffers were changed. Fractions ( $12,0 \text{ cm}^3$ ) were collected at a flow rate of  $1,0 \text{ cm}^3$  per minute. Enzyme and protein assays were performed as described for Figure 10. CMCCase (o), xylanase ( $\square$ ), protein (---).

( $0,5 \text{ mol dm}^{-3} \text{ NaCl}$ ), appearing in fractions marked D3 and D4. Xylanase and cellulase activities co-chromatographed in both peaks D3 and D4 and both fractions showed a marked similarity in composition as judged by electrophoresis in SDS-containing gels. Fraction D4, however, appeared to contain a larger proportion of a low molecular weight protein, indicating that some separation had occurred on the ion-exchange resin. Fraction D3 contained the greater share of both enzyme activity and protein and was therefore chosen for further purification by electrofocusing.

#### 4.2.3. Electrofocusing of D3 in the LKB 8101 column

Fraction D3 was separated into 4 major protein fractions by electrofocusing in a sucrose gradient over the pH range 2,5 to 5,0 (Fig. 12). Although xylanase and cellulase activities appeared to have been resolved (Fig. 13), none of the separated protein fractions was homogeneous on SDS-containing gels (Plate 1). SDS gel electrophoresis of fraction E3, which contained all the recoverable CMCase activity of D3, revealed two major bands corresponding to components of molecular masses  $5,7 \times 10^4$  and  $7,2 \times 10^4$  respectively (Plate 1).

#### 4.2.4 Purification of E3 on Bio-Gel P-100

The comparatively large differences in molecular masses between the components of E3 suggested that this fraction could be further refined by molecular exclusion chromatography. The resin selected was Bio-Gel P-100 which fractionates in the molecular mass range  $5 \times 10^3$  to

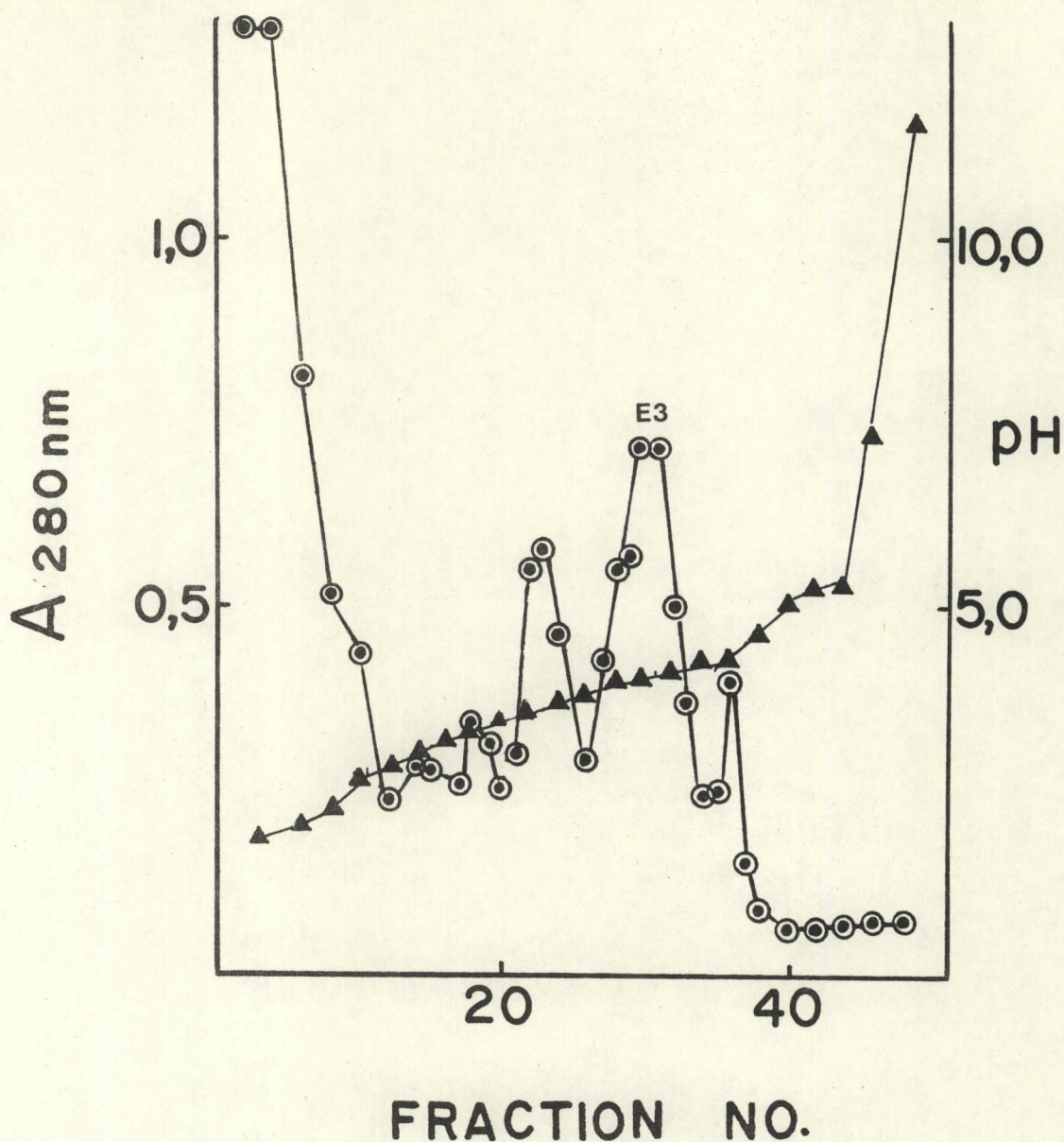


Figure 12. Electrofocusing D3 in a sucrose gradient using the LKB 8101 column: protein (●) and pH (▲) profile. Approximately one-third of fraction D3 (see Figure 11) was washed on an Amicon PM 10 ultrafilter in  $0,002 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, and reduced to a final volume of  $6,6 \text{ cm}^3$  prior to electrofocusing in the pH range 2,5 to 5 at 15 W and  $4^\circ\text{C}$  for 16 hours. After electrofocusing was complete, water was pumped in at the top of the column (the cathode) and fractions ( $2,4 \text{ cm}^3$ ) were collected at a flow rate of  $1,0 \text{ cm}^3$  per minute. The pH of each fraction was measured, at the running temperature, with a glass electrode and protein was determined by the absorbance at 280 nm.

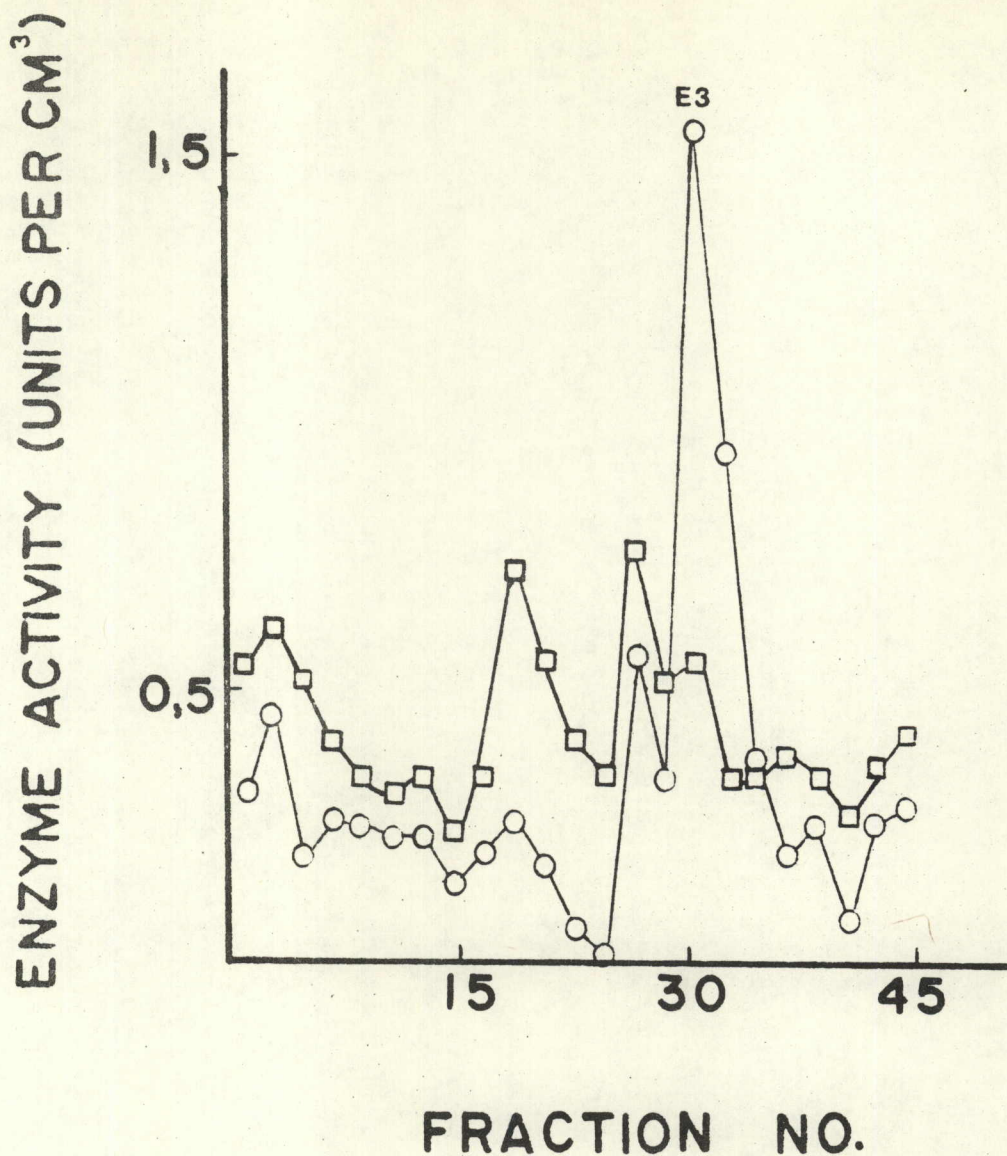


Figure 13. Electrofocusing D3 in a sucrose gradient using the LKB 8101 column: CMCase (○) and xylanase (□) activities. Enzyme solutions were diluted 10 fold before assay. Enzyme solution (0,002 cm<sup>3</sup>) was incubated with the appropriate substrate stock solutions (0,25 cm<sup>3</sup>) at 50°C for 1 hour before measuring reducing sugar. Each activity determination was corrected for a blank value obtained after incubating enzyme in the absence of substrate, but otherwise under the same experimental conditions. Other experimental details were as for Figure 11.

$1 \times 10^5$  and which was therefore deemed capable of resolving the components of E3. Upon chromatography on a column of Bio-Gel P-100 (2,5 x 100 cm), E3 exhibited a single CMCCase activity peak (Fig. 14), but further examination of the components of this peak, by SDS gel electrophoresis, showed the continued presence of the two major protein bands first seen following electrofocusing of E3. However, compared with E3, the higher mass component (molecular mass  $7,2 \times 10^4$ ) was present in a much reduced proportion (Fig. 14). In contrast, the pooled Bio-Gel column fractions (40 to 49), eluting before the CMCCase activity, appeared to consist mainly of the larger component, with only a trace of the smaller protein (molecular mass  $5,7 \times 10^4$ ) (Fig. 14).

The results suggested that only one CMCCase component (of molecular mass  $5,7 \times 10^4$ ) was present in E3 and that the enzyme could, in principle, be separated from contaminating protein by molecular exclusion chromatography.

#### 4.2.5 Disadvantages of the electrofocusing step

It was noticed that fractions eluted from the electrofocusing column appeared to contain high levels of reducing sugar. Further investigation traced the probable cause to the presence of an invertase in fraction E3 that was catalysing hydrolysis of the sucrose used in constructing the density gradient. The high reducing sugar background interfered with subsequent enzyme assay of the fractions, moreover its presence implied that the gradient was being disturbed. It was therefore desirable to remove the invertase prior to electrofocusing.

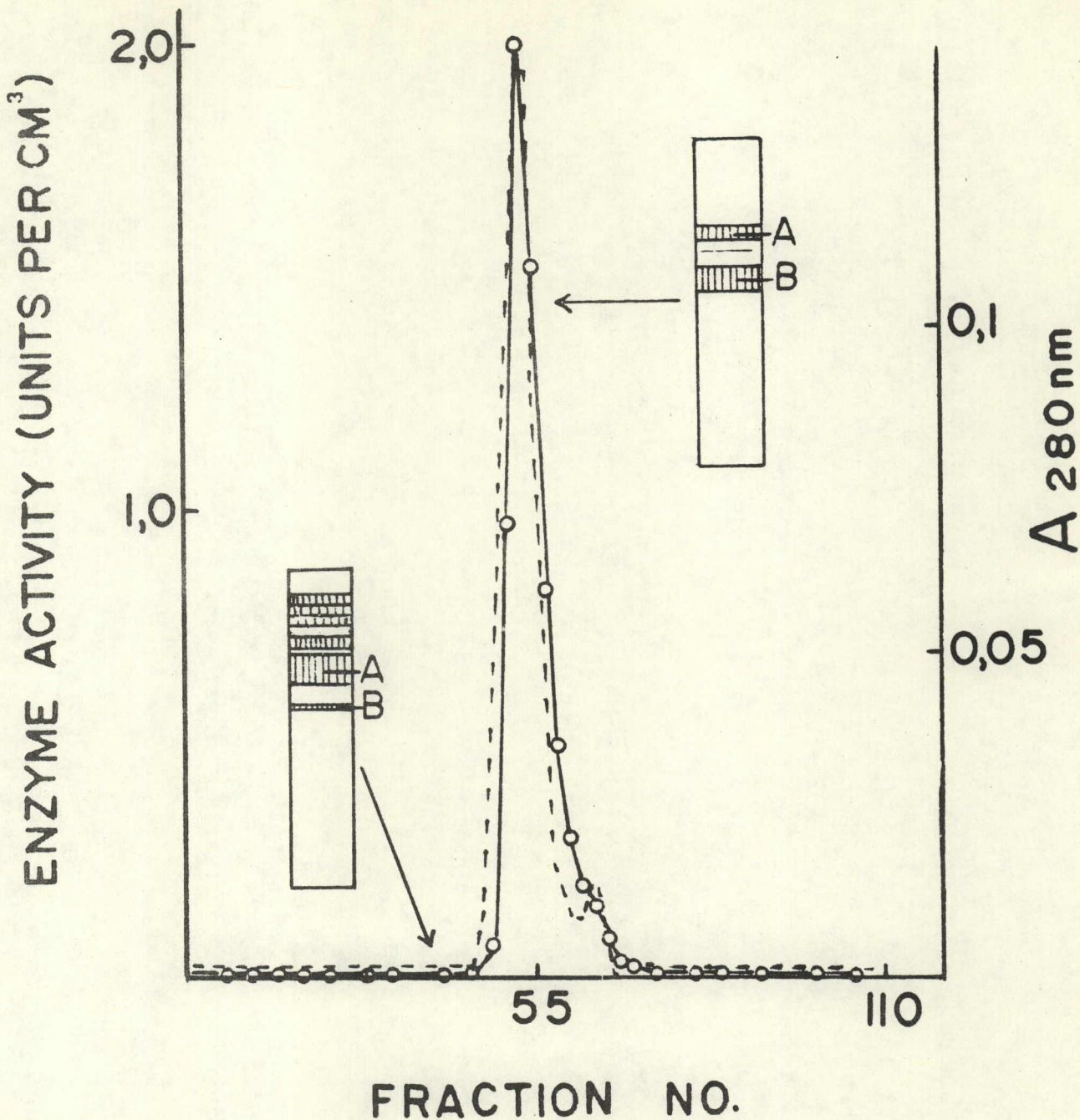


Figure 14. Chromatography of E3 on Bio-Gel P-100. A sample of E3 was washed free of Ampholytes on an Amicon PM 10 ultrafilter with  $0,05 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6. The sample ( $5,0 \text{ cm}^3$ ) was chromatographed on a Bio-Gel P-100 column ( $92 \times 2,6 \text{ cm}$ ) previously equilibrated, and subsequently eluted, with the same buffer at room temperature. Fractions ( $3,6 \text{ cm}^3$ ) were collected at a flow rate of  $9,5 \text{ cm}^3$  per hour. CMCcase activity ( $\circ$ ) was assayed by the release of reducing sugar after incubating enzyme ( $0,01 \text{ cm}^3$ ) with CMC stock solution ( $0,3 \text{ cm}^3$ ) at  $50^\circ\text{C}$  for 1 hour. Rectangles on either side of the peak represent SDS electrophoretograms of selected pooled fractions. A denotes a protein of molecular mass  $7,2 \times 10^4$  and B a protein of molecular mass  $5,7 \times 10^4$ .

Following chromatography of E3 on Bio-Gel P-100, it was established that invertase activity was associated with heavier material eluting before the CMCase activity, indicating that invertase could be completely removed from the CMCase by molecular exclusion chromatography.

#### 4.2.6 Re-appraisal of the purification scheme

On re-examination of the scheme for the purification of A1, the steps of which have been summarised in Fig. 9, several aspects appeared unsatisfactory. For example, reference to Fig. 11 shows that an incomplete separation was achieved following ion exchange chromatography of A1. Most of the protein and enzyme activity was poorly resolved, eluting at high ionic strength as a single asymmetrical peak (D3 and D4). In addition, since in both ion-exchange chromatography and electrofocusing charge differences between proteins form the basis for separation, the sequential application of the two techniques seemed redundant. Finally, examination of the appropriate patterns on SDS-containing gels (Plate 1) shows that components of D3, separated by electrofocusing, differed from one another quite markedly in molecular mass, i.e. electrofocusing coincidentally appeared to have fractionated components according to their molecular masses. It seemed, therefore, that A1 could be resolved more efficiently by direct application of molecular exclusion chromatography. Although this expectation was largely fulfilled, electrofocusing still had to be retained as a further purification step (see below). The conclusion that electrofocusing coincidentally separated components according to

molecular weight was therefore only partly true.

#### 4.2.7 Chromatography of A1 on Bio-Gel P-150

In an attempt to maximise the efficiency of the molecular exclusion step, the length of the gel bed was increased from 100 cm to 145 cm and a gel of larger pore size (P-150) was selected. Details of the subsequent resolution of A1 on Bio-Gel P-150 are presented in Fig. 15. The fraction marked P3, comprising the pooled column fractions 76 to 79 contained a high proportion of the CMCCase activity and ran as one major and two minor bands by SDS gel electrophoresis (Plate 2A). CMCCase activity was also found in fractions 84 to 91, and this fraction was designated P4 (Fig. 15).

#### 4.2.8 Analytical electrofocusing of P3

Despite its high degree of homogeneity, P3 manifested xylanase activity in addition to the CMCCase activity, suggesting either the presence of a xylanase impurity (with a molecular mass similar to that of the CMCCase) or, alternatively, the existence in P3 of a single enzyme with both xylanase and CMCCase activities. It therefore became necessary to establish whether the xylanase and CMCCase activities could be separated. Earlier work suggested that these activities could be resolved by electrofocusing (Fig. 13), however, a firm decision could not be reached at the time, owing to interference in the enzyme assays by high background levels of reducing sugar. An alternative solution to the problem was sought through analytical electrofocusing in

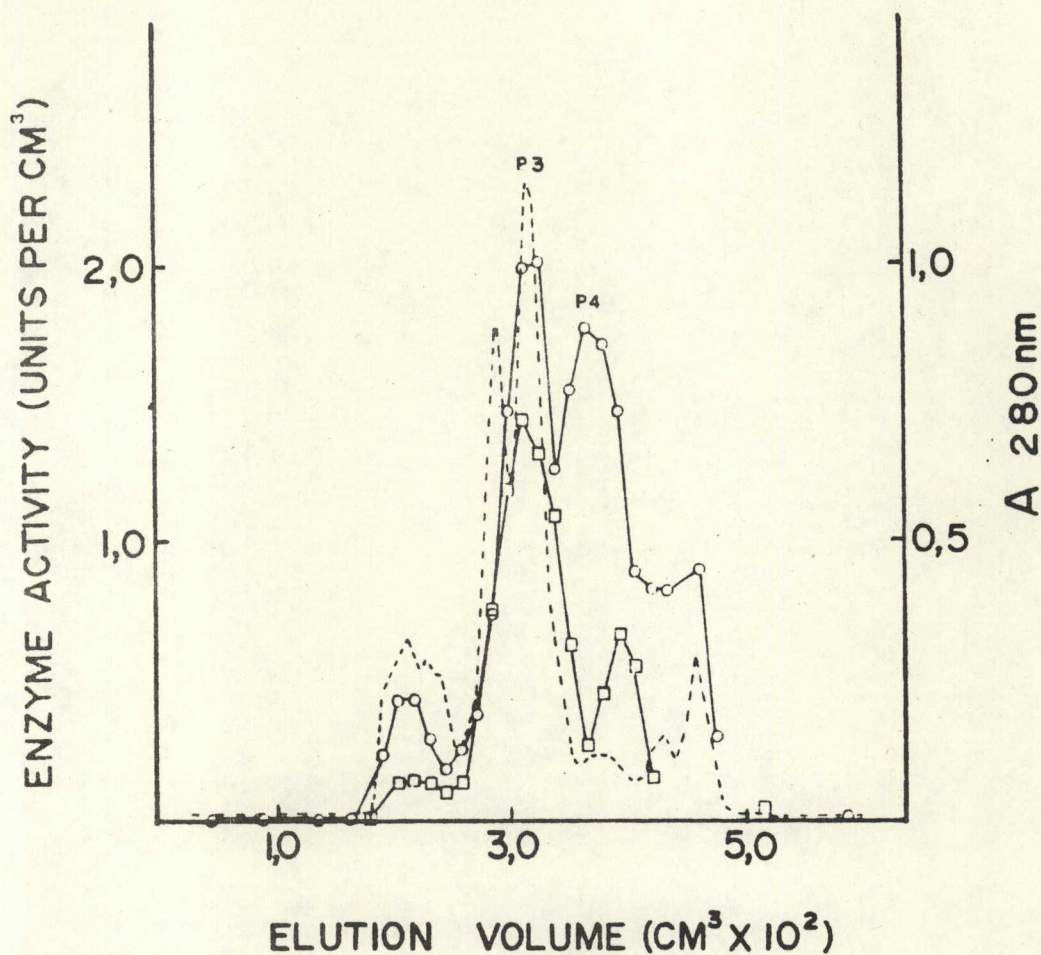


Figure 15. Chromatography of A1 on Bio-Gel P-150. A sample of A1 derived from ca. 18 g of acetone powder was washed with  $0,05 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, on an Amicon PM 10 ultrafilter, reduced to a volume of 10 cm and applied to a column (140 x 2,5 cm) of Bio-Gel P-150 equilibrated in the same buffer at room temperature. Elution was with the equilibrating buffer and fractions ( $4,5 \text{ cm}^3$ ) were collected at a flow rate of  $7,2 \text{ cm}^3$  per hour. The concentrations of the substrate solutions were  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) and  $5,0 \text{ g dm}^{-3}$  xylan (Sigma) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffered at pH 4,9 and 4,5 respectively. CMCcase (○) and xylanase (◻) activities were assayed by the release of reducing sugar after incubating enzyme ( $0,02 \text{ cm}^3$ ) with substrate solution ( $0,3 \text{ cm}^3$ ) at  $50^\circ\text{C}$  for 1 hour. Protein (---) was determined by absorbance at 280 nm.

polyacrylamide gel. A sample of P3 was duly focused in a glass backed 5% polyacrylamide gel slab over the pH range 2,5 to 5,0. The gel was subsequently cut into strips and, after extraction in buffer, the enzyme activities associated with individual strips was determined. The results showed that xylanase and cellulase activities were separable (Fig. 16) and, consequently, the possible existence of a single enzyme able to attack both CMC and xylan was discounted.

The success obtained in resolving small samples of P3 by electrofocusing in polyacrylamide gels argued in favour of using electrofocusing for the further resolution of P3 on a preparative scale. Prior chromatography on Bio-Gel P-150 would have resulted in the separation of CMCase and invertase activities, thus removing a major obstacle to electrofocusing in a sucrose gradient. However, flat bed electrofocusing in a Sephadex G-50 gel support offered the combined advantages of a comparatively large sample load and relatively sharp resolution and consequently became the chosen method.

#### 4.2.9 Preparative electrofocusing of P3

Fig. 17 presents details of the protein content and enzyme activities of gel segments cut from the flat bed after focusing over the pH range 2,5 to 5,0. As expected, xylanase and cellulase activities (which co-chromatographed on the Bio-Gel P-150 column) were separated, and the CMCase fraction, F2, now free of xylanase activity, was subsequently found to be homogeneous by SDS gel electrophoresis and by non-SDS gel electrophoresis run at pH 8,9 (see Plate 2, B and C).

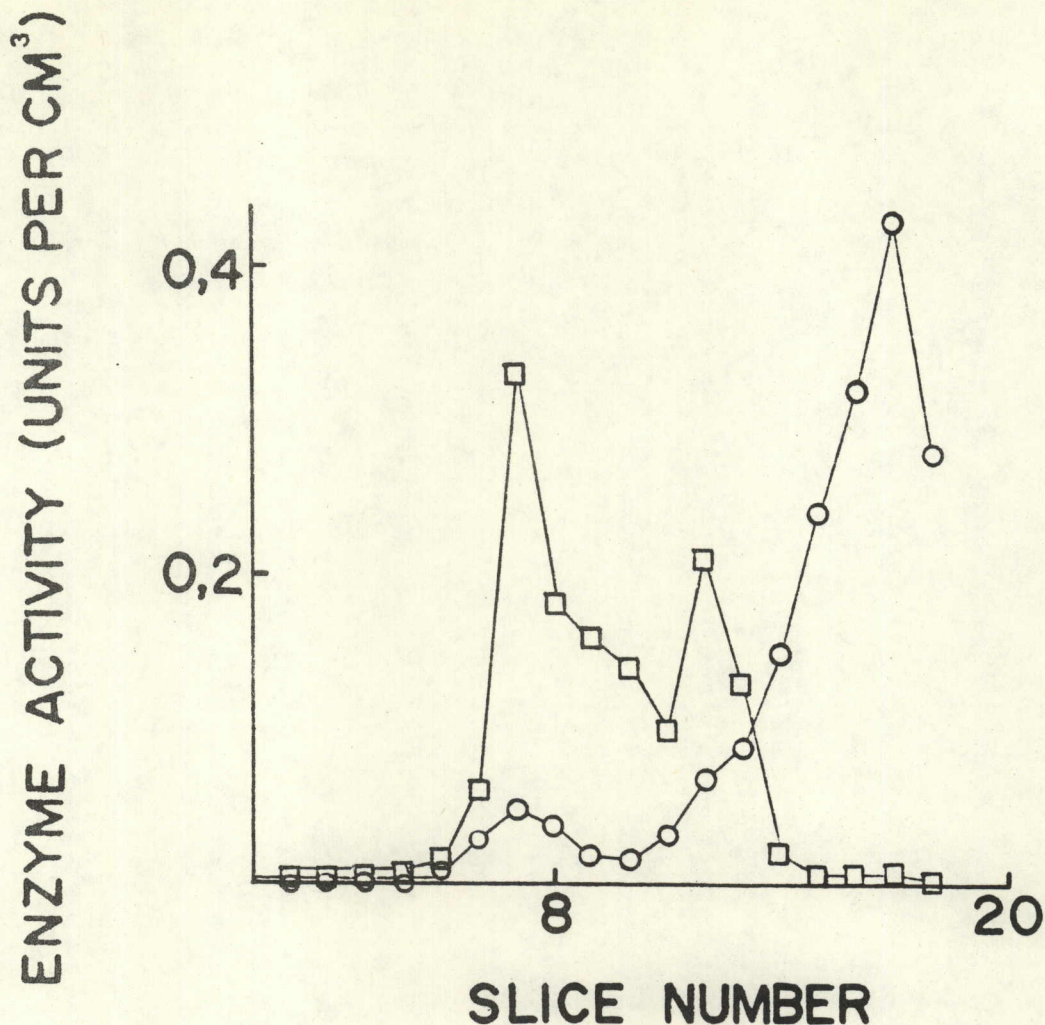


Figure 16. Analytical electrofocusing of fraction P3. A sample of P3 containing ca.  $3,7 \times 10^{-4}$  g of protein was focused in a polyacrylamide gel slab at  $0,09 \text{ W cm}^{-2}$  and  $6^\circ\text{C}$  over the pH range 2,5 to 5,0. The gel was subsequently cut into 1,0 cm wide strips that were soaked overnight in  $0,1 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, containing  $0,2 \text{ g dm}^{-3}$  sodium azide ( $2,0 \text{ cm}^3$ ). Enzyme activity, released into the soaking buffer, was assayed by the production of reducing sugar in mixtures containing enzyme ( $0,05 \text{ cm}^3$ ) and either  $5,0 \text{ g dm}^{-3}$  CMC 4 H1F (CMCase) or  $5,0 \text{ g dm}^{-3}$  Sigma xylan (xylanase) buffered in  $0,05 \text{ mol dm}^{-3}$  sodium acetate at pH 4,5 ( $0,15 \text{ cm}^3$ ). CMCase (○), xylanase (□).

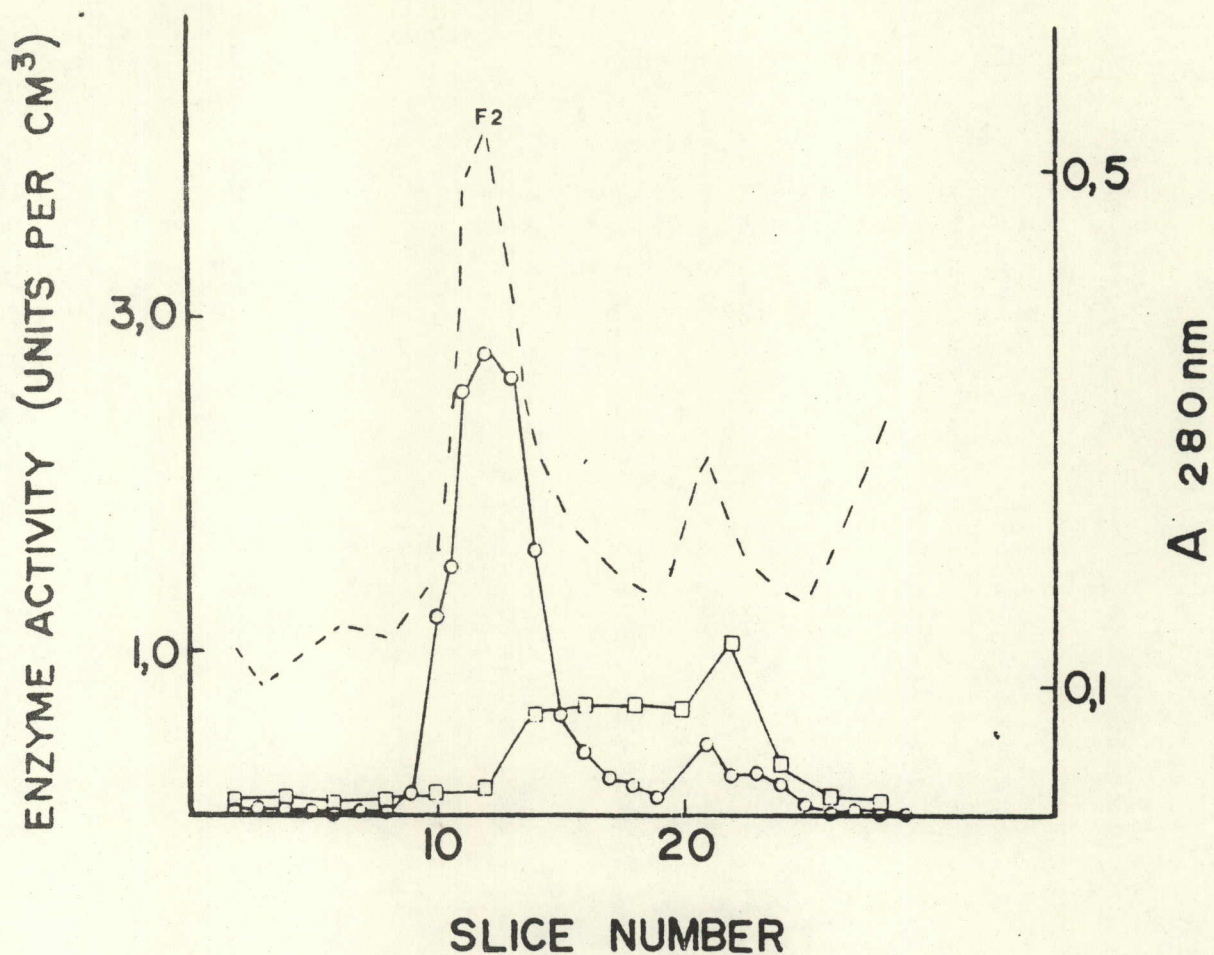


Figure 17. Flat bed electrofocusing of fraction P3. A sample of P3 ( $6,0 \times 10^{-3}$  g) was electrofocused in the pH range 2,5 to 5,0 using the LKB 2117 Multiphor according to the manufacturer's instructions. On completion of electrofocusing, the bed was cut into 1 cm broad segments and the gel eluted with  $0,13 \text{ g dm}^{-3}$  sodium phosphate buffer pH 6,5 ( $3,25 \text{ cm}^3$ ). The substrate solutions used to measure enzyme activity were  $5,0 \text{ g dm}^{-3}$  CMC 4 H1F in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, and  $5,0 \text{ g dm}^{-3}$  xylan (Sigma) in the same buffer at pH 4,9. Enzyme ( $0,01 \text{ cm}^3$ ) was incubated with substrate solution ( $0,25 \text{ cm}^3$ ) and reaction allowed to proceed at  $50^\circ\text{C}$  for 30 minutes before measuring reducing sugar production. CMCCase (○), xylanase (□), protein (---).

### 4.3 Properties of the purified CMCase F2

#### 4.3.1 Assessment of the nature and purity of F2

Although homogeneous by both SDS and non-SDS gel electrophoresis, F2 could be resolved into 3 separate proteins by electrofocusing in polyacrylamide gels. Comparison with pI marker proteins focused in the same gel showed that the pI values of the separate components of F2 were between 4,3 and 4,5 (Plate 3). Staining with Schiff's-periodate reagents (101) following electrofocusing in polyacrylamide gels, indicated that all 3 components of F2 were glycoproteins. Purification data for F2 are presented in Table 3.

#### 4.3.2. Estimation of the molecular mass of F2

The molecular mass of F2 was estimated both by molecular exclusion chromatography and by SDS gel electrophoresis. The estimation of molecular mass by molecular exclusion chromatography was made on fraction P3, (which contained the CMCase F2, later resolved from other P3 components by electrofocusing), using a Bio-Gel P-150 column. The column was calibrated by chromatography of proteins of known molecular mass and the resulting (linear) plot of log molecular mass vs  $V_e/V_o$  (Fig. 18) used (75) to estimate a molecular mass for P3 of  $5,4 \times 10^4$ . An independent estimation of the molecular mass of P3 (and consequently F2) was thus obtained and seen to be in good agreement with the molecular mass of  $5,7 \times 10^4$  obtained for F2 by SDS gel electrophoresis.

TABLE 3. SPECIFIC ACTIVITIES OF FRACTIONS DURING CMCase PURIFICATION.<sup>a</sup>

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FRACTION	SPECIFIC ACTIVITY
	(units per $1,0 \times 10^{-3}$ g protein)
ACETONE POWDER	3.28
A1	4.47
P3	5.40
F2	2.50

---

<sup>a</sup>CMCase activity was determined at 50°C using 20 g dm<sup>-3</sup> CMC (Sigma, D.S. 0,7) in 0,05 mol dm<sup>-3</sup> sodium acetate buffer, pH 4,9. Initial velocity was calculated from the linear portion of the reaction progress curve.

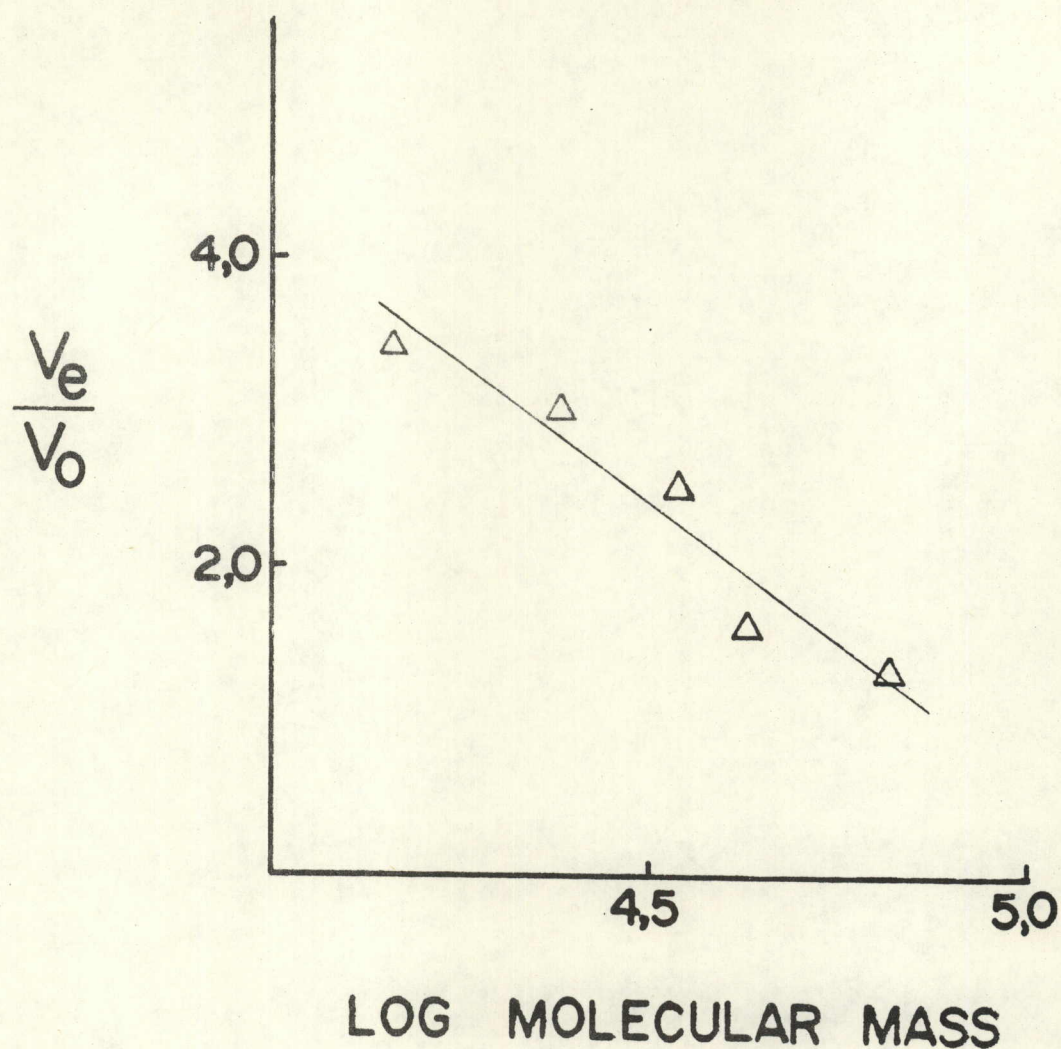


Figure 18. Calibration of the Bio-Gel P-150 column. The following proteins (ca. 0,025 g of each) were used to calibrate the Bio-Gel P-150 column (molecular masses are given in brackets after the protein): bovine serum albumin ( $6,6 \times 10^4$ ): ovalbumin ( $4,5 \times 10^4$ ): pepsin ( $3,47 \times 10^4$ ): trypsinogen ( $2,4 \times 10^4$ ): lysozyme ( $1,43 \times 10^4$ ). Details of sample application, column operation and protein measurement were as for Figure 15, except that the fraction size was  $9,0 \text{ cm}^3$ . Protein in the eluted fractions was determined by the absorbance at 280 nm.

The close agreement between these independent molecular mass determinations suggested a lack of subunit structure for the CMCase (F2) since, had subunits existed, treatment with SDS would likely have caused their disaggregation resulting in a reduction of the apparent molecular mass determined by SDS gel electrophoresis.

#### 4.3.3 Kinetic properties

pH Optimum and stability. The optimum pH for the CMCase activity of F2 over a 20 minute assay at 50°C was 4,0. The enzyme was stable at room temperature for 18 hours over the pH range 2,2 to 8,0 but at high pH values residual activity, measured at pH 4,5, decreased rapidly (Fig. 19). Pre-incubation of the enzyme at 50°C for 1 hour resulted in more than 50% loss of activity at and above pH 6. At this higher temperature the enzyme also appeared unstable at pH 2,2, losing ca. 50% of its initial activity.

Temperature optimum and stability. Maximal CMCase activity was observed at ca. 50°C following incubation of enzyme with substrate for 20 minutes. Pre-incubation of the enzyme at pH 4,5 for 20 minutes, at and above 70°C, resulted in a loss of all measurable activity (Fig. 20).

Specificity. F2 catalysed hydrolysis of both CMC and phosphoric-acid swollen cellulose, but showed no tendency to degrade Avicel, a highly crystalline hydrocellulose. No significant activity was detected on other substrates tested except in the case of galactomannan which

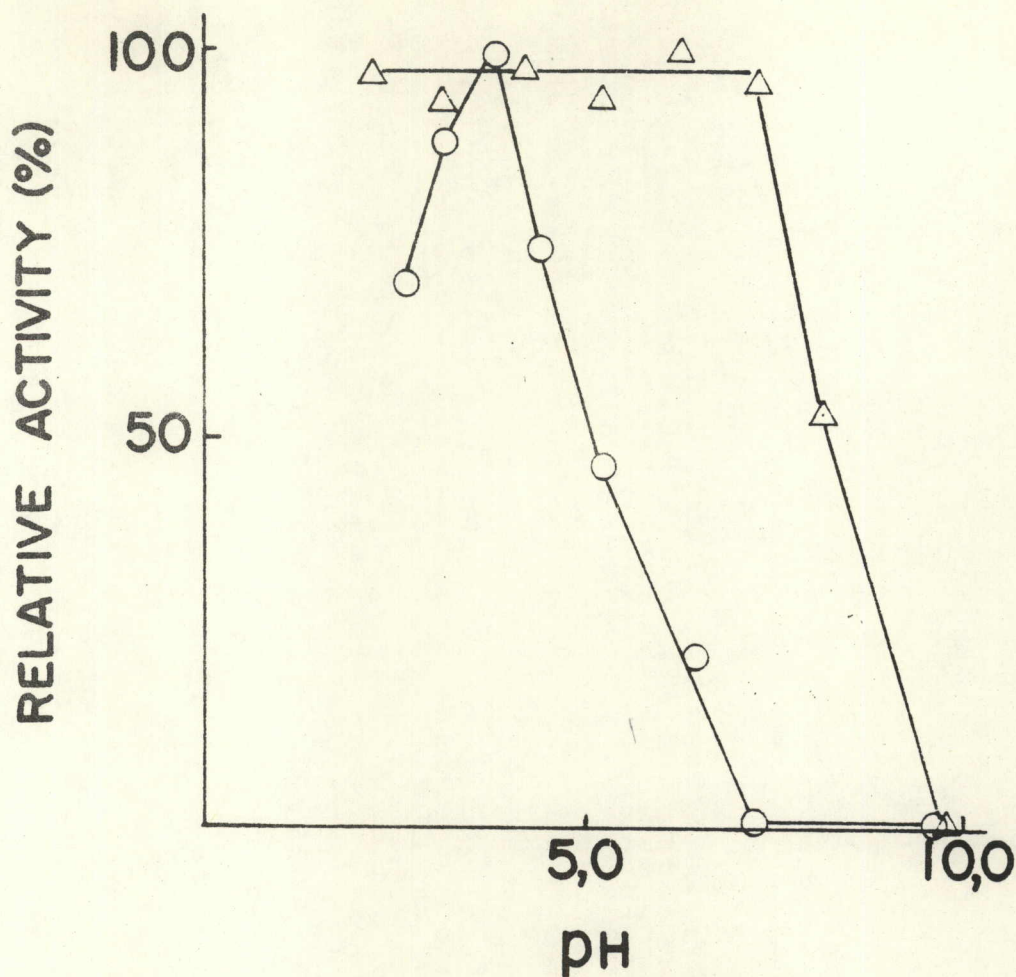


Figure 19. pH optimum and stability of F2. The pH optimum was determined using  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) and a reaction time of 20 minutes. Half strength McIlvaine buffers were employed at pH 2,2 to 3,2 and  $0,05 \text{ mol dm}^{-3}$  glycine-NaOH buffer at pH 10 (58). pH stability was assessed by preincubating enzyme at different pH values for 18 hours at room temperature before assaying residual activity in  $0,5 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5. The pH optimum and the residual activity were determined at  $50^\circ\text{C}$ . pH optimum (O), stability ( $\Delta$ ).

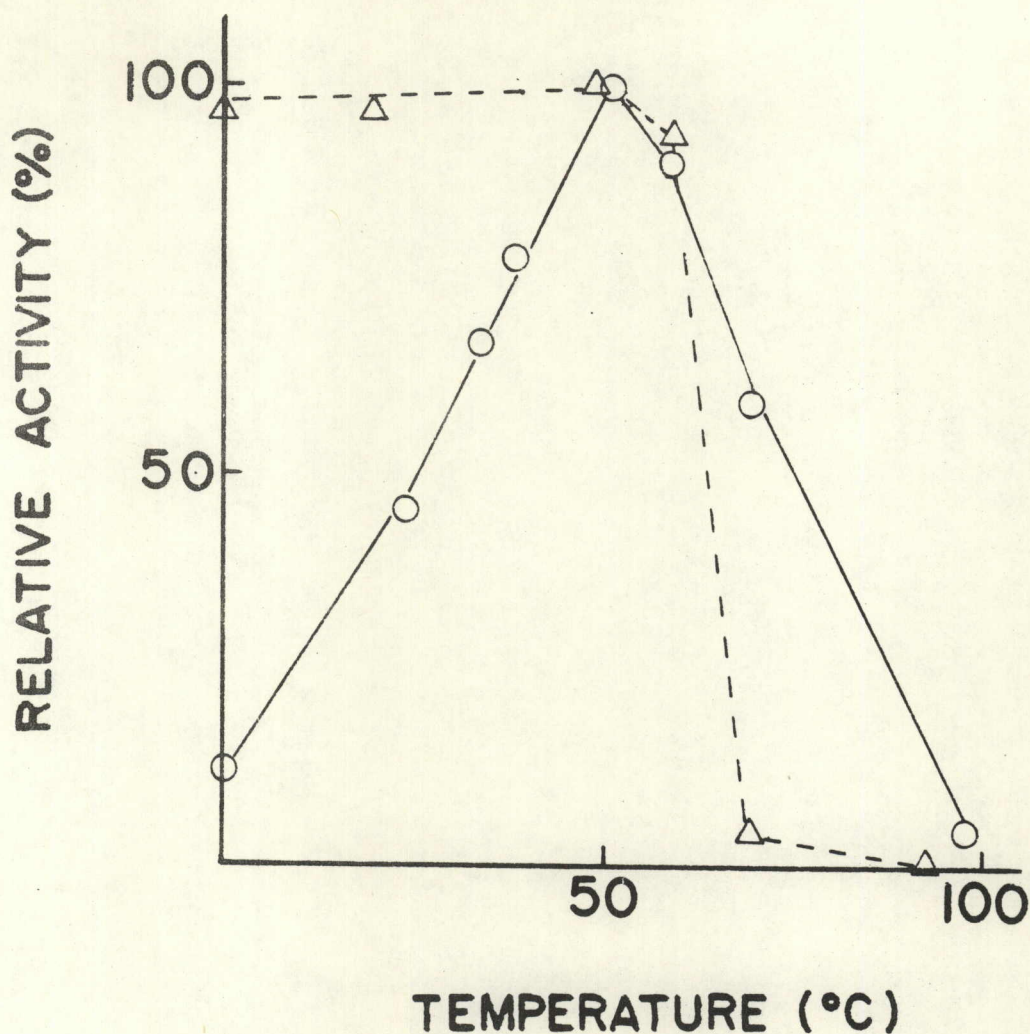


Figure 20. Temperature optimum and thermal stability of F2. Assays were conducted using  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,9, and a reaction time of 20 minutes. Thermal stability was established by preincubating enzyme in the same buffer at pH 4,5 over the range 0 to  $93^{\circ}\text{C}$  for 20 minutes before measuring residual activity at  $50^{\circ}\text{C}$ . Temperature optimum (O), stability ( $\Delta$ ).

yielded ca. 30% of the reducing sugar generated from CMC under the same conditions.

Determination of the Michaelis constant (Km) for F2. The Km for F2 was measured from the initial velocity of attack on CMC 4 HIF (Hercules) over the substrate concentration range 0,5 to 5,0 g dm<sup>-3</sup>. Enzyme solution was diluted until the reaction progress curve for the lowest substrate concentration was linear for at least 2 minutes, and average velocity over 2 minutes was taken as a measure of the initial velocity at this and higher substrate concentrations. Linear regression analysis, applied to the results of six separate velocity determinations, yielded a Km of 2,06 g dm<sup>-3</sup>. No linearisation equation was used and the regression was not weighted. The multiple correlation coefficient was 0,996.

#### 4.3.4 Mechanism of action using oligosaccharide substrates

Synthesis of reduced and non-reduced cellooligosaccharides. The separation on a carbon-celite column of a mixture of cellooligosaccharides is shown in Fig. 21. Individual components up to celloheptaose are seen to be completely resolved. Fig. 22 shows the result of a plot of suspected degree of polymerisation of the cellooligosaccharides vs. log t<sub>R</sub> based on data from high performance liquid chromatography. The straight line relationship obtained in Fig. 22 confirmed the identities of each oligosaccharide.

Following reaction of oligosaccharides with NaBH<sub>4</sub>, the Nelson-

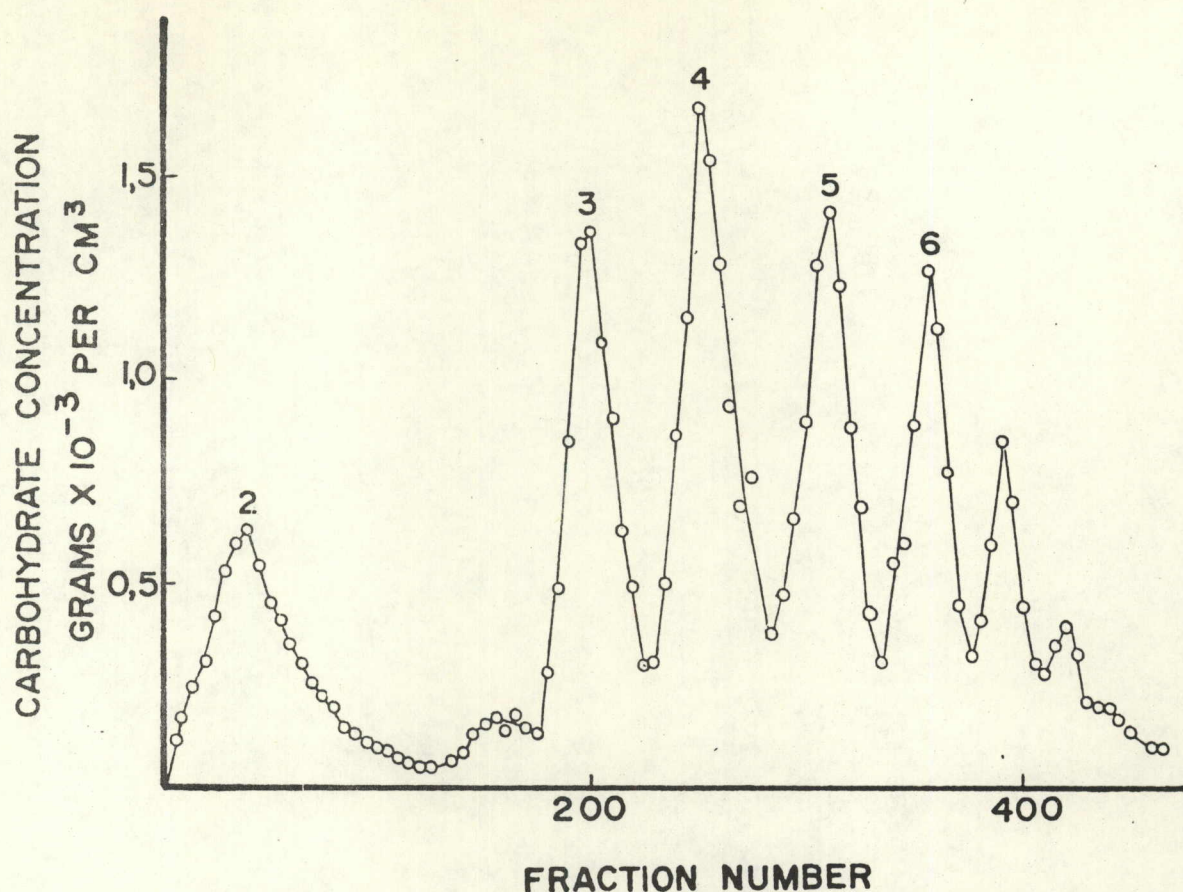


Figure 21. Separation of cellooligosaccharides on a carbon-celite column. Cellooligosaccharides, prepared from Avicel by the method of Miller (76), were chromatographed on a carbon-celite column (5,5 x 91,0 cm). After applying the sample, glucose was eluted from the column with water (2,5 dm<sup>3</sup>) and elution continued with a linear ethanol-water gradient. The donor vessel initially contained 40% (v/v) ethanol (3,0 dm<sup>3</sup>) and the receiving vessel distilled water (3,0 dm<sup>3</sup>). The flow rate was 2,0 cm<sup>3</sup> per minute and the initial fraction size was 220 drops (13,0 cm<sup>3</sup>). The number of drops per fraction was increased to 320 at fraction 209 and 360 drops at fraction 356. At fraction 303 the contents of the donor flask were replaced by 50% (v/v) ethanol (2,0 dm<sup>3</sup>) and 30% (v/v) ethanol (1,0 dm<sup>3</sup>) was added to the receiving vessel. Carbohydrate was determined by the method of DuBois *et al.* (66). Numbers above the peaks refer to D.P.

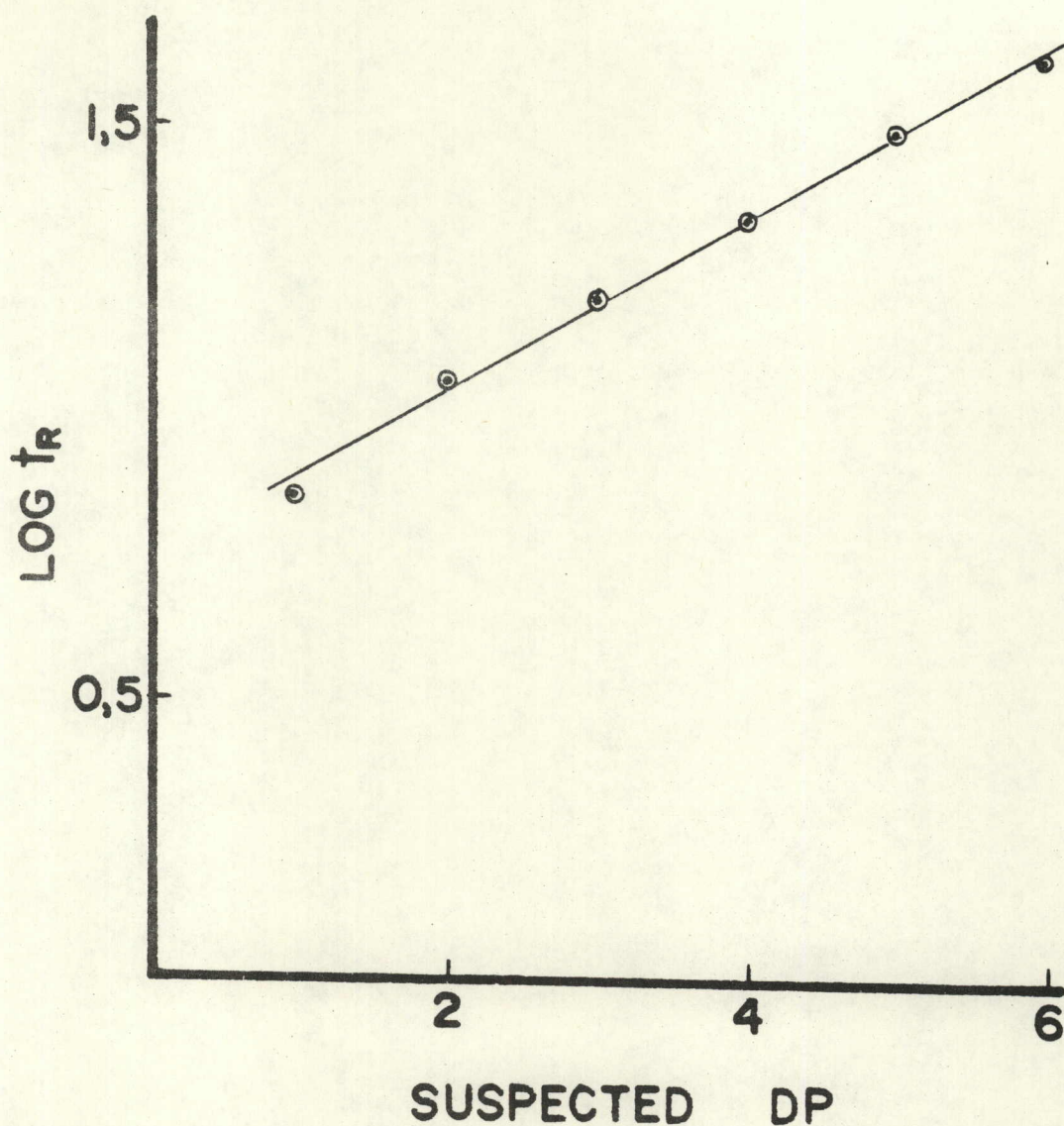


Figure 22. Identification of cellooligosaccharides using high performance liquid chromatography. Cellooligosaccharides were chromatographed on a Waters analytical carbohydrate column using a Varian Model 5 000 Liquid Chromatograph equipped with a refractive index detector. Pressure was maintained at 88 bar and elution was performed isocratically with acetonitrile water (63:37) at a flow rate of 3,0 cm<sup>3</sup> per minute. The symbol  $t_R$  represents the adjusted retention time and is defined by:  $t_R = (t_S - t_W)$  where  $t_S$  and  $t_W$  are the retention times of the sample and water respectively. Note that  $t_R$  is analogous to the expression  $(1 - R_f)/R_f$  for paper chromatography since the  $R_f$  for a sample chromatographed on the column is logically defined by  $t_W/t_S$  i.e. the ratio of the mobility of the sample to that of a non-retarded compound (water). Then,  $(1 - R_f)/R_f = (t_S - t_W)/t_W$  and since, for a homologous series of oligosaccharides, a plot of  $\log (1 - R_f)/R_f$  vs. D.P. is linear (section 2.13.2) it follows that a plot of  $\log t_R$  vs. D.P. will also be linear.

Somogyi reducing sugar method was used to test for completeness of reduction. A positive test ( $< 10\%$  of the control) was given only in the case of cellobiose, indicating that more than 90% of the cellobiose was reduced and that reduction of the longer chain sugars was essentially complete. However, as judged from the thin layer chromatogram, there appeared to be some unreduced cellopentaose present in the cellopentaitol sample (Plate 5 A). Owing to the insolubility of cellohexaose, its conversion to the alditol derivative was not attempted.

Degradation of cellooligosaccharides. During a 2 hour incubation period F2 degraded cellooligosaccharides of D.P.  $\geq 3$  in each case to a mixture of cellobiose and cellotriose, together with trace amounts of glucose. Under similar conditions cellotetraitol and cellopentaitol were each degraded to a mixture of both reduced and non-reduced forms of cellobiose and cellotriose. Cellotriitol by itself was unattacked, but a mixture of cellotriitol and cellotriose was partially degraded to a mixture of cellobiose and cellobiitol.

During shorter incubation times (10 to 20 minutes), cellotetraitol was degraded largely to cellotriitol (Plate 4) and cellopentaitol was degraded to both reduced and non-reduced forms of cellobiose, cellotriose and cellotetraose, (Plate 5 A). Early products of cellohexaose breakdown were cellobiose, cellotriose and cellotetraose. Apparently no cellopentaose was generated (Plate 5 B).

Oligosaccharides of D.P. 2 to 5 were incubated individually with

PNG in the presence of F2 and the subsequent release of p-nitrophenol measured spectrophotometrically at 401 nm. PNG itself was unattacked, however a weak reaction occurred between PNG and each of the oligosaccharides cellotetraose and cellopentaose. No reaction was observed between PNG and either cellobiose or cellotriose.

Effect of oligosaccharide concentration on the activity of F2. A study of the influence of oligosaccharide concentration on their degradation rate was undertaken to determine the influence of the degree of polymerisation on  $K_m$ . Several authors (35,39,102) have noted that, in the case of endocellulases,  $K_m$  usually decreases with increasing D.P. and this might be interpreted as indicating a preference of the enzyme for highly polymerised substrates. In the present study, a similar correlation was sought in the case of F2. Since reaction rate was to be measured by the rate of release of reducing products by the Nelson-Somogyi method, it was advantageous to employ non-reducing substrates (e.g. cellotetraitol and cellopentaitol). In this way substrate would not interfere with measurement of the reaction products, and the sensitivity of the assay would be retained. A plot of substrate concentration vs. F2-catalysed degradation rate is presented for each of the substrates cellotetraitol and cellopentaitol in Fig. 23. In both cases reaction rate was calculated from linear progress curves and therefore approximates closely the true initial velocity. The rate of cellotetraitol degradation seemed relatively unaffected by cellotetraitol concentration (Fig. 23), whereas cellopentaitol degradation was subject to marked substrate inhibition (Fig 23). Since reaction rate did not continue to increase with sub-

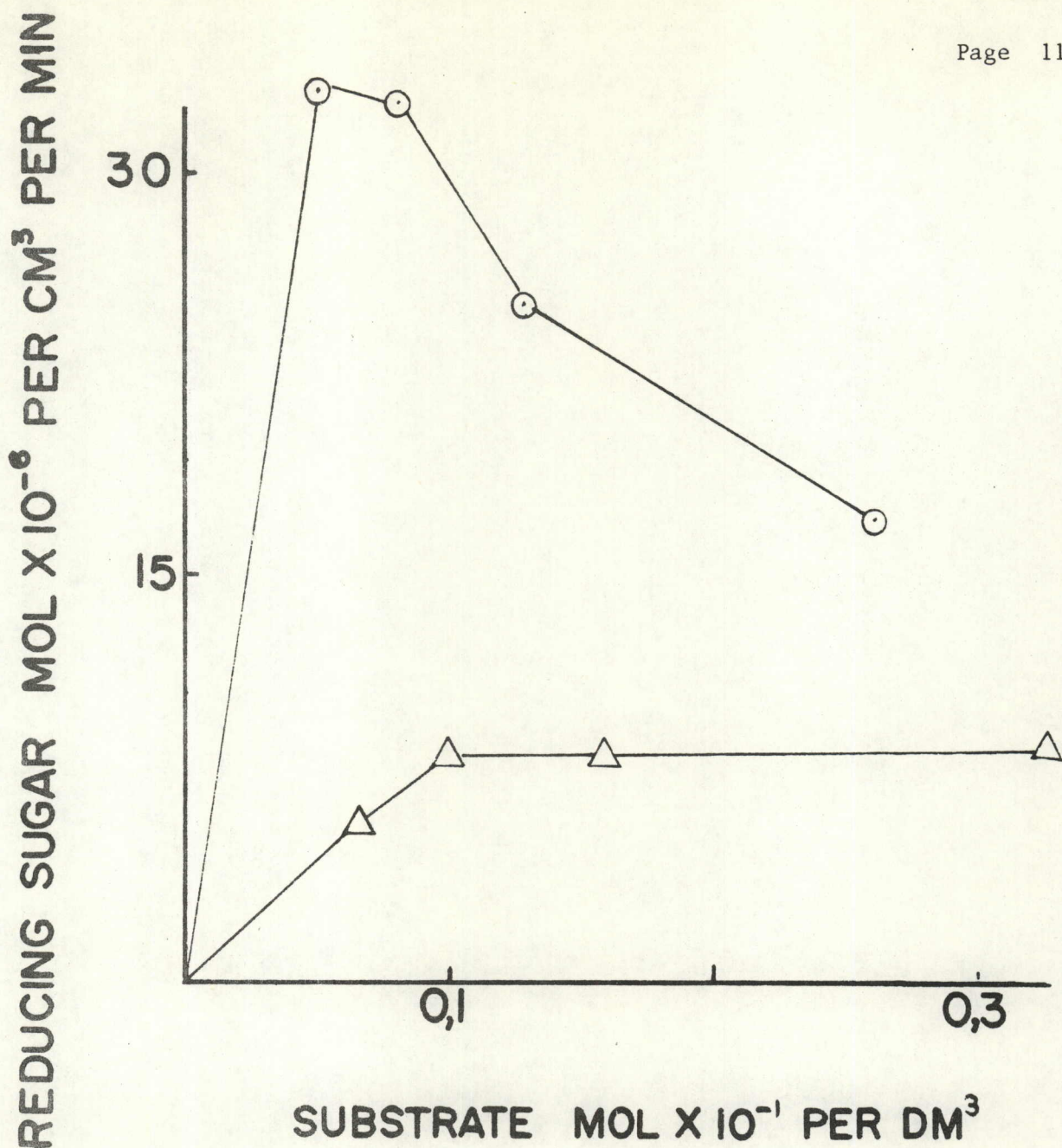


Figure 23. F2-catalysed degradation of cellotetraitol and cellopentaitol: effect of substrate concentration. Cellotetraitol or cellopentaitol ( $5,2 \times 10^{-3}$  to  $3,3 \times 10^{-2} \text{ mol dm}^{-3}$ ) were incubated with F2 in  $0,05 \text{ mol dm}^{-3}$  ammonium acetate, pH 4,5. The reaction was allowed to proceed at  $51^\circ\text{C}$  for 4 minutes before determining the release of reducing sugar. The progress curves for the degradation of both substrates at the lowest substrate concentration were linear for at least 4 minutes. Cellotetraitol, ( $\Delta$ ) and cellopentaitol ( $\circ$ ).

strate concentration in either case, attempts to measure  $K_m$  using oligosaccharides were abandoned.

#### 4.4 Properties of the partially purified CMCCase P4

In addition to P3 a second CMCCase, P4, was fractionated on the Bio-Gel P-150 column following chromatography of A1, (Section 4.2.7). P4 exhibited 3 bands on SDS-containing gels and 3 (or possibly 4) bands following electrofocusing in polyacrylamide gel.

Weak xylanase activity was discerned in the fractions constituting P4 during assay of the Bio-Gel P-150 effluent (Fig. 15) and, after concentrating the pooled P4 fractions, the presence of xylanase activity was confirmed. In addition the concentrated P4 fraction was able to catalyse hydrolysis of ONX. Results obtained from the subsequent electrofocusing of P4 suggested that both xylanase and xylosidase activities were associated with protein(s) distinct from the CMCCase protein(s) (Fig. 24).

Even though P4 was demonstrably an impure enzyme preparation, several properties of the CMCCase(s) in P4 were deduced. The results obtained are compared with the corresponding data for F2 in Table 4.

#### 4.5 Modes of attack of F2 and P4 on CMC

Although CMCases, by definition, are all capable of degrading CMC, their modes of attack on this substrate are often quite distinct.

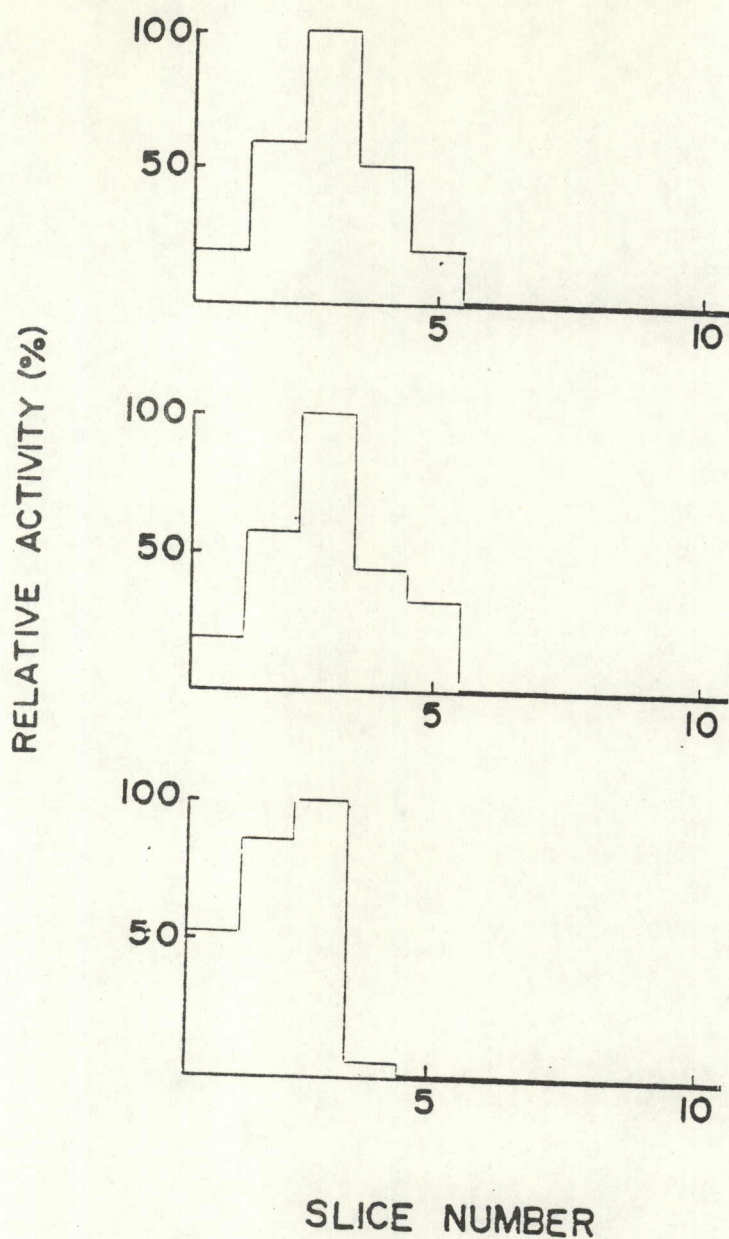


Figure 24. Analytical electrofocusing of fraction P4. A sample of P4 was focused over the pH range 3,5 to 9,5 in a ready-made polyacrylamide gel slab (LKB) at  $0,09 \text{ W cm}^{-2}$ . Strips ( $1,0 \times 2,0 \text{ cm}$ ) were cut from the gel and soaked overnight in  $0,5 \text{ mol dm}^{-3}$  sodium acetate, pH 4,5, containing  $0,2 \text{ g dm}^{-3}$  sodium azide ( $2,3 \text{ cm}^3$ ). Polysaccharide degrading activity released from the gel strips was assayed by measuring the production of reducing sugar from  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) (CMCase) or  $5,0 \text{ g dm}^{-3}$  purified Sigma xylan (xylanase). Activity on *o*-nitrophenyl- $\beta$ -D-xyloside (ONX) was measured by the increase in absorbance at 401 nm (see section 2.4.2) following incubation of F2 with  $10 \text{ g dm}^{-3}$  ONX. In all cases substrates were buffered in  $0,05 \text{ mol dm}^{-3}$  sodium acetate containing  $0,2 \text{ g dm}^{-3}$  sodium azide at pH 4,5. Enzyme ( $0,05 \text{ cm}^3$ ) was incubated with substrate ( $0,5 \text{ cm}^3$ ) at  $45^\circ\text{C}$  for 12 hours before measuring product formation.

TABLE 4. A COMPARISON OF SOME PROPERTIES OF F2 AND P4.

PROPERTY	F2	P4
MOLECULAR MASS <sup>a</sup>	5,4 X 10 <sup>4</sup>	4,4 X 10 <sup>4</sup>
pH OPTIMUM <sup>b</sup>	ca.4	ca.4
pH STABILITY <sup>c</sup>	UNSTABLE ABOVE pH 7	LESS THAN 10% LOSS OF ACTIVITY UP TO pH 10
TEMPERATURE OPTIMUM <sup>d</sup>	50°C	50 to 70°C
THERMAL STABILITY <sup>e</sup>	UNSTABLE ABOVE 60°C	STABLE UP TO 70°C
K <sub>m</sub> (g dm <sup>-3</sup> ) <sup>f</sup>	2,06	3,35
MODE OF ACTION ON CMC	LESS RANDOM	MORE RANDOM

a: determined on Bio-Gel P-150

b: 20 minute reaction time at 50°C

c: preincubation at room temperature for 18 hours

d: 20 minute reaction time at pH 4,9

e: preincubation at pH 4,5 for 20 minutes

f; substrate: CMC 4 H1F

CMCase attack on polymeric substrates (such as CMC) is normally endo in character, that is internal (1→4)-β-D-glycosidic bonds are preferentially cleaved resulting initially in a rapid decrease in molecular weight of the substrate and generation of comparatively large oligosaccharide products. Some CMCase however, whilst still attacking internal bonds, preferentially degrade those bonds situated nearer a terminus of the polymer, effecting a less marked reduction in substrate molecular mass, at the same time, generating a proportion of comparatively small oligosaccharide products.

Owing to their depolymerising action, CMCase cause a decrease in the viscosity of the CMC solutions. The greater the tendency of a CMCase to cleave more central bonds, the greater will be the rate of viscosity decrease per unit of enzyme added. It should therefore be possible, in theory, to classify CMCase according to the rate at which a fixed amount of enzyme decreases the viscosity of a CMC solution under defined conditions. However, the problem might arise as to how to measure the correct amount of enzyme required for the standard viscosity determination since, at present, no universally accepted unit of CMCase activity appears to exist.

An alternative approach, independent of enzyme concentration, consists of plotting the generation of reducing sugar (on the abscissa) against the corresponding specific fluidity,  $\phi_{sp}$ , of a CMC solution as enzymic degradation proceeds.  $\phi_{sp}$  denotes the inverse of the specific viscosity  $\eta_{sp}$ , i.e.  $\phi_{sp} = \eta_{sp}^{-1}$ . Experiment has shown that a straight line results from this plot and the slopes of the lines obtained from

data on different CMCase has been used as a means of classifying these enzymes (39,41,103). The success of the method depends on the observation that oligosaccharides of comparatively high degree of polymerisation contribute less on a molar basis to the measured reducing sugar than do smaller oligosaccharides. A CMCase with comparatively high affinity for the centrally situated bonds of CMC will initially cause a rapid increase in fluidity of the solution while, at the same time, the large molecular weight products formed will contribute little to the measured reducing sugar. The resulting reducing sugar vs.  $\phi_{sp}$  plot would therefore have a comparatively steep slope.

In contrast, a CMCase with preference for bonds nearer a CMC terminus has less effect on  $\phi_{sp}$  but generates some low molecular weight products with an associated high reducing sugar activity. The reducing sugar vs.  $\phi_{sp}$  plot in this latter case would show a comparatively shallow slope. Note that a single enzyme cleavage of CMC results in the formation of two oligosaccharide products. In the case of a CMCase showing preference for central bonds of the CMC, two medium-sized oligosaccharides are formed per cleavage. A CMCase with greater affinity for bonds nearer a CMC terminus produces one large and one small oligosaccharide per cleavage. Compared with an oligosaccharide of medium size, a small oligosaccharide has an enhanced reducing activity and a large oligosaccharide a decreased reducing activity. It is implicit in the rationale of the previous paragraph that the enhanced reducing activity of the small oligosaccharide outweighs the decreased reducing activity of its larger companion. In other words,

reducing activity must fall off rapidly above a certain limiting oligosaccharide size, this limit being less than or equal to half the size of an undegraded CMC molecule.

Although the plot of reducing sugar vs.  $\phi_{sp}$  offers a basis for comparing the modes of action of different CMCase, its scope of application is limited by a lack of consensus on the choice of experimental parameters such as temperature and the method for estimating reducing sugar. The plot may, however, be applied successfully to enzyme reactions determined under the same conditions and this has been done for the CMCase isolated in this study, namely F2 and P4 (Fig. 25).

The results presented in Fig. 25 indicate that F2 appears to attack bonds in CMC which lie closer to a CMC terminus than do those attacked by P4.

#### 4.6 Pitfalls in the assay of CMCase activity

The assay of CMCase activity, by measuring the enzyme-catalysed release of reducing sugars from CMC, is a widely adopted procedure and is used extensively in the work reported in this thesis. There are, however, several pitfalls associated with this method of assay that arise from the nature of both the substrate and the enzymes employed. For example, comparison of the activities of different enzyme preparations normally requires that the respective enzyme dilution curves be linear and hence that the measured reaction velocity be

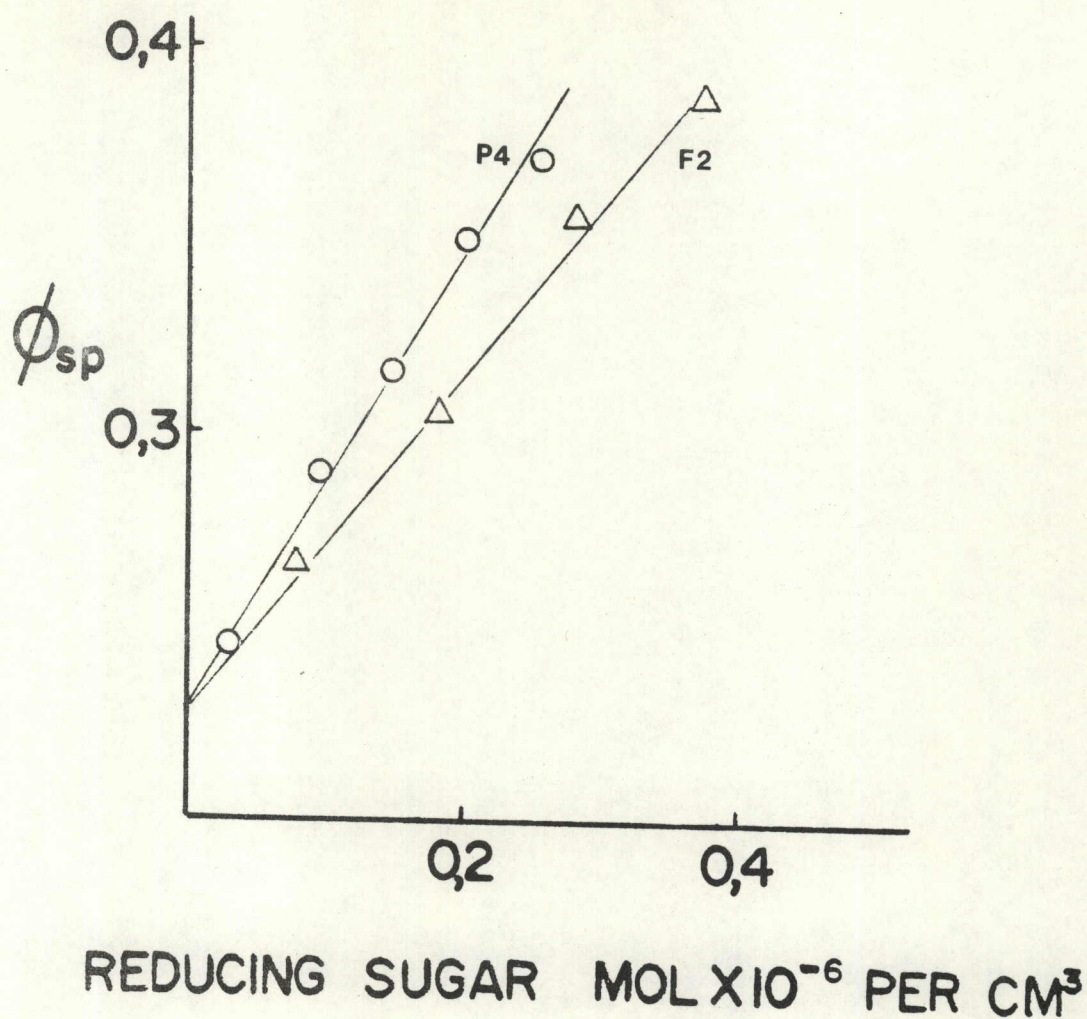


Figure 25. Relationship between the change in specific fluidity and the production of reducing sugar by F2 and P4. Enzyme solution was incubated with  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, at  $25^\circ\text{C}$ . At intervals, samples were removed for the estimation of reducing sugar. The time taken for the reaction mixture to drain from a capillary tube ( $15 \times 0,5 \text{ cm}$ ) was simultaneously measured and used to calculate specific fluidity ( $\phi_{sp}$ ).

proportional to enzyme concentration. It has, however, been shown (104, 105) that non-linear dilution curves are often obtained for CMCases. Thus Miller, Blum, Glennon and Burton (104) obtained anomalous S-shaped dilution curves which were ascribed to the complexities of both substrate and enzyme. These authors suggested that standard dilution curves be constructed for each individual enzyme preparation from which enzyme units could be related to activity in much the same way as suggested for proteinases (105). An alternative approach (106) has been to derive a non-linear equation to be applied to the enzyme activity at any given dilution to yield activity at any other desired dilution.

These considerations are relevant not only to those situations in which CMCASE activities from different organisms are being compared, but also to the calculation of recovery and purification data during the isolation of a single CMCASE.

Another difficulty relates peculiarly to the latter situation. CMCASES often occur as families of physicochemically different enzymes but having the same nominal activity, since they are not distinguished by the assay system. In such a case, the calculation of recovery and degree of purification based on activity measurements would seem to be at variance with the normally accepted meanings of the terms. The manifestation of synergistic effects by CMCASES (107) serves to further complicate the issue.

In much of the work published to date no explicit evidence has

been offered to the effect that any of these difficulties has been avoided, and the validity of much of the published data is thus called into question.

In this section, results obtained with both a crude cellulase preparation from T. reesei (a gift from Dr. M.H. Mandels), and a partially purified CMCCase from S. rolfsii, P3 (section 4.2.7), are used to define the conditions under which accurate determination of enzyme concentration can be made from activity measurements. In addition, attention is drawn to the many incorrect inferences that have been drawn in the past from data on CMCases.

#### 4.6.1 Causes of non-linearity of progress and dilution curves

An early observation made in this study was that the non-linear enzyme dilution curve was a secondary effect resulting from a non-linear progress curve, even under conditions where substrate concentrations remained in apparent excess (Figs. 26 and 27).

The surprisingly rapid onset on non-linearity in the progress curves (Fig. 26) bore further investigation and the following possible causes were examined: enzyme instability, cryptic substrate limitation and product inhibition.

Enzyme stability. Pre-incubation for 0,5 hours of the S. rolfsii CMCCase under the experimental conditions failed to impair its subsequent activity, indicating that the enzyme was stable. The

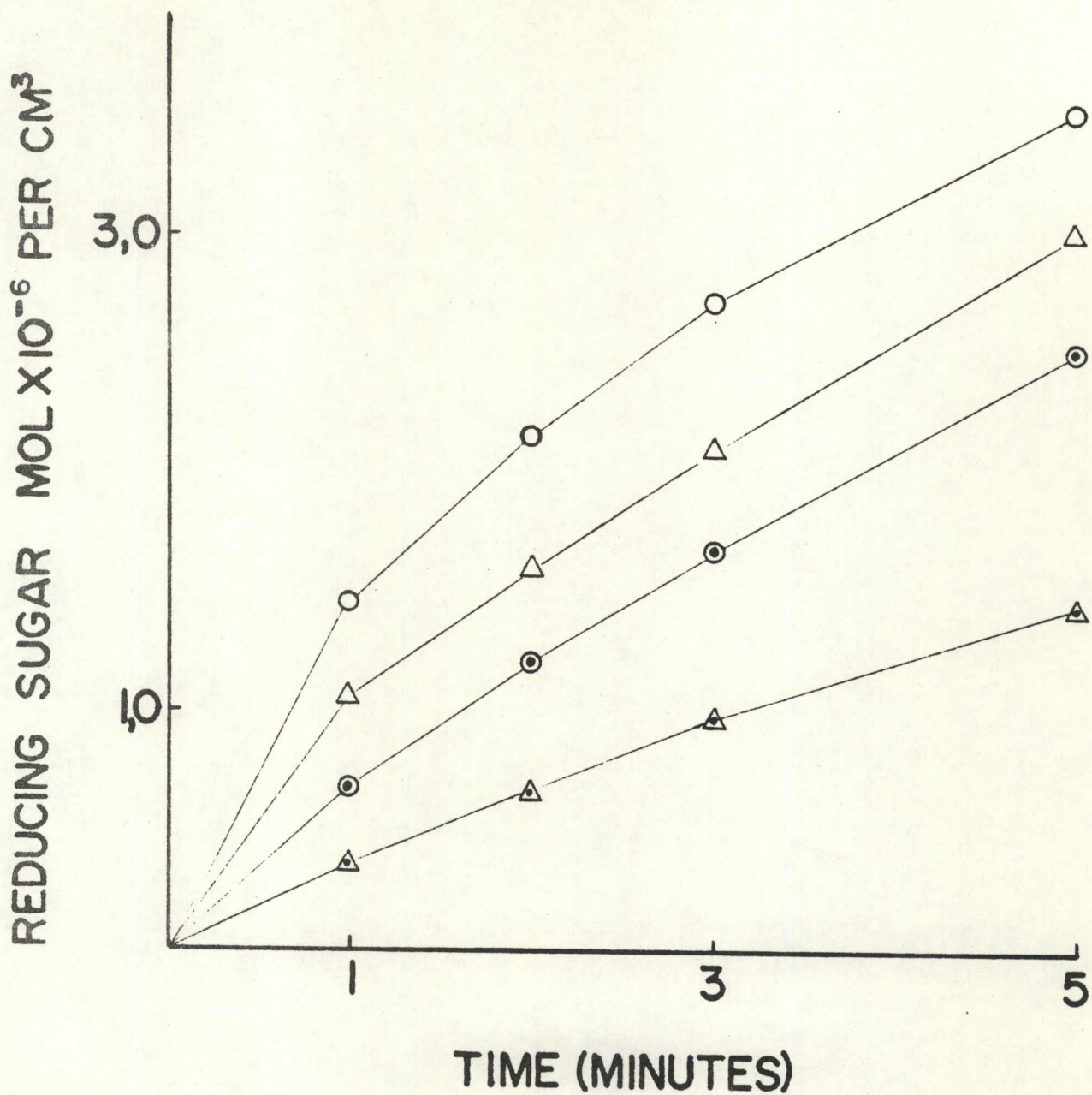


Figure 26. Non-linear progress curves for P3. The substrate was  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,075 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,7. The final protein concentrations ( $\text{g} \times 10^{-6}$  per  $\text{cm}^3$ ) were: 26,25 (○) 15,75 (△) 10,5 (⊙) and 5,25 (◐). The temperature was  $53^\circ\text{C}$ .

REDUCING SUGAR MOL X 10<sup>-6</sup> PER CM<sup>3</sup> PER MIN

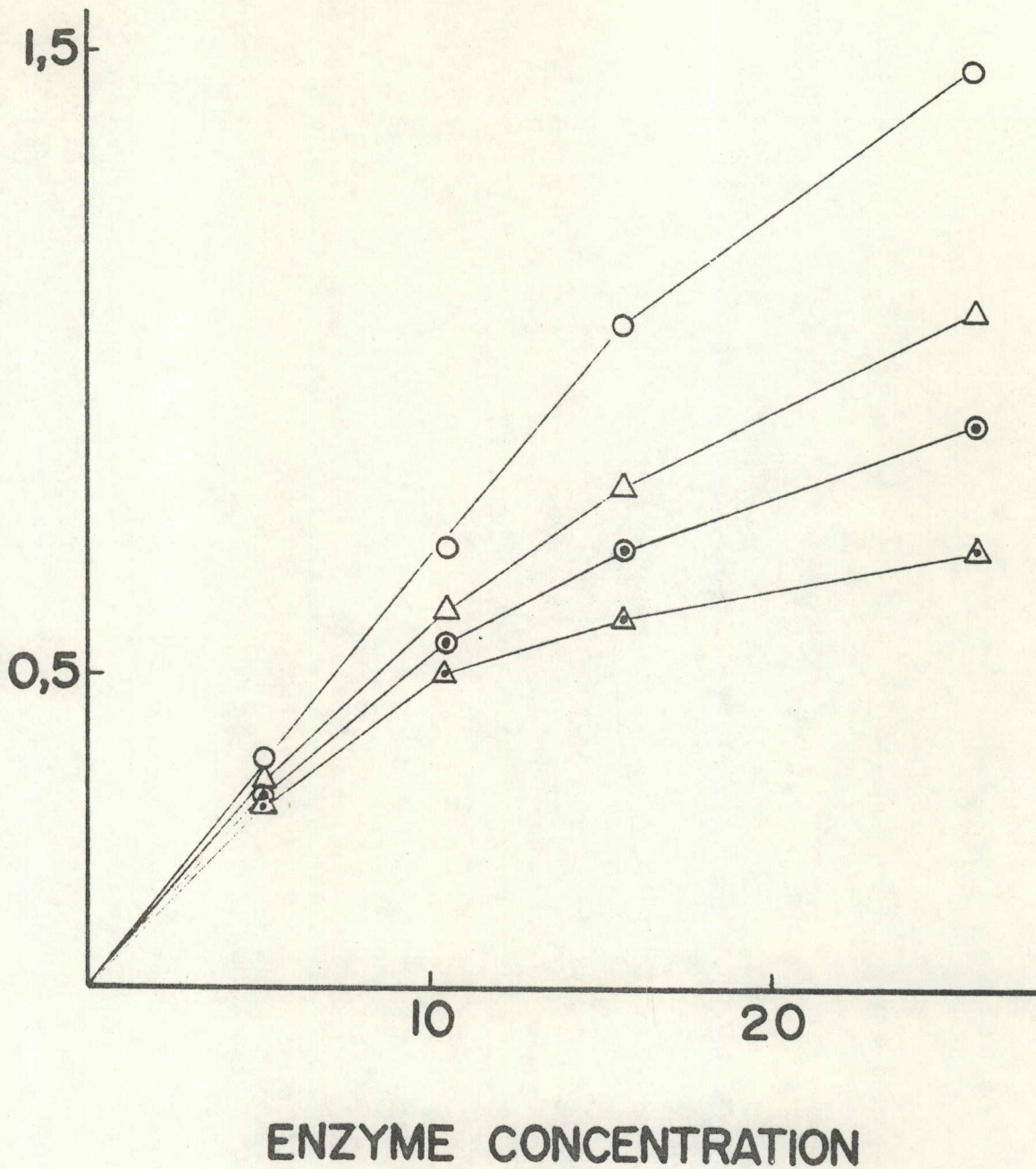


Figure 27. Non-linear enzyme dilution curves for P3. The curves were plotted from the data of Figure 26. The average velocity was calculated over 1 (○) 2 (△) 3 (⊙) and 5 (◡) minutes.

stability of the T. reesei preparation may also be inferred (96).

Cryptic substrate limitation. Although the progress curves became non-linear despite an apparent excess of substrate, a cryptic substrate limitation was considered possible since, owing to the substituents on CMC, not all the bonds might be susceptible to enzymic hydrolysis. This possibility was tested by comparing the progress curves for the P3 catalysed hydrolysis of two CMC preparations, one of D.S. 0,4 (4 HF) and the other of D.S. 0,7 (7 HF). The results, shown in Fig. 28, indicate that non-linearity could be associated with CMC of high D.S., suggesting a possible limitation in the number of susceptible bonds.

Product inhibition. The possible occurrence of product inhibition was tested by comparing the activity of P3 in the presence and absence of added cellobiose, used here as a model for the hydrolysis products (Fig. 29). Added cellobiose resulted in a substantial reduction of activity (23% at 1 minute), indicating the possible occurrence of product inhibition. However, a re-examination of Fig. 28 reveals that the progress curve for hydrolysis of CMC 4 HF was linear where the CMC 7 HF hydrolysis curve was non-linear despite the greater accumulation of product in the former case. It therefore seems that cryptic substrate limitation might be a primary cause of non-linearity in the CMC case progress curve whereas product inhibition could play a secondary role.

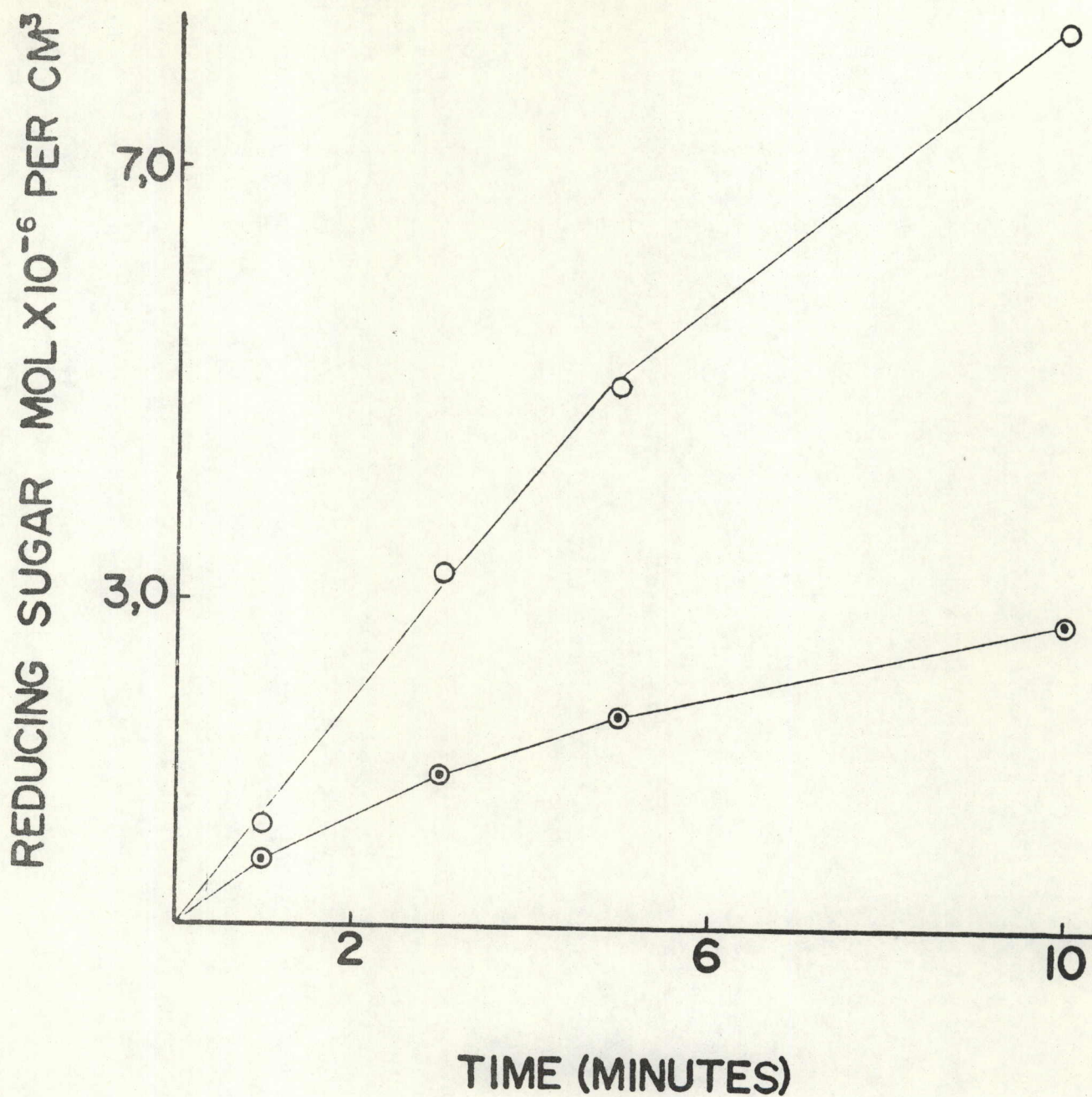


Figure 28. Progress curves for the P3-catalysed hydrolysis of different CMC preparations. The substrate concentration was  $5,0 \text{ g dm}^{-3}$  in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, and the temperature was  $50^\circ\text{C}$ . CMC 4 H1F (O) and CMC 7 HF ( $\odot$ ).

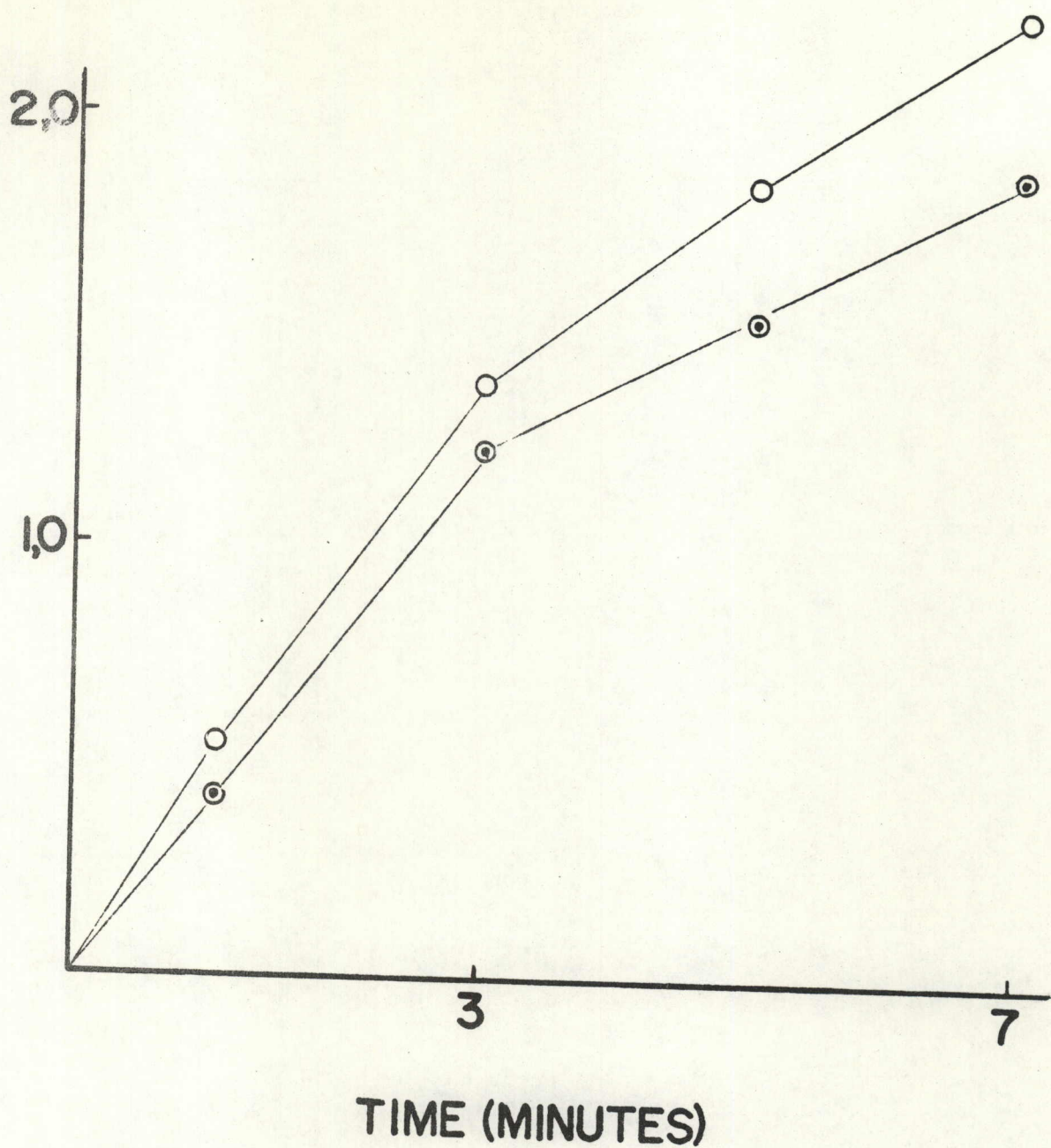
REDUCING SUGAR MOL X 10<sup>-6</sup> PER CM<sup>3</sup>

Figure 29. Cellobiose inhibition of P3. The substrate was 20 g dm<sup>-3</sup> CMC (Sigma, D.S. 0,7) in 0,05 mol dm<sup>-3</sup> sodium acetate buffer, pH 4,9, and the temperature was 50°C. Added cellobiose (1,1 g dm<sup>-3</sup>) (⊙) and control (O).

#### 4.6.2 Attainment of linear progress and dilution curves

Taking the above considerations into account, the problem of obtaining linear dilution curves was eventually solved by confining attention to the initial stages of the reaction only (Figs. 30 and 31). However, in the initial stages of the reaction, very little product has accumulated and, consequently, comparatively large samples (0,5 to 1,0 cm<sup>3</sup>) had to be withdrawn from the reaction mixture for assay of reducing sugar. In addition, it was noted that at low product concentration, the DNS method of reducing sugar measurement (108) yielded a non-linear dilution curve under conditions where a linear dilution curve could be obtained using the Nelson-Somogyi assay (Fig. 32). Non-linearity of the dilution curve with the DNS assay probably reflects the susceptibility of glucose to partial destruction by alkaline oxidation which also results in non-linear standard curves at low glucose concentrations. As recommended by Miller (108) addition of a small amount of glucose to the assay medium may have overcome this effect but still, the Nelson-Somogyi procedure is preferred, particularly when using CMC of high D.S.

#### 4.7 Discussion

##### 4.7.1 Purity and physicochemical properties of the CMC<sub>Case</sub> F2

Fraction F2 was homogeneous by both SDS and non-SDS gel electrophoresis but showed as 3 separate glycoproteins following both preparative and analytical electrofocusing. Although the 3 components

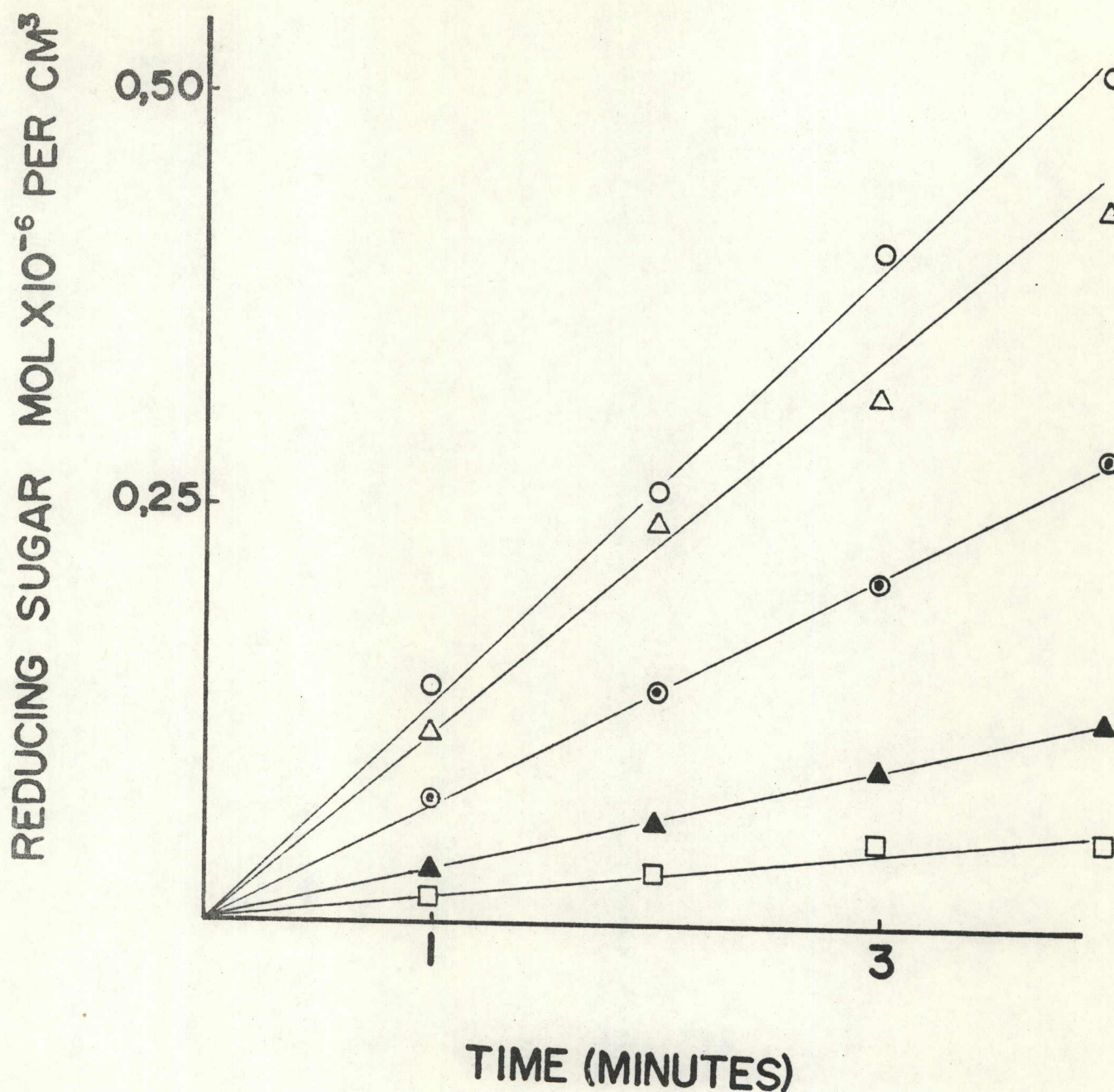
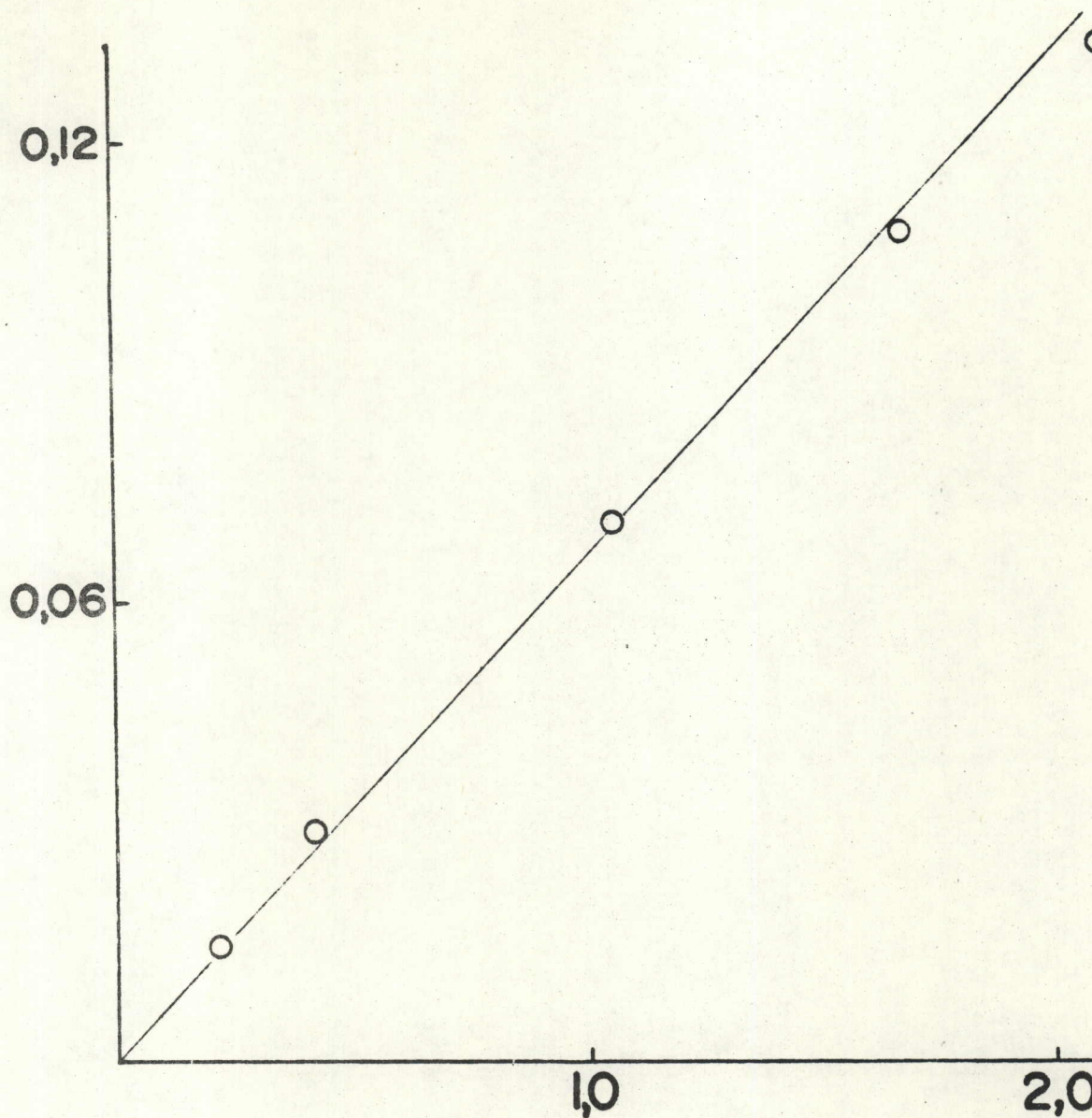


Figure 30. Linear progress curves for P3. Aliquots ( $0,5 \text{ cm}^3$ ) were assayed for reducing sugar. The substrate was  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,9. The temperature was  $50^\circ\text{C}$ . Final protein concentrations ( $\text{g} \times 10^{-6}$  per  $\text{cm}^3$ ) were: 2,1 (○) 1,68 (Δ) 1,05 (⊙) 0,42 (▲) and 0,21 (□).

REDUCING SUGAR MOL X 10<sup>-6</sup> PER CM<sup>3</sup> PER MIN

ENZYME CONCENTRATION

Figure 31. Linear enzyme dilution curve for P3. Average velocity was calculated from the data of Figure 30.

REDUCING SUGAR MOL X 10<sup>-6</sup> PER CM<sup>3</sup> PER MIN

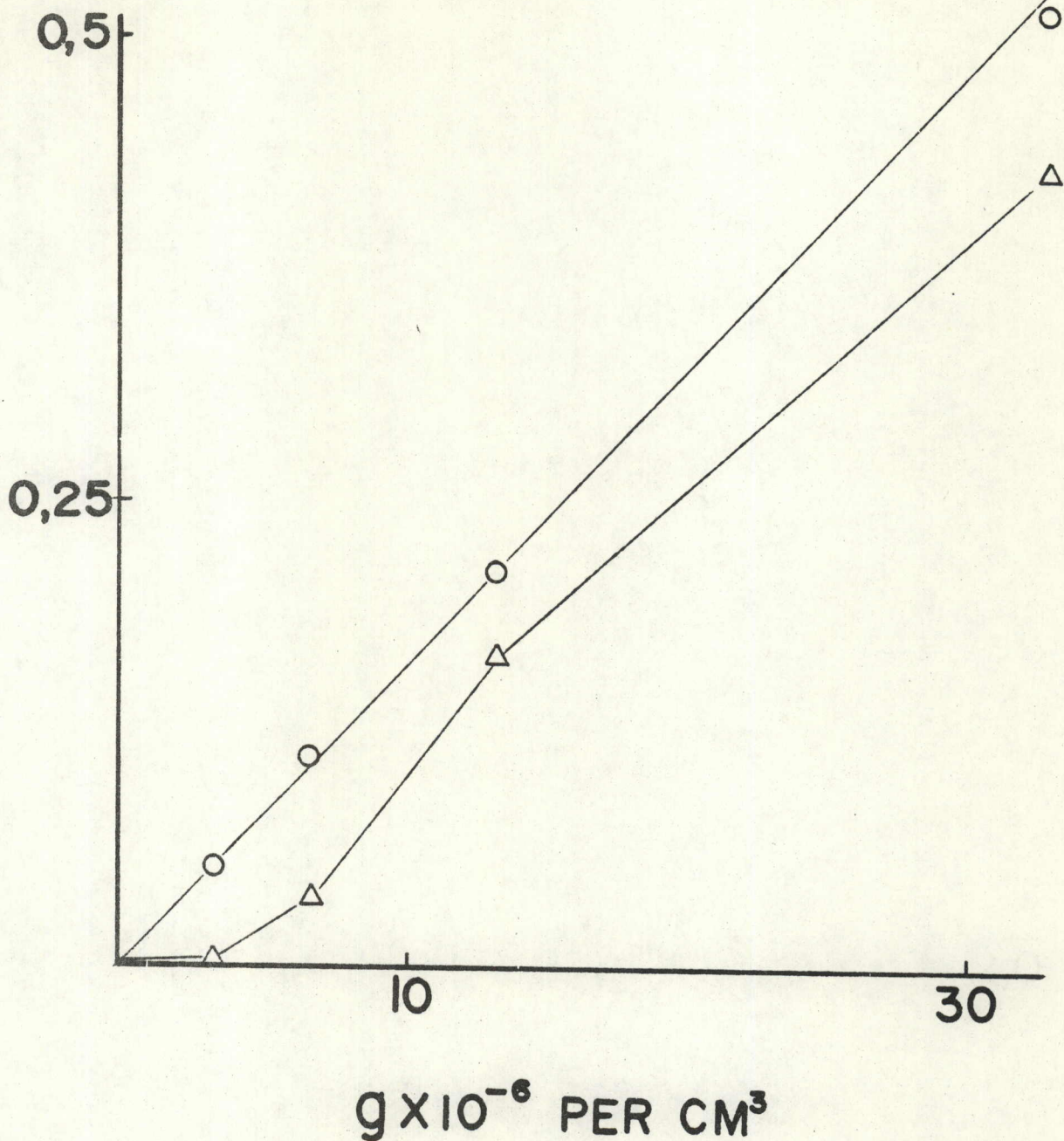


Figure 32. Effect of the reducing sugar assay on the shape of dilution curves for *T. reesei* crude cellulase. The average velocity was taken over 4 minutes. The substrate was 5,0 g dm<sup>-3</sup> CMC 4 H1F in 0,05 mol dm<sup>-3</sup> sodium acetate buffer, pH 4,5 and the temperature was 50°C. Nelson-Somogyi (section 2.5) (○) and DNS (108) (Δ). The horizontal axis shows freeze-dried culture filtrate in g x 10<sup>-3</sup> per cm .

could be resolved by preparative electrofocusing, the resulting bands were both convoluted and close together. Therefore, in the work reported here, the individual components of fraction F2 could not be isolated and characterised. The proximity of the bands to each other is reflected in the single, symmetrical protein peak obtained from analysis of the slices cut from the gel bed following preparative electrofocusing (Fig. 17). However, Fig. 17 also shows that both the protein and the carboxymethylcellulase activity peaks of fraction F2 coincided and were superimposable. This indicates that the specific activity was constant over the width of the protein peak and thus constitutes evidence that all 3 bands were carboxymethylcellulases. It is therefore likely that the 3 components of fraction F2 represent multiple forms of a single enzyme. In this context it should be noted that highly purified cellulases have been shown elsewhere to exhibit multiple bands on both SDS containing (35) and non-SDS containing gels (109).

On the other hand, its ability to catalyse galactomannan degradation argues against the purity of fraction F2. There is evidence that cellulase and xylanase activities can reside in a single protein (49,110) but apparently no report exists of an enzyme exhibiting activity on both carboxymethylcellulose and galactomannan. A firm decision on the nature of the constituents of F2 cannot be made until the components have been individually isolated and characterised. Although the levels of specific activity obtained for the various fractions during purification have been presented (Table 3), during the course of purification of F2 it was observed that several other

physicochemically distinct CMCase were present in S. rolfsii culture filtrates. Owing to this multiplicity of nominally equivalent enzymes, purification data based on measurements of specific activity are probably meaningless (58).

The physicochemical properties of fungal CMCase exhibit considerable diversity. Molecular masses range from ca.  $2 \times 10^4$  (111) to  $6 \times 10^4$  (112) with a majority lying between  $3,5 \times 10^4$  and  $4,5 \times 10^4$ , pH optima vary from 3,7 (113) to 5,5 (17) but most commonly lie between 4 and 5, and pI values are normally found in the interval 2,6 (113) to 7,5 (111). Temperature optima are comparatively high (45 to 60°C) (109,114) but these values generally depend on the reaction time and since there is no consensus on the duration of reaction, data from different sources are not always directly comparable.

A similar problem arises when attempting to compare the pH and thermal stabilities of fungal CMCase where no agreement exists on preincubation times prior to measuring residual activity. As a general rule however, the enzymes appear stable between pH 4 and 6 and seem to tolerate, for short periods, temperatures as high as 50 to 60°C. It is especially difficult to compare  $K_m$  values for different enzymes since  $K_m$  is often determined under a variety of conditions of both pH and temperature and employing a range of substrate types. In particular, the type of CMC used has been shown to significantly influence the measured  $K_m$  (115).

Notwithstanding the difficulties inherent in defining a "typical

CMCase", F2 apparently does not exhibit any markedly unusual physico-chemical properties.

#### 4.7.2 Degradation of oligosaccharides by F2

The mode of action of F2 on oligosaccharide substrates deserves special comment. The degradation of cellotriose and cellotetraose to cellobiose and cellotriose, without significant concomitant glucose production, suggests the operation of transglycosylation. It is not possible with the limited data available in this study to arrive at an unambiguous mechanism for the transglycosylation process. However, some insight may be gained from examination of the early products of enzyme attack.

This approach focuses attention on the initial substrate conversions and thus minimises complications introduced by transglycosylation reactions between the products. In addition, it was expedient to employ reduced oligosaccharides, thereby obtaining information on which bonds were preferentially cleaved. Cellotriitol is a major product of cellotetraitol degradation at early times (10 to 20 minutes), which suggests that during cellotetraitol breakdown, a glucose monomer is cleaved from the non-reduced end of the tetrasaccharide. Further, since no free glucose can be detected amongst the degradation products, the monomer presumably remains enzyme bound awaiting reaction with a further molecule of cellotetraitol.

The apparent contamination of the cellopentaitol sample by

unreduced cellopentaose complicates interpretation of the degradation path of cellopentaol. The early appearance of cellotetraitol (without concomitant glucose production) suggests that, as in the case of cellotetraitol breakdown, a glucosyl transfer mechanism might accommodate the empirical observations. In addition di-, tri- and tetrasaccharide products all appear as early products of pentasaccharide degradation. Since similar products also appear during cellohexaose breakdown (see below), the proposed glucosyl transfer between pentasaccharides to produce a hexasaccharide intermediate is consistent with the data. It is assumed that the mechanism of degradation of cellotetraose and cellopentaose would be similar to that of their reduced analogues.

The absence of cellopentaose from amongst the early degradation products of cellohexaose suggests that glucosyl transfer does not occur during enzymic attack on cellohexaose. Indeed, transglycosylation need not be invoked at all to explain the products of cellohexaose degradation. Thus cleavage of the central bond of cellohexaose would yield cellotriose, and separate cleavage of either of the bonds adjacent to the central bond would yield a mixture of cellobiose and cellotetraose.

In deciding on a possible mechanism for cellotriose degradation, the following observations must be accommodated: (a) cellotriose by itself undergoes transglycosylation to cellobiose, (b) cellotriitol by itself is unattacked, and, (c) a mixture of cellotriose and cellotriitol reacts in the presence of F2 to produce cellobiitol. Two

alternative mechanisms present themselves. Two cellotriose molecules (with free reducing ends) may condense "head-to-tail" to form cellose, eventually yielding cellobiose and cellotriose. Alternatively, by analogy with the proposed degradation mode of cello-tetraose and cello-pentaose, a glucose monomer is cleaved from cello-triose and remains enzyme bound. This enzyme-bound glucose reacts either with a further cellotriose molecule, or with cellotriitol, to produce a tetrasaccharide eventually yielding di- and trisaccharide products.

If the latter mechanism is favoured, the non-reactivity of cello-triitol by itself could be explained by postulating that the presence of a terminal sorbitol residue prevents cleavage of glucose from the non-reduced end of the substrate molecule. However, since a mixture of cellotriose and cellotriitol interact, it seems unlikely that cello-triitol is intrinsically unreactive, and hence, if cellotriitol cannot be fragmented, by implication neither can cellotriose. Therefore, the more likely mechanism appears to be a head-to-tail condensation.

Further evidence of a unique mechanism for cellotriose degradation is provided by the observation that F2 was able to catalyse *p*-nitrophenol release from PNG in the presence of either cello-tetraose or cello-pentaose, but not in the presence of cellotriose. It is proposed that cello-tetraose and cello-pentaose catalyse PNG hydrolysis through their ability to promote the formation of an enzyme-glucose complex. Cellotriose, on the other hand, if it is degraded via a head-to-tail mechanism, would not promote formation of an

enzyme-glucose complex and would therefore not catalyse PNG hydrolysis. A mechanism for cellotriose degradation involving head-to-tail condensation appears not to have been suggested previously.

However, a glucosyl transfer mechanism for cellulase, such as suggested here for cellotetraose and cellopentaose degradation, has been proposed before to account for transglycosylation of *p*-nitrophenyl- $\beta$ -D-cellobioside (38). Shoemaker and Brown (39), however, considered cellobiosyl transfer the likely mechanism of transglycosylation catalysed by a cellulase, endoglucanase III, from *T. viride*. Glucosyl rather than cellobiosyl transfer appears better to fit the data on transglycosylation reactions catalysed by fraction F2 from *S. rolfsii*.

#### 4.7.3 Pitfalls in the assay of CMCase activity

The results of this study suggest that linear CMCase dilution curves can be obtained provided these are constructed using true initial velocities. Although this is recognised as a general principle for most enzymes (62) the point deserves special emphasis in the present context. The non-continuous nature of the reducing sugar assay makes it best suited to the measurement of average velocity and the latter is equal to initial velocity only when the progress curve is linear. However, owing to cryptic substrate limitation (and possibly product inhibition) the linear portion of the CMCase progress curve is restricted to the very early stages of the reaction, when comparatively little product has accumulated, and its detection will therefore

require a particularly sensitive assay technique. A further difficulty resides in the product detection system. It is important to realise that product estimation by most colorimetric reducing sugar assays is non-stoichiometric since the aldehyde group reacts differently depending on the residue to which it is attached (116). Consequently, a strictly proportionate increase in product will only be measured where the composition of the product remains invariant. In the case of CMCases, however, the product array almost certainly undergoes changes as the reaction proceeds and a non-stoichiometric assay is therefore an unreliable means of estimating product concentration except over limited reaction times. Some authors (115,117) have sought to interpret CMCCase progress curves in terms of the integrated Michaelis-Menten equation, employing a non-stoichiometric assay, but this approach is invalid since an accurate estimation of (relative) product concentration cannot be made. The situation is complicated further by a lack of certainty regarding initial (available) substrate concentration. Under these conditions, no correlation between experimental progress curves and the integrated equation can really be expected.

Although a linear dilution curve is a primary requirement in monitoring enzyme purification, a remaining problem is that of finding a meaning for such data based on enzyme activity and applied to a system consisting of a multiplicity of "nominally-equivalent, simultaneously-occurring enzymes". The name neso-enzymes or nesozymes is suggested for such a group of enzymes as distinct from the terms iso-enzymes or isozymes, which designation implies a common mode of

action on the substrate. In the usual calculation of specific activity it is implicitly assumed that the measured activity is due to a single enzyme. Furthermore, in calculating a purification factor from the ratio of specific activities before and after a purification step, the same assumption is made.

In the case of CMCases, however, this assumption is not valid, as these enzymes almost always exist as families of physicochemically distinct species whose different modes of action are not distinguished by the assay system, i.e. nesozymes. The measured index of activity, namely increase in reducing sugar with time, is therefore a function of the activities of all the individual components of the mixture. If the enzymes act independently, the total activity might simply be made up of the sum of the activities of the individual species. Since evidence has, however, been published for the synergistic action of mixtures of CMCases (107), the total activity will probably in general be a more complex function of the individual activities.

In practice then, the measured specific activity obtained with a mixture of nesozymes will be some function approximating an average of that of the isolated components. The individual specific activities of certain of these components could in principle therefore be lower (or higher) than the average value. In consequence it is theoretically possible to obtain a declining specific activity for an enzyme fraction which is actually becoming increasingly purified of other components. Although the foregoing is an extreme example, it nevertheless illustrates that, in similar circumstances, neither the purification

factor nor the yield of an isolated CMCase calculated in the usual way need reflect the actual values which might in practice have been attained.

In conclusion, the assay of CMCases based on release of reducing sugars can be made to yield valid measurements of activity provided assay conditions are carefully prescribed. The accurate measurement of initial velocity will generally require: (i) dilute enzyme preparations, (ii) a substrate of low D.S. (e.g. 0,4), (iii) a sensitive sugar reagent (e.g. Nelson-Somogyi), and, (iv) maximisation of product concentration by withdrawing comparatively large volumes from the reaction mixture for subsequent assay. However, because of its non-stoichiometric nature and the complications introduced by the existence of nesozymes, the assay is best suited to situations in which neither the enzyme nor the product composition changes significantly.

In this thesis initial velocity was used in the determination of  $K_m$  and in calculating enzyme purification factors. In these instances appropriate steps were taken to avoid the pitfalls associated with the assay of CMCase activity. It is stressed, however, that despite these precautions, the nesozyme nature of S. rolfsii CMCases renders meaningless the purification data of Table 3.

## CHAPTER 5

SCHUTZ'S LAW AND THE ACTION OF CELLULASES

In Chapter 4 mention was made of the non-linear dilution curves often obtained for CMCases. Examination of the data obtained by the author for S. rolfsii CMCases (e.g. Fig. 33 and 34) showed that the dilution curves often obeyed the relationship

$$\text{reaction rate} \propto \sqrt{(\text{enzyme concentration})} \quad \{1\}$$

Similarly, the S. rolfsii CMCase progress curves could often be described by the relationship

$$\text{product concentration} \propto \sqrt{(\text{time})} \quad \{2\}$$

The occurrence of kinetics described by the relationships {1} and {2} has been noted before in connection with a variety of enzyme catalysed reactions (118) and these reactions are said to obey Schutz's law.

A more detailed consideration of the kinetics of CMCases from S. rolfsii led the author to conclude that substrate limitation was the major factor responsible for the observed non-linear kinetics (Chapter 4). It was found that linear dilution curves could be obtained if these were constructed using true initial reaction velocities. However, whereas it is clear that non-linearity of the progress and dilution curves could result from substrate limitation effects, there

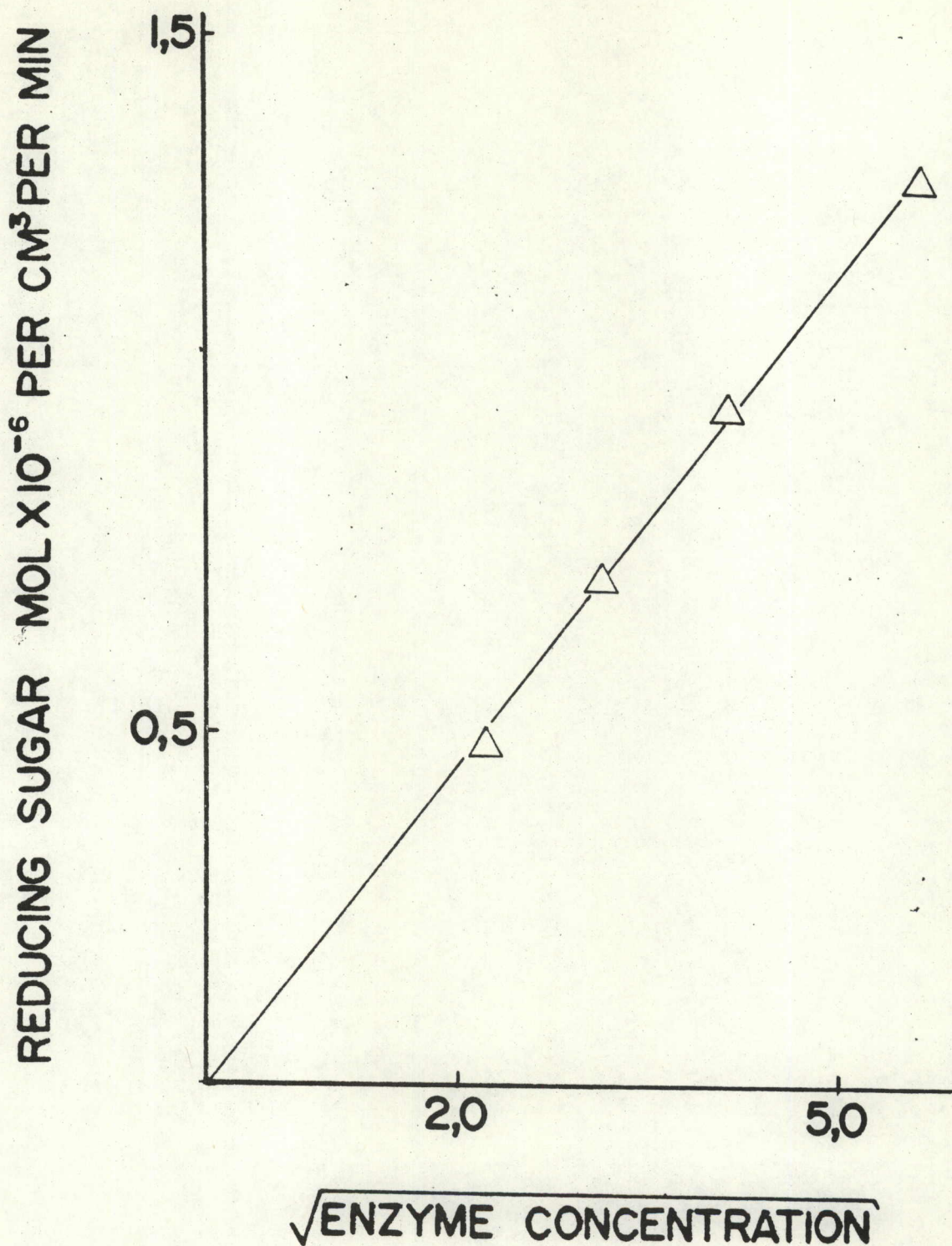


Figure .33. Curvilinear relationship between enzyme concentration and average reaction velocity (over 3 minutes) for fraction P3 acting on CMC stock solution. Enzyme concentration refers to  $g \times 10^{-6}$  protein in the reaction mixture. The reaction temperature was  $50^{\circ}C$ .

REDUCING SUGAR MOL X 10<sup>-6</sup> PER CM<sup>3</sup>

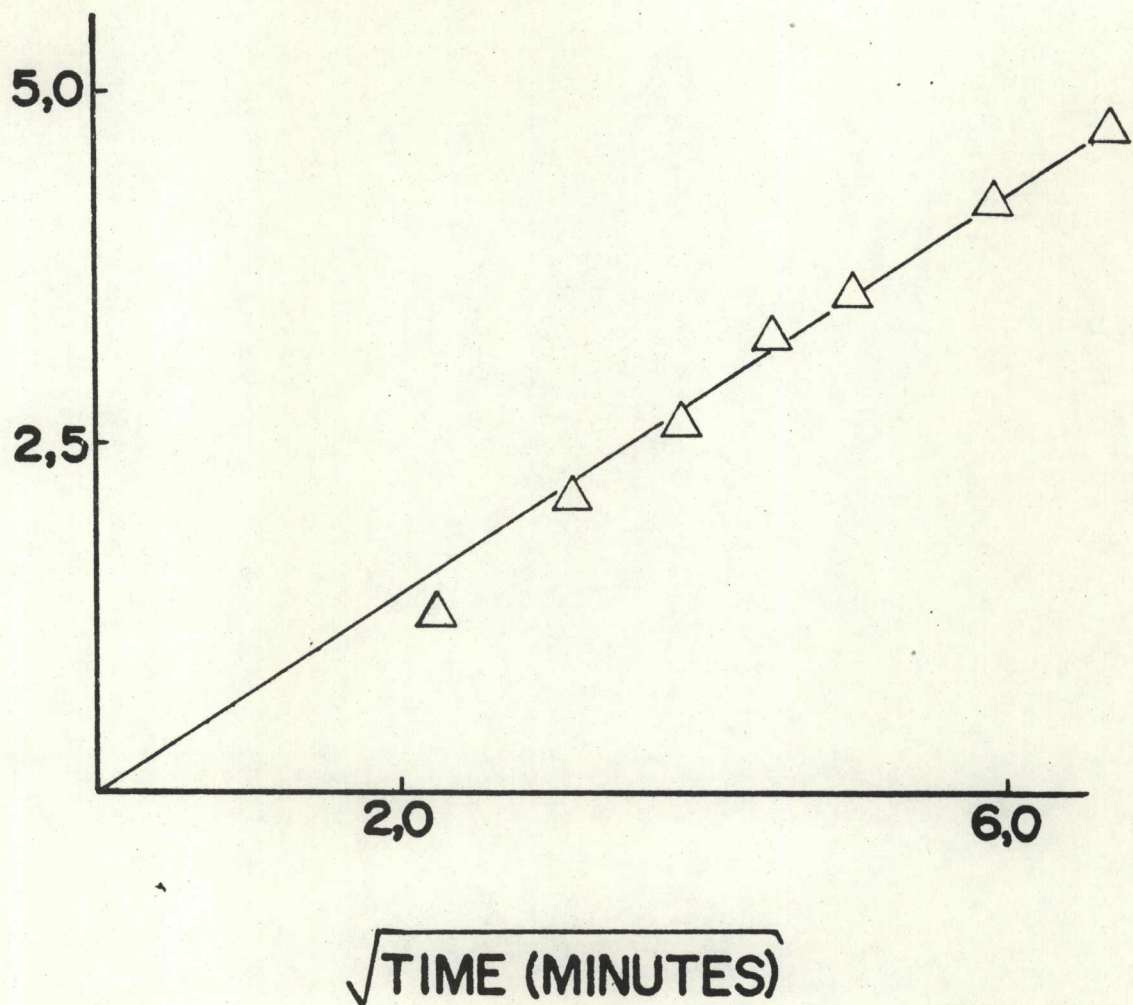


Figure 34. Curvilinear relationship between time and product formation for fraction P3 acting on CMC (Sigma, D.S. 0,7). Enzyme ( $2,0 \times 10^{-5}$  cm<sup>3</sup> containing  $1,05 \times 10^{-5}$  g protein) was mixed with  $20 \text{ g dm}^{-3}$  CMC in  $0,2 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5 ( $2,0 \text{ cm}^3$ ) and the resulting mixture incubated at  $49^\circ\text{C}$ . At 5 minute intervals samples ( $0,1 \text{ cm}^3$ ) were withdrawn for determination of reducing sugar.

is no apparent reason why the resulting curves should specifically follow Schutz's law. In other words, the characteristic shapes of the progress and dilution curves are not adequately explained by the simple effects of substrate limitation.

Moreover, examination of the cellulase literature brought to light several further examples of the applicability of Schutz's law to cellulase kinetics (33,92,119-121). Schutz's law, therefore, appeared to be of real significance in the action of cellulases, and this justified its further investigation, especially in view of the current interest in the bioconversion of cellulose in South Africa and elsewhere (1).

Schutz's law has been described by one source (122) as "evidently not of fundamental significance" and "unable to throw light on the nature of enzyme action", while several modern textbooks omit comment on Schutz's law and the impression might be gained that it is of historic interest only. However, as mentioned above, this law has been frequently cited in comparatively modern literature in the context of studies on cellulases and, since there is no consensus on the underlying causes of the kinetic behaviour described by Schutz's law (Schutz kinetics), dismissal of the law as of no fundamental significance may be somewhat premature.

Schutz's law was originally deduced from the work of Emil Schutz (123) who observed that, during pepsin hydrolysis of egg albumin, product concentration after a given time (i.e. average reaction rate)

was proportional to the square root of the total quantity of added enzyme.

Mathematically this finding can be expressed as :

$$x = a \text{ constant} \sqrt{E_0} \quad \{3\}$$

where x represents product concentration at a specified time and  $E_0$  is total enzyme concentration. The constant term is of course dependent on time elapsed and further independent observations led to the formulation of the Schutz-Borissov-Somjloff law (118), also known as the Schutz-Arrhenius law (122), which includes time (t) as a variable,

$$x = a \text{ constant} \sqrt{E_0 t} \quad \{4\}$$

Rather confusingly, other definitions of Schutz's law exist in the literature (62,122), one such (63) being

$$v = a \text{ constant} \sqrt{E_0} \quad \{5\}$$

where v depicts velocity.

Equation {5} is different in meaning from {4}, this difference being first highlighted by Bodansky (124). The difference is also implicit in an earlier treatment given by Langmuir (125) who differentiated x with respect to t in {4} to obtain

$$v = \frac{dx}{dt} = \text{a constant} \frac{E_0}{x} \quad \{6\}$$

Whereas {5} implies that velocity is proportional to the square root of the enzyme concentration and is independent of product concentration, {4} implies that velocity is proportional to enzyme concentration raised to the first power and is inversely proportional to product concentration ({6}). For the sake of clarity, in this Chapter, equation {4} will be considered to represent Schutz's law. It should be noted, however, that {4} embodies two aspects, the dependence of  $x$  upon  $\sqrt{E_0}$  (at constant  $t$ ) and upon  $\sqrt{t}$  (at constant  $E_0$ ).

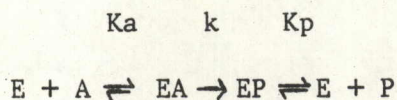
The aim in this Chapter is to show that Schutz kinetics may be explained in terms of product inhibition or diffusion limited reactions either of which, if applicable, might provide some insight into the factors involved in the enzymic degradation of cellulose.

### 5.1 Product inhibition models

Arrhenius (126) deduced equation {4} from consideration of the law of mass action, but his derivation predates currently accepted models of enzyme action and fails to take account of the enzyme-substrate complex. An alternative deduction was made by Langmuir (125) based on adsorption theory and which dealt with enzymes in terms of non-specific surface catalysis. Derivations more in keeping with present-day conceptions of enzyme action are offered below.

### 5.1.1 Competitive inhibition

In the following model, E, A and P represent enzyme, substrate and product, respectively,  $K_a$  and  $K_p$  represent the dissociation constants of the enzyme-substrate and enzyme-product complexes, and  $k$  is the catalytic rate constant:



Assuming rapid equilibrium conditions and that the equilibrium constant for the overall reactions is large, manipulation of the integrated Michaelis-Menten equation for this case (122) yields :

$$\frac{k K_p E_0 t}{K_p - K_a} = \frac{K_a (K_p + a_0)}{K_p - K_a} \ln \left( \frac{a_0}{a_0 - x} \right) + x \quad \{7\}$$

in which  $a_0$  represents initial substrate concentration.

Expanding the logarithmic terms as a power series, {7} becomes

$$k K_p E_0 t = K_p (K_a + a_0) \left( \frac{x}{a_0} \right) + K_a (K_p + a_0) \left( \frac{x^2}{2 a_0^2} \right) + \frac{K_a (K_p + a_0) R_2}{K_a (K_p + a_0) R_2} \quad \{8\}$$

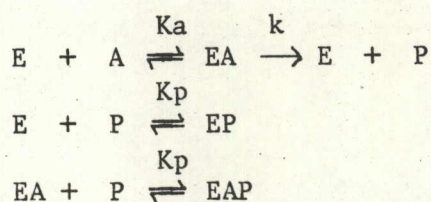
Under conditions where the  $x^2$  term dominates (see Appendix 1), {8} reduces to

$$x \approx \sqrt{2 k K_p a_0^2 / K_a (K_p + a_0)} \sqrt{E_0 t} \quad \{9\}$$

which is a form of Schutz's law.

5.1.2 Non-competitive inhibition

Using the same symbols as above and assuming that the dissociation constants for both enzyme-product complexes (EP and EAP) are the same, the applicable model is:



The integrated Michaelis-Menten equation applicable to the above scheme (122) becomes, after suitable rearrangement,

$$\frac{k K_p E_0 t}{K_p - K_a} = \frac{K_a (K_p + a_0)}{K_p - K_a} \ln \left( \frac{a_0}{a_0 - x} \right) + x + \frac{x^2}{2(K_p - K_a)} \quad \{10\}$$

By employing almost identical reasoning to that used for the competitive case, {10} can be reduced to

$$x \approx \sqrt{2 k K_p a_0^2 / K_a (K_p + a_0) + a_0^2} \sqrt{E_0 t} \quad \{11\}$$

which is another form of Schutz's law.

Examination of the above derivations reveals that in both cases Schutz's law will approximate the integrated Michaelis-Menten equation for all values of  $x$  in an interval (see Appendix 1). Whereas in the competitive case  $K_p < K_a$  is a necessary condition for the existence of  $x$  in this interval, no such condition appears to be necessary when the

inhibition is non-competitive. Neither is it necessary for the following, somewhat less rigorous, derivation based on the differential form of the Michaelis-Menten equation (62) which, upon rearrangement, becomes

$$\frac{dx}{dt} = \frac{kE_0t}{1+(x/k_p)} \cdot \frac{(a_0-x)}{(a_0-x)+K_a}$$

Under conditions such that  $x \gg K$  and  $(a - x) \gg K$ , this reduces to

$$\frac{dx}{dt} = \frac{k k_p E_0 t}{x}$$

On integration, this equation yields

$$x = \sqrt{2 k k_p} \sqrt{E_0 t} \quad \{12\}$$

a form of Schutz's law which approximates {11} if  $a_0$  is sufficiently large.

Some evidence exists for the occurrence of product inhibition during cellulase action (26,41,127,128) both exo- (26,127) and endocellulases (41). It is possible therefore that some form of product inhibition could account in certain cases for the operation of Schutz's law during cellulase action. Cellobiose inhibition of exocellulase is thought to be competitive but, if the relevant integrated equation ({7}) is to be approximated by Schutz's law, then  $K_p \ll K_a$ . This condition seldom obtains in real systems but cellulases may constitute an exception, since the ratio of  $K_p/K_a$  for a cellobiohydrolase (competitively inhibited by reaction product) has

been shown (127) to be as low as  $2,2 \times 10^{-2}$ . The possibility exists that further cellulases may be found manifesting even lower values of this ratio. Non-competitive product inhibition, which places no constraints on the relative magnitudes of  $K_p$  and  $K_a$ , offers a more general model for Schutz kinetics than does competitive product inhibition. In at least one reported case, the progress curves for cellulase-catalysed degradation of cellulose did show evidence of a non-competitive inhibition by reaction products (128) but, here again, it is not certain to what extent this observed behaviour is typical of cellulases in general. There is unfortunately a scarcity of suitable published information for testing the contending models.

Contrary to the above findings, Flora (120) claims to have ruled out product inhibition as a cause of Schutz kinetics, and suggests the need for alternative explanations.

## 5.2 Diffusion-limited reaction model

Two attempts have been made by Moelwyn-Hughes (129,130) to explain Schutz's law in terms of diffusion-limited catalysis. In the earlier treatment (129), the rate of reaction was assumed to be governed by the rate at which substrate diffused to the enzyme surface through a mixture of substrate and product molecules -- a plausible explanation of Schutz's law, particularly in cases where the viscosity of the medium is comparatively high. This mechanism could apply to the action of endocellulases on soluble (usually highly viscous) cellulose derivatives; and it is of interest to note that the hydrolysis of

carboxymethylcellulose by an enzyme from S. rolfsii apparently obeys Schutz's law (Figs. 33 and 34).

Moelwyn-Hughes' later analysis (130) assumes that diffusion of the enzyme through the substrate molecules is the rate determining step. The enzyme is viewed as performing a three-dimensional random walk and, in so doing, collides with and transforms any substrate molecules in its path. The amount of substrate transformed is proportional to the product of the volume swept out by the enzyme and the number of substrate molecules per unit volume. Moelwyn-Hughes assumes that the swept volume is proportional to the enzyme's displacement, which in turn is proportional to  $\sqrt{t}$ . Denoting the viscosity of the medium by  $\eta$ , the displacement by  $S$  and the radius of cross-section of the enzyme by  $r$ , the reaction progress curve is given by :

$$\begin{aligned} x &= \text{a constant } E_0 (a_0 - x) \pi r^2 S && \{13\} \\ &= \text{a constant } E_0 (a_0 - x) \sqrt{t/\eta} \end{aligned}$$

Under conditions where both  $(a_0 - x)$  and  $\eta$  remain essentially constant, {13} expresses the time-dependent aspect of Schutz's law.

It is not clear why Moelwyn-Hughes uses displacement rather than total path length in calculating the volume swept by the enzyme. There seems to be no reason why the enzyme should not be active over the entire distance it moves. Further, since the total path length is directly proportional to time, the rate of substrate conversion should,

in the absence of other effects, be approximately constant, resulting in a roughly linear progress curve.

### 5.2.1 Computer model

In an attempt to resolve these conceptual difficulties, a computer-simulated model of a two-dimensional random walk was developed to test Moelwyn-Hughes' idea. In the model, a Cartesian field represented two-dimensional space and the position of substrate molecules was given on the field by each point  $(x,y)$ , where  $x$  and  $y$  were positive or negative integers. A hypothetical enzyme molecule, placed initially at  $(0,0)$ , was allowed to move at random over the field in discrete steps  $< 0,02$  units in length; substrate conversion was assumed to have occurred if the enzyme approached any substrate location to within a distance of  $0,3$  units. After conversion, the place formerly occupied by the substrate molecule was left empty.

After allowing the enzyme to move a total of  $5 \times 10^5$  steps, the simulation was terminated without having shown any evidence that the course of substrate degradation obeyed Schutz's law (Fig 35). This result does not support Moelwyn-Hughes' contention that in a reaction governed by the enzyme's rate of diffusion, the extent of substrate conversion is necessarily proportional to the enzyme's displacement. Despite the unacceptability of a random-walk mechanism in two or three dimensions, adaptation of the concept to a one-dimensional case might be one way of explaining the Schutz kinetics observed during the degradation of insoluble cellulose, particularly in those cases where

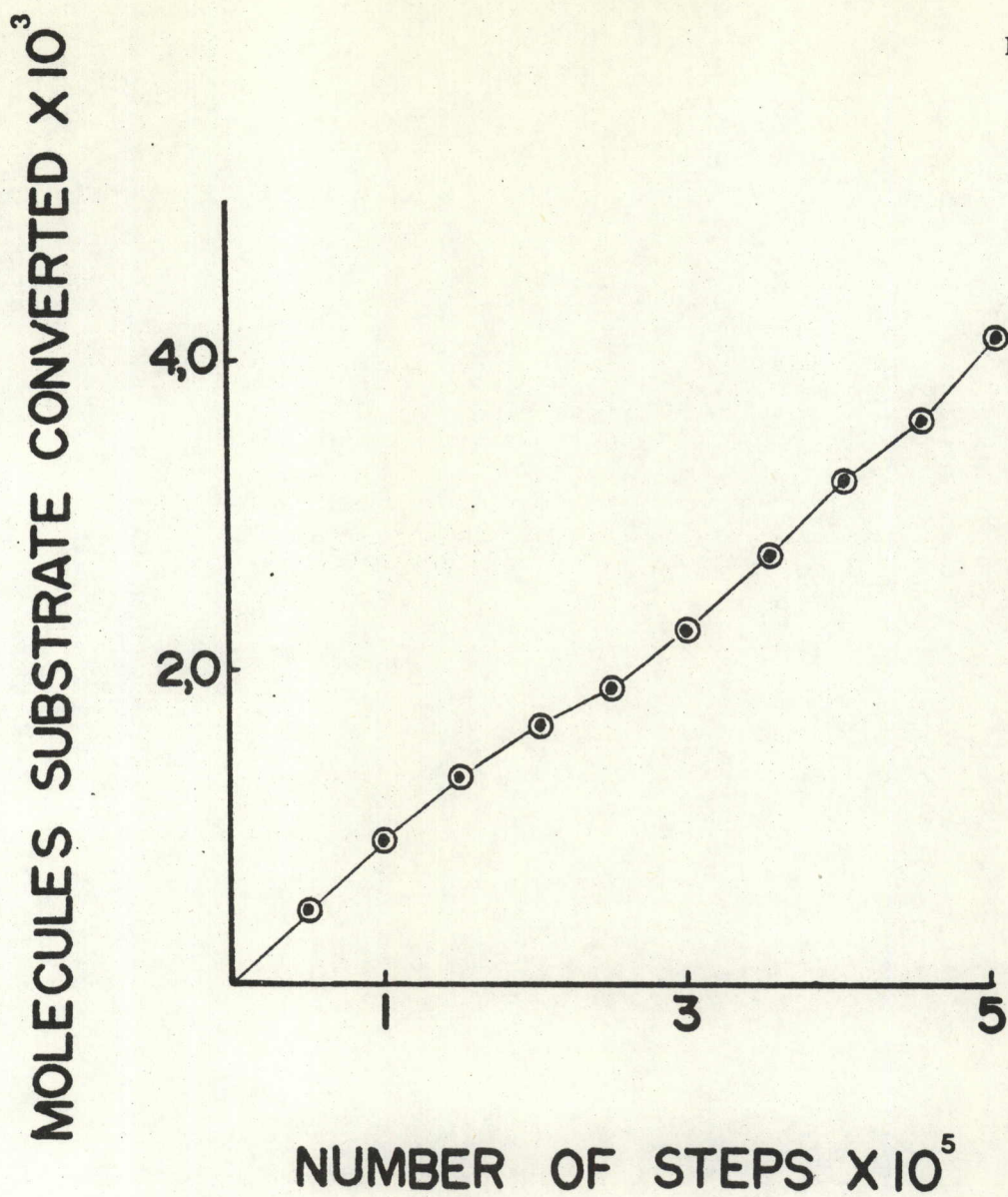


Figure 35. Two-dimensional random walk model for enzyme action.

the possibility of product inhibition has been excluded.

### 5.2.2 A new model for cellulose degradation

Flora (120) concluded that Schutz kinetics are a reflection of the insolubility of the substrate and Lindner et al. (31) have proposed a model, which is also dependent upon the substrate insolubility, but incorporates an adaptation of the concepts of Moelwyn-Hughes. The following assumptions apply to this model:

1. degradation can occur only at a finite number of locations on the cellulose surface,
2. enzyme molecules adsorbed at these labile areas are able to move over the substrate surface parallel to the cellulose fibres in a manner consistent with a one-dimensional random walk,
3. degradation of the fibres is unidirectional,
4. substrate transformation is catalysed by a single mobile entity, consisting either of a single enzyme such as an Avicelase or of an enzyme complex formed by combination of endo- and exocellulases (131).

The enzyme molecule is visualised as moving back and forth at random along a straight line, catalysing hydrolysis of only those bonds which lie ahead of an initiation point (e.g. a chain end). The total

distance travelled by the particle will be proportional to time, but much of this time is spent either in traversing elements of the path behind the initiation point, or in retracing sections lying ahead of the initiation point which have already been degraded; both regions which do not contribute to (further) product formation. In these circumstances, product formation will be commensurate with only the net unidirectional distance moved by the enzyme particle from its starting point (i.e. the displacement). The average net distance can be shown (132) to be proportional to  $\sqrt{t}$ , hence product formation will likewise be proportional to  $\sqrt{t}$ , a result in accordance with Schutz's law.

The above assumptions are not altogether arbitrary. For example, the possibility that adsorbed cellulases might be mobile on the cellulose surface may be deduced from the fact that hydrolysis can proceed despite the finding that cellulases are all but irreversibly bound to the cellulose surface (133). Since the substrate presumably cannot move, the enzymes must. In this context it may be noted that experimental evidence for the mobility of a surface-bound protease has been presented by McLaren and Estermann (134) and the theoretical possibility of such motion has been restated in a recent review (135). The assumption of linear degradation is in accord with evidence provided by electron microscopy (92). Thus, among the changes noted in the gross structure of crystalline cellulose following enzyme attack, is the appearance of rifts in the cellulose surface which all run in a direction parallel to the long axis of the crystallite aggregates (92). In another case, a longitudinal splaying of cellulose fibrils has been observed (133). Both of these observations may be explained in terms

of the linear hydrolysis of individual fibril components. If the cellulases acted in a non-directional manner, any rifts might be expected to be at random, or alternatively, the entire surface might be eroded away without any rift formation at all.

Finally, the assumption of unidirectional degradation is supported by the biochemical evidence (26,136) that at least one component of the cellulosic complex, namely cellobiohydrolase, is obliged to attack cellulose unidirectionally, starting at the non-reducing end of the polymer and proceeding towards the reducing terminus (49). The presence of this enzyme is apparently required to effect large-scale fibril splaying (133) and might be similarly implicated in rift formation.

### 5.3 Discussion

Different explanations of Schutz kinetics are clearly possible, the more conventional of these being in terms of product inhibition. One alternative interpretation (129) is most readily applicable in cases where the reaction mixture is very viscous. Indeed, as mentioned in section 5.2, and as seen from Figs. 33 and 34, the kinetics of hydrolysis of carboxymethylcellulose by a cellulase from S. rolfsii did obey Schutz's law. The author later realised that, owing to the non-stoichiometric nature of the CMCase assay used, some uncertainty necessarily attends the measurement of product concentration. Therefore the applicability of Schutz's law to CMCcase kinetics has not been demonstrated unequivocally in this thesis.

However, in other studies (92,120,121) demonstrating the applicability of Schutz's law to cellulase action, enzyme activity was assayed by a direct measurement of the solubilisation of crystalline cellulose. This type of assay appears to be free of the complications introduced by non-stoichiometric product measurement. It is in the case of crystalline cellulose degradation that the random walk model proposed by Lindner et. al. (31) is most likely to apply.

It would be hazardous to invoke any particular mechanism before the alternatives are fully explored experimentally. Unfortunately, the nature of the substrates and the products formed militate against the design of experiments which would give unequivocal answers and this matter requires further attention. Meanwhile, it would seem inappropriate to dismiss Schutz's law as having no fundamental importance or to interpret the curvilinear progress curves typical of Schutz kinetics as necessarily indicating inhibition by reaction product.

## CHAPTER 6

THE PURIFICATION AND CHARACTERISATION OF A XYLANASE FROMSCLEROTIUM ROLFSSII

It will be recalled from section 4.2.1 that two enzyme fractions, A1 and A2, were obtained following chromatography of the acetone powder on Avicel. Each fraction exhibited both CMCase and xylanase activities and the isolation and characterisation of a CMCase from A1 has been described in Chapter 4.

In the present Chapter, attention is focused on fraction A2 which, although it apparently contained less protein than A1, nevertheless exhibited substantial cellulase and xylanase activities (Fig. 10). During the course of enzyme purification, however, it became evident that the bulk of the recoverable protein in A2 was associated with xylanase activity. Attention was therefore concentrated on the purification and characterisation of xylanases from A2.

There are several factors that make the characterisation of xylanases more difficult than that of cellulases. In the first place, highly purified xylan substrates are not commercially available, whereas highly purified cellulose preparations are. Secondly, purified xylooligosaccharides are normally heteropolymers whose structures are not always rigorously defined. Cellulose, in contrast, is a linear homopolysaccharide whose chemical structure is well characterised. Thirdly, the identification of xylooligosaccharides, produced either by enzyme

or acid catalysed hydrolysis of xylan, is generally more difficult than the identification of cellooligosaccharides. Thus, while the products of cellulose hydrolysis are invariably linear cellooligosaccharides, xylan hydrolysis yields both linear homooligosaccharides and branched heterooligosaccharides. In addition, only the monomeric components of xylan appear to be available commercially, whereas both glucose and cellobiose are available for use as chromatographic standards.

In addition to the isolation and characterisation of a xylanase, both the purification of xylan and the identification of linear xylooligosaccharides received attention in this study. Results are presented detailing the purification, properties and mode of action of a xylanase, U1. Additional results confirm the effectiveness of methods, developed by the author, to purify a commercial xylan preparation and to identify linear xylooligosaccharides.

## 6.1 Enzyme purification

### 6.1.1 Chromatography on DEAE-Sephadex A-50

Chromatography of A2 on DEAE-Sephadex resulted in partial separation of CMCase and xylanase activities and yielded a fraction, D3', containing the bulk of both the recoverable protein and xylanase activity (Fig. 36).

Fraction D3' was resolved into four protein components by SDS gel electrophoresis (Plate 6) and therefore required further purification.

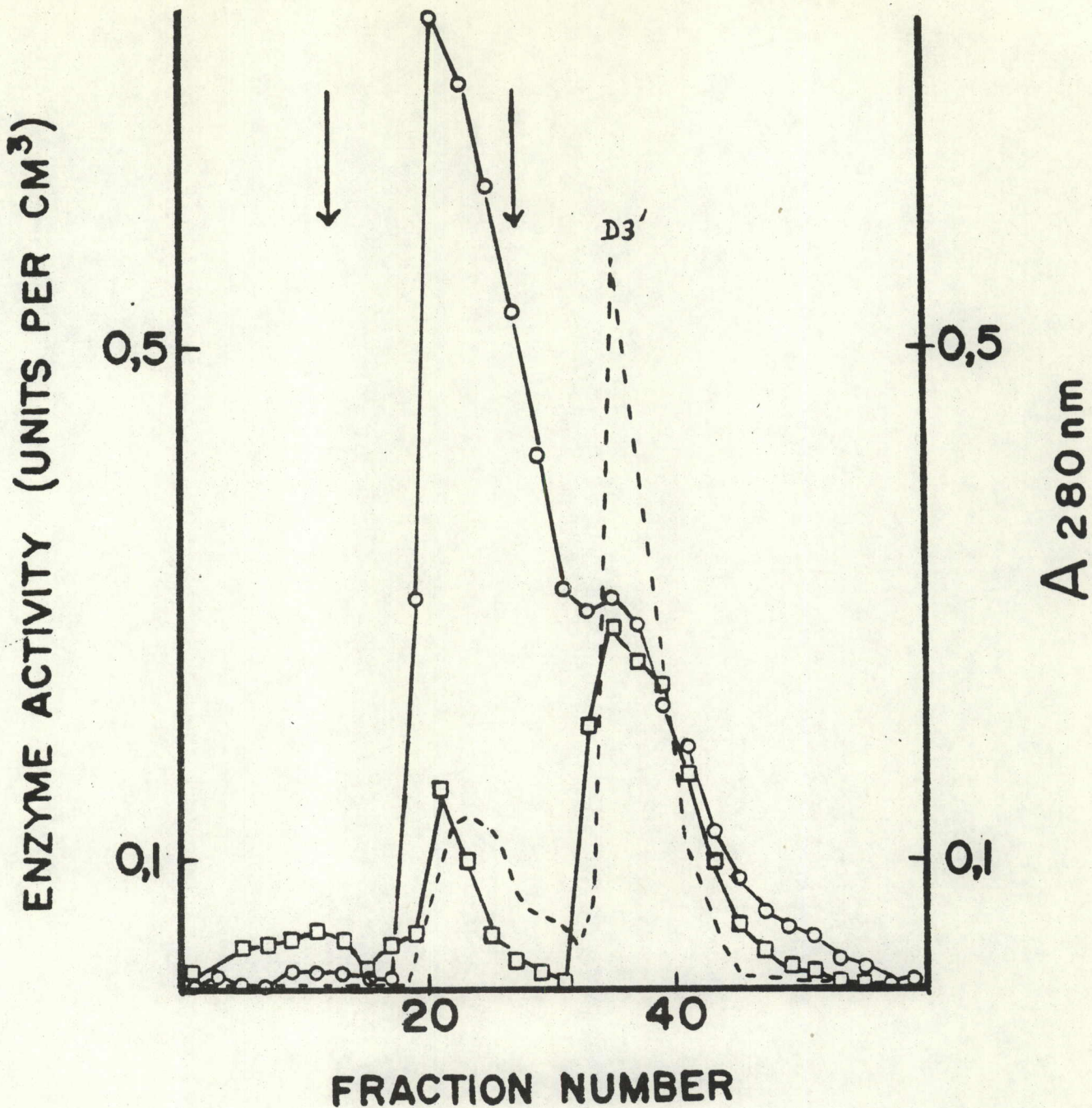


Figure 36. Resolution of A2 on DEAE-Sephadex A-50. Fraction A2, comprising material from ca. 20 g of acetone powder, and dissolved in  $0,01 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, ( $24 \text{ cm}^3$ ) was chromatographed on a column ( $21 \times 2,5 \text{ cm}$ ) of DEAE-Sephadex A-50. The resin was pre-equilibrated in the same buffer and maintained at ca.  $4^\circ\text{C}$  during chromatography. Protein was eluted from the column with, in sequence, the equilibrating buffer,  $0,05 \text{ mol dm}^{-3}$  sodium phosphate buffer, pH 6,6, and this last buffer incorporating initially  $0,5 \text{ mol dm}^{-3}$  sodium chloride and later  $1,0 \text{ mol dm}^{-3}$  sodium chloride. (At the higher sodium chloride concentration no detectable enzyme activity eluted). Changes in the eluant composition are indicated by arrows. Fractions ( $12 \text{ cm}^3$ ) were collected at a flow rate of  $1,0 \text{ cm}^3$  per minute. The substrate solutions used to measure enzyme activity were  $20 \text{ g dm}^{-3}$  CMC (Sigma, D.S. 0,7) in  $0,1 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, and  $5,0 \text{ g dm}^{-3}$  xylan (Sigma) in the same buffer at  $0,05 \text{ mol dm}^{-3}$  with respect to acetate. Enzyme ( $0,1 \text{ cm}^3$ ) was mixed with substrate solution ( $0,2 \text{ cm}^3$ ) and incubated at  $41^\circ\text{C}$  for 0,5 hours before measuring the release of reducing sugar. Protein(---) was determined by the

### 6.1.2 Electrofocusing of D3' in the LKB 8101 column

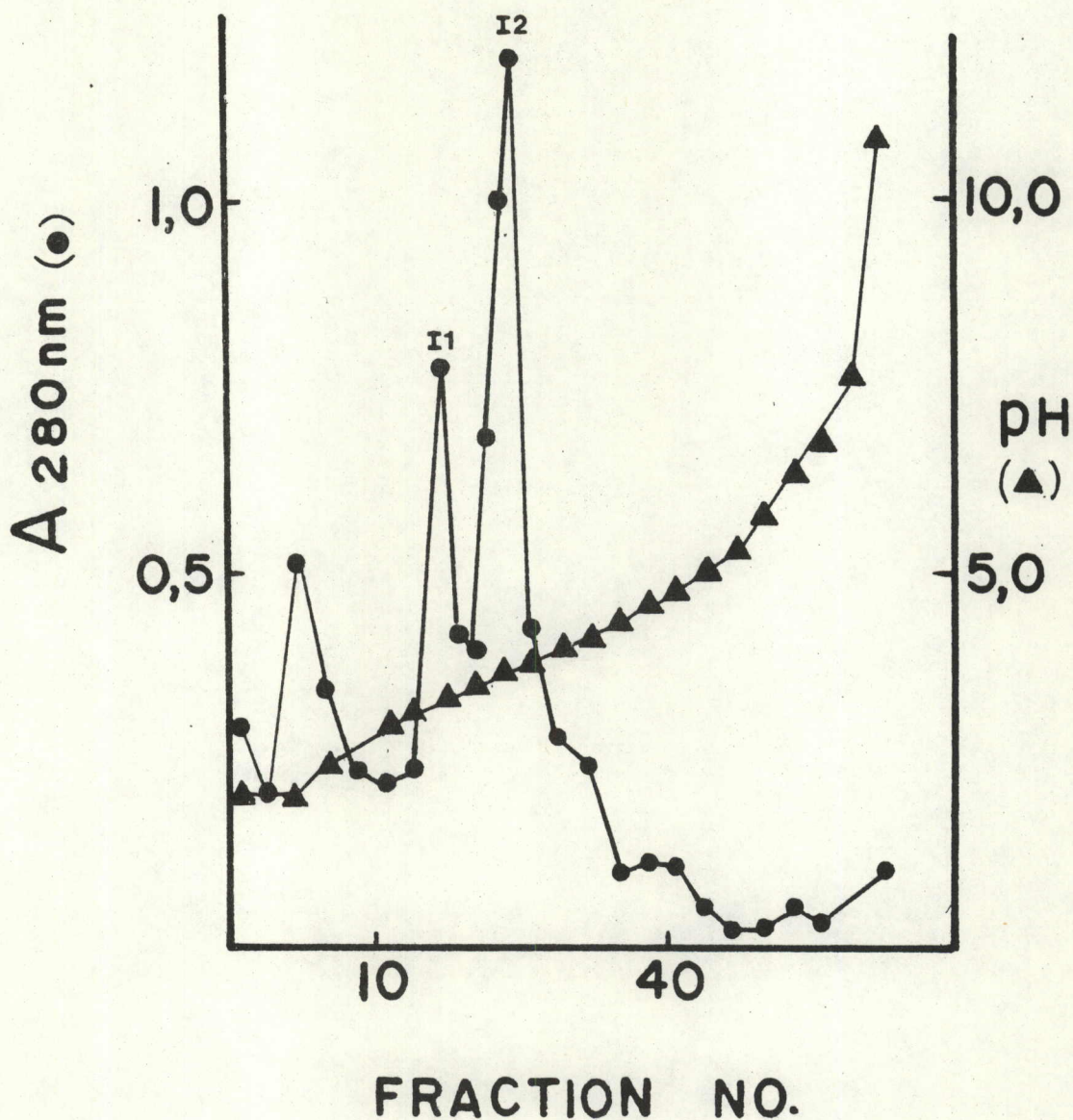
Electrofocusing of D3' in a sucrose gradient over the pH range 3 to 10 established that all the recoverable protein focused at comparatively low pH values (ca. pH 3 to 4). Consequently, further quantities of D3' were focused in the same column using a gradient from pH 2.5 to 5.0. Focusing in the narrower gradient yielded two protein fractions, I1 and I2, (Fig. 37), neither of which was homogeneous by SDS gel electrophoresis (Plate 6). However, SDS gel electrophoresis further revealed that the four protein bands originally present in D3' had been resolved partially into two separate pairs of bands by narrow range electrofocusing. The upper two bands of D3' appeared in fraction I1 and the lower two in fraction I2 (Plate 6).

Enzyme assay revealed that all the recoverable xylanase activity was in fraction I2.

### 6.1.3 Resolution of I2 by electrophoresis

Fraction D3' had previously been subjected to electrophoresis on an analytical scale in polyacrylamide gel at pH 8,9 and the gel subsequently cut into 0,2 cm thick slices. Assay of the enzyme activity in individual slices revealed the presence of two distinct xylanase components.

The success achieved in resolving xylanase components of D3' on an



**Figure 37.** Electrofocusing of  $D_3'$  in the LKB 8101 column. A sample of  $D_3'$ , washed free of electrolytes on an Amicon PM 10 ultrafilter was electrofocused in a sucrose gradient at 15 W for 19 hours over the pH range 2,5 to 5,0. Proteins were eluted from the column by pumping water in at the top (cathode) at a flow rate of  $2,0 \text{ cm}^3$  per minute. The fraction size was  $2,4 \text{ cm}^3$ . The pH of the fractions was measured at the running temperature with a glass electrode and protein was determined by the absorbance at 280 nm.

analytical scale suggested that electrophoresis be used on a preparative scale to further purify I2 (which contained all the recoverable xylanase activity of D3'). The results of the preparative electrophoresis of I2 employing the LKB Uniphor are presented in Fig. 38. As anticipated, I2 was resolved into two xylanase components (U1 and U2 of Fig. 38).

It was observed that some degree of physical association existed between U1 and U2. Thus, evidence from both SDS and non-SDS gel electrophoresis suggested that U2 contained residual U1. In addition, both U1 and U2 liberated the same products from Sigma larchwood xylan. It therefore seemed possible that the observed xylanase activity of U2 could have been a result of contamination by associated U1. Because of doubts surrounding the nature and purity of U2, attention was concentrated on characterising U1.

## 6.2 Properties of the purified xylanase U1

### 6.2.1 Purity, molecular mass and isoelectric point

U1 was homogeneous by SDS gel electrophoresis (Plate 7), and comparison with SDS molecular mass markers indicated a molecular mass for U1 of  $5,3 \times 10^4$ . Relevant purification data are presented in Table 5.

Reference to Fig. 37 shows that fraction I2, which contained U1, focused in the pH interval 3,4 to 3,8. The isoelectric point of U1

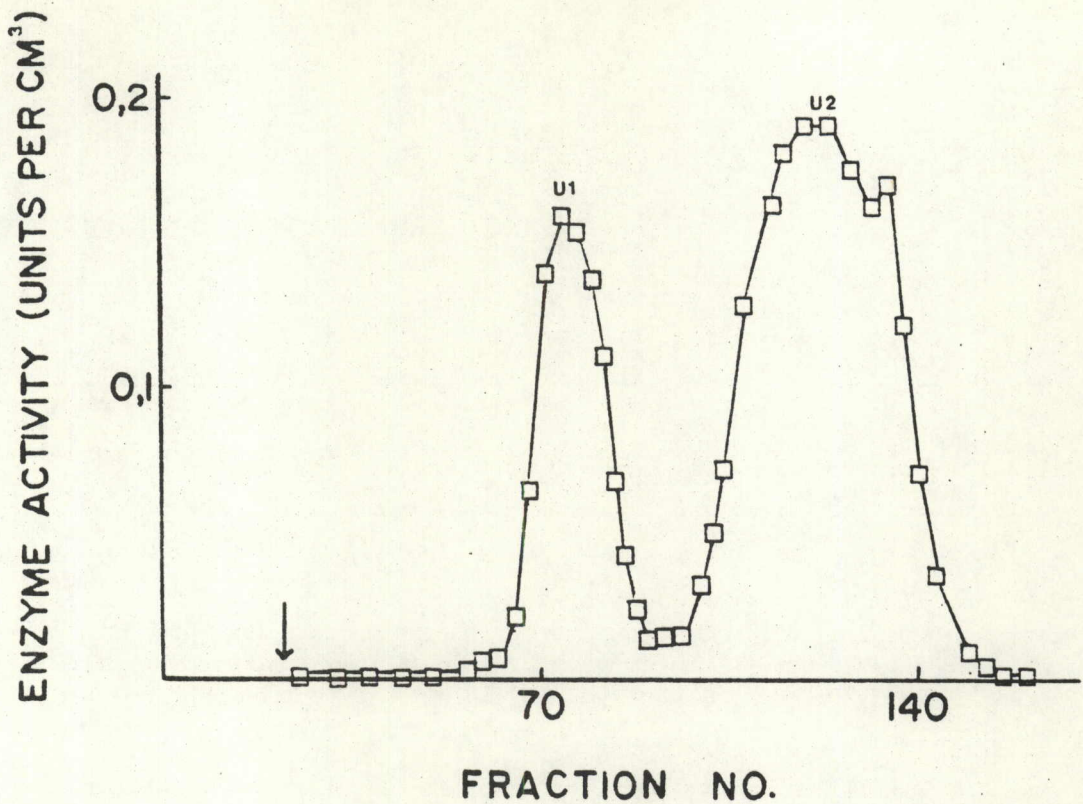


Figure 38. Resolution of I2 by preparative electrophoresis using the LKB 7900 Uniphor. A sample of I2 was subjected to electrophoresis on a column (10 x 2,5 cm) of 7,5 per cent polyacrylamide gel at pH 9,5 and an initial power of 20 W. Fractions (3,6 cm<sup>3</sup>) were collected at a flow rate of 14,5 cm<sup>3</sup> per hour. Protein (---) was detected by the absorbance at 280 nm. Xylanase activity (□) was assayed by incubating enzyme (0,1 cm<sup>3</sup>) with xylan stock solution (0,2 cm<sup>3</sup>) at 43°C for 1 hour before measuring reducing sugar release.

TABLE 5. SPECIFIC ACTIVITIES OF FRACTIONS DURING THE PURIFICATION OF U1.<sup>a</sup>

FRACTION	SPECIFIC ACTIVITY (units per $1,0 \times 10^{-3}$ g protein)
ACETONE POWDER	4,9
A2	6,1
D3	13,2
I2	11,1
U1	29,3

<sup>a</sup> Xylanase activity was determined at 42°C using xylan stock solution with reduced xylan concentration ( $2,5 \text{ g dm}^{-3}$ ). Initial velocities were estimated from the slopes of the tangents to the progress curves at time zero.

therefore lay between these same limits. During purification of U1 several batches of I2 were prepared by electrofocusing, making it possible to calculate an average isoelectric point for I2 of 3,6 with a standard error of the mean of 0,13.

### 6.2.2 Kinetic properties

pH optimum and stability. U1 exhibited maximum activity at a pH of between 4 and 5 over a ten minute reaction period. The curve of pH vs. activity showed a second, somewhat reduced maximum at pH 8,5 (Fig. 39). Preincubation of U1 for one hour at pH values below 6 resulted in significant loss of activity (Fig. 39).

Temperature optimum and thermal stability. The temperature optimum of U1 over a 10 minute reaction period was 70°C and this optimum was unchanged on increasing the reaction period to 20 minutes (Fig. 40). This result suggests that U1 was comparatively stable at temperatures as high as 70°C. However, closer examination of Fig. 40 reveals that, at temperatures >50°C, the longer reaction time was not accompanied by a proportional increase in product concentration. It therefore seemed that U1 was not stable at higher temperatures.

The stability of U1 was re-tested by preincubating the enzyme for one hour at temperatures between 0 and 70°C and pH 4,5 before measuring residual activity at 43°C (Fig. 40). The data show that the enzyme was apparently unstable at temperatures above 30°C.

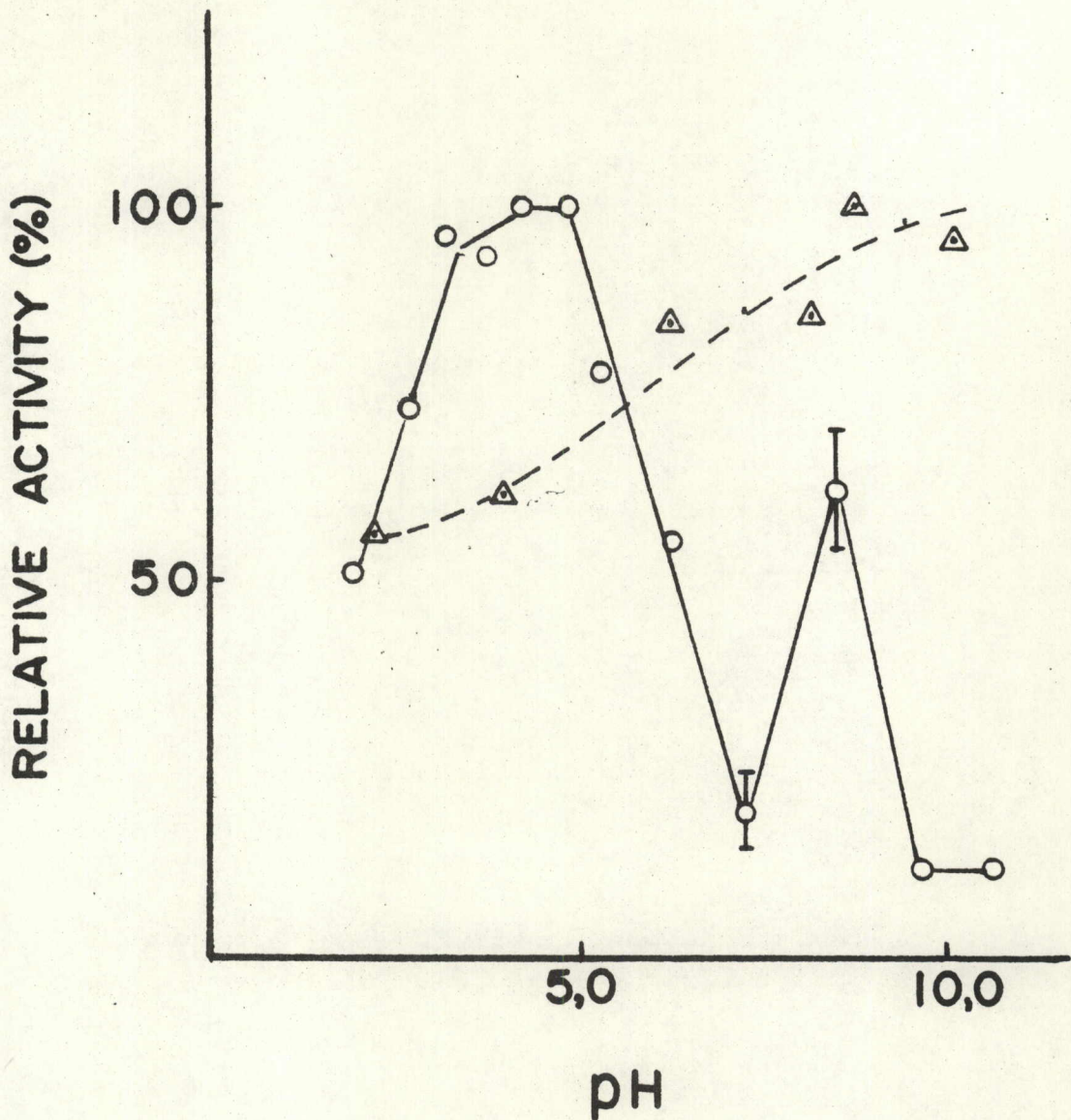


Figure 39. pH optimum and stability of U1. Assays were conducted using  $5,0 \text{ g dm}^{-3}$  xylan (Sigma) and a reaction time of 10 minutes. Glycine-HCl buffer ( $0,05 \text{ mol dm}^{-3}$ ) was used at pH 1,9, half strength McIlvaine buffers were employed at pH 2,7 to 7,2 and  $0,05 \text{ mol dm}^{-3}$  glycine-NaOH was the buffer at higher pH values when measuring the pH optimum. pH stability was assessed by incubating enzyme in one tenth strength McIlvaine buffers (pH 2,2 to 6,3) or  $0,02 \text{ mol dm}^{-3}$  glycine-KOH buffer (pH 8,3 to 10,2) at  $43^\circ\text{C}$  for one hour before measuring residual activity in  $0,06 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,4. The pH optimum was determined at  $40^\circ\text{C}$  and residual activity at  $43^\circ\text{C}$ . pH optimum (O), stability ( $\Delta$ ).

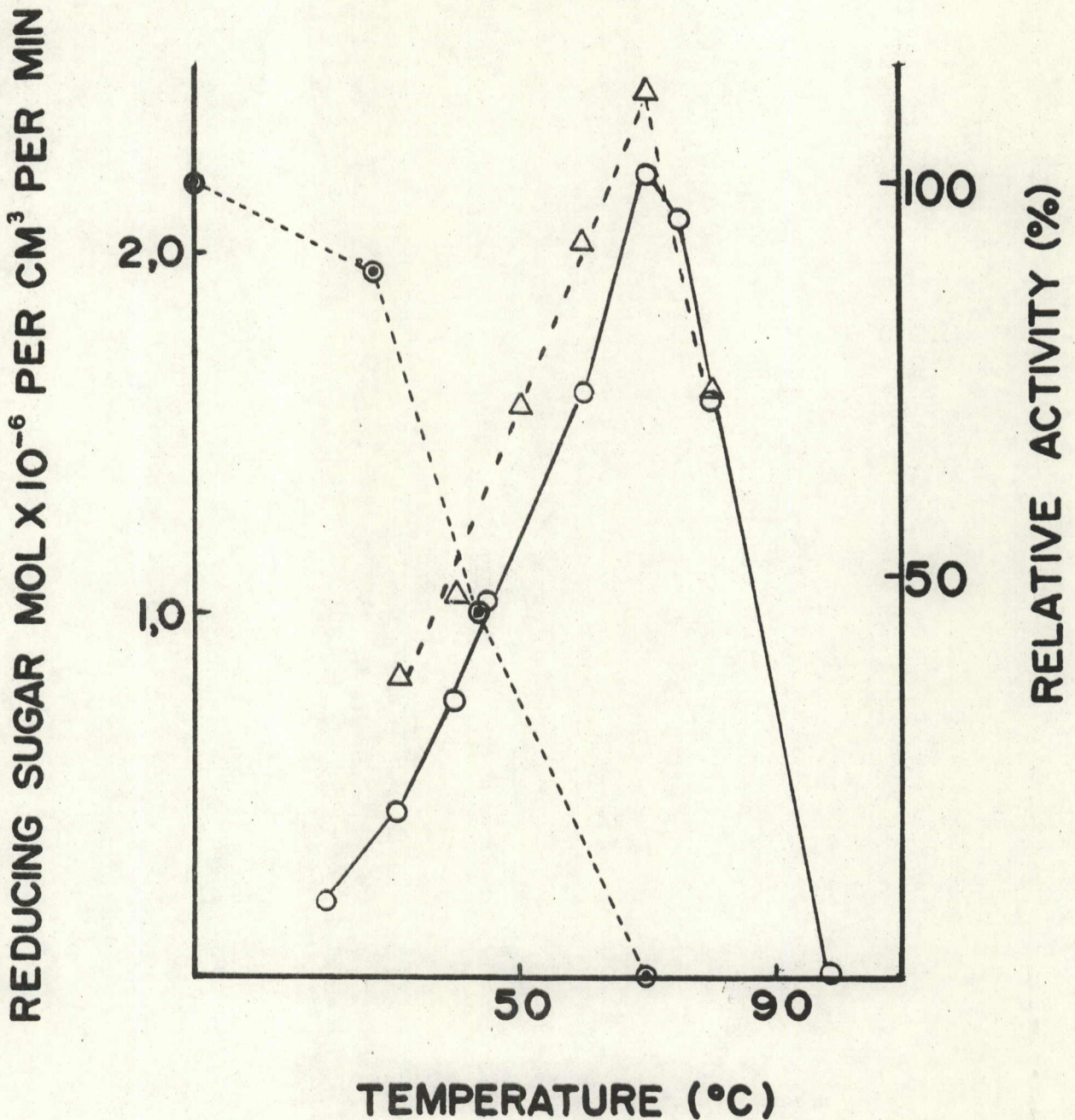


Figure 40. Temperature optimum and thermal stability of Ul. Assays were conducted using xylan stock solution. The temperature optimum was determined for reaction times of 10 (○) and 20 (△) minutes. Thermal stability (⊙) was assessed by preincubating Ul in  $0,05 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, for one hour at temperatures between 0 and  $98^\circ\text{C}$ . Residual activity was determined at  $43^\circ\text{C}$  employing a reaction time of 30 minutes.

Substrate specificity. U1 catalysed the degradation of xylans from several different sources (see section 6.3.3) and, in addition, catalysed the release of o-nitrophenol from ONX. No significant activity was observed on the following substrates (all from Sigma): CMC, arabinogalactan, galactomannan and PNG.

The effect of substrate concentration on the activity of U1. The effect of substrate concentration on the rate of U1-catalysed xylan degradation was investigated using purified Sigma Larchwood xylan at concentrations between 0,91 and 13,6 g dm<sup>-3</sup>. The initial reaction velocity appeared to be comparatively insensitive to changes in substrate concentration (Fig. 41).

### 6.3 Mode of action

#### 6.3.1 Purification of Sigma larchwood xylan

Following hydrolysis of Sigma larchwood xylan in 0,5 mol dm<sup>-3</sup> sulphuric acid, hydrolysates were chromatographed on paper and found to contain only xylose, arabinose and glucose. Correspondence with Sigma confirmed that the xylan preparation was an arabinoxylan, which explains the presence of arabinose in the xylan hydrolysates. However, the presence of glucose in the hydrolysates was puzzling since glucose is not a known constituent of larchwood arabinoxylan. The glucose could not be removed by washing the xylan in water before hydrolysis and the author concluded that the xylan was contaminated by an insoluble glucan. Support for this conclusion was found in the report

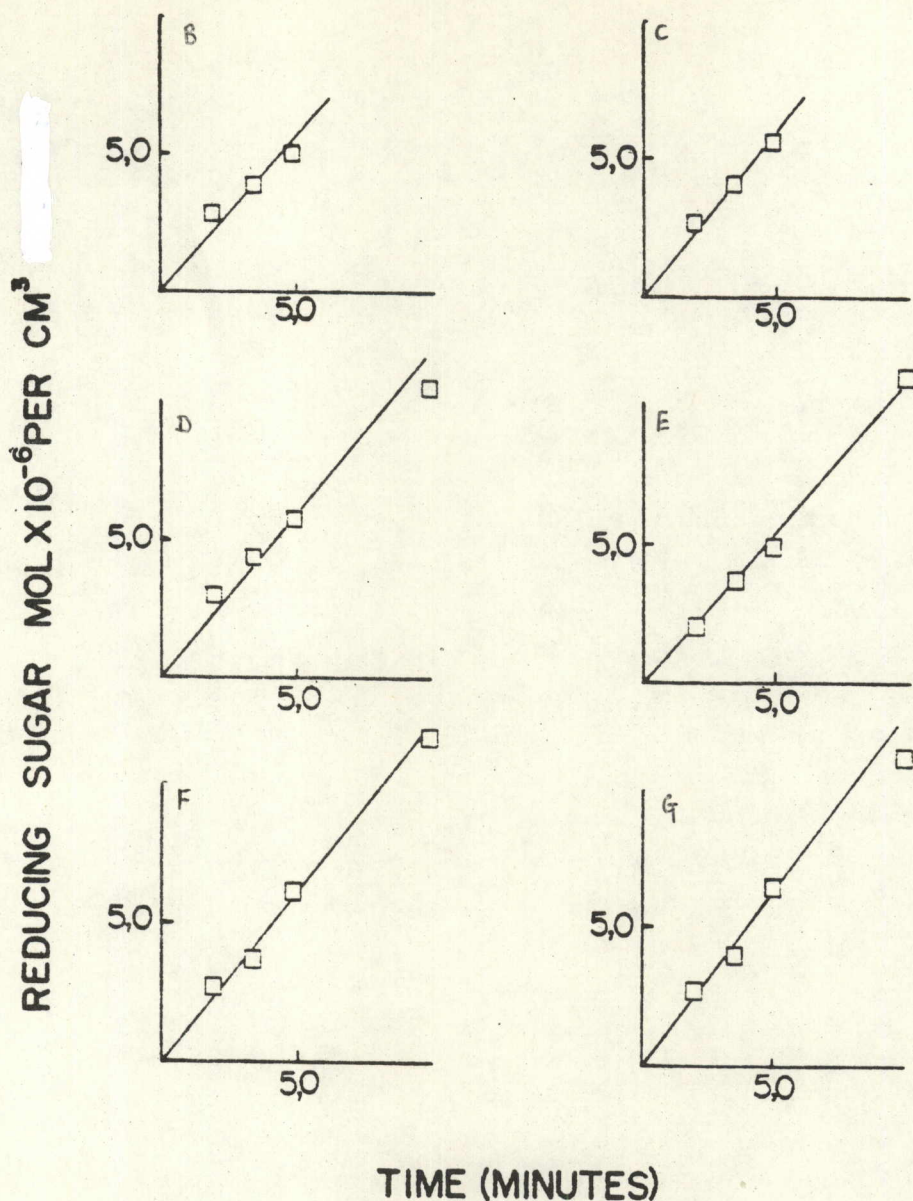


Figure 41. The effect of substrate concentration on the initial velocity of UI-catalysed xylan degradation. Concentrated xylan stock solution (made with purified xylan) was diluted with  $0,05 \text{ mol dm}^{-3}$  sodium acetate, pH 4,5, to obtain the desired substrate concentration range. The reaction temperature was  $53^{\circ}\text{C}$ . At the indicated times samples were removed for the determination of reducing sugar. Xylan concentrations in  $\text{g dm}^{-3}$  were : B , 1,0 ; C , 1,1 ; D , 1,8 ; E , 4,5 ; F , 9,0 ; G , 13,6.

of Taiz and Honigman (83). The mass ratio of xylose:glucose was determined by quantitative paper chromatography before and after purification. Before purification the ratio was ca. 4 and this fell to a value of ca. 0.1 following purification, indicating that almost all the glucan had been removed (Table 6). The mass of xylan recovered after purification was ca. 10% of that of the crude starting material.

#### 6.3.2 Isolation and identification of xylooligosaccharides generated by acid hydrolysis

Xylooligosaccharides for use as substrates of U1 in mode of action studies were generated by controlled hydrolysis of purified Sigma xylan using trifluoroacetic acid (see section 2.12.2). Paper chromatography of the hydrolysate revealed the presence of several compounds assumed to comprise both branched (arabinose containing) and unbranched (linear) xylooligosaccharides. The molecular structure of branched oligosaccharides is comparatively difficult to establish and in the absence of a sure knowledge of their structure they were of little use as enzyme substrates for mode of action studies. Therefore no attempt was made to isolate the branched oligosaccharides and further efforts were directed to isolating and identifying the linear xylooligosaccharides.

Comparisons of xylose and arabinose spot sizes and intensities on chromatograms of xylan hydrolysates indicated a mass ratio of xylose to arabinose in Sigma xylan of ca. 8:1, suggesting the existence of relatively little branching in the xylan polymer. Therefore it seemed

TABLE 6. PURIFICATION OF SIGMA XYLAN.<sup>a</sup>

XYLAN SAMPLE	NUMBER OF DETERMINATIONS	METHOD OF CARBOHYDRATE MEASUREMENT	RATIO OF GLUCOSE TO XYLOSE
UNPURIFIED	2	NELSON-SOMOGYI	0,26
UNPURIFIED	3	DU BOIS <u>et al.</u>	0,22
PARTIALLY PURIFIED	3	DU BOIS <u>et al.</u>	0,05
PURIFIED	3	NELSON-SOMOGYI	0,008

<sup>a</sup> The final barium chloride concentrations during precipitation were 50 g dm<sup>-3</sup> (partially purified sample) or 60 g dm<sup>-3</sup> (purified sample). Hydrolysates were analysed by quantitative paper chromatography and carbohydrate determined by either the Nelson-Somogyi method or by the method of DuBois et al. (66). Recovery of carbohydrate from the paper was calculated using glucose, and found to be between 89% and 114%.

reasonable to assume that the products of acid hydrolysis would consist predominately of homooligosaccharides of xylose. Consequently attention was focused on the major hydrolysis products only.

Partial separation of xylooligosaccharide mixtures was achieved by chromatography on Sephadex G-15 (Fig. 42), however complete resolution required the additional application of preparative paper chromatography. Following their isolation, the identities of individual oligosaccharides were deduced from the data presented below.

Monosaccharide composition. Individual xylooligosaccharides were hydrolysed in sulphuric acid and the resulting hydrolysates analysed qualitatively by paper chromatography. Apart from traces of arabinose observed in hydrolysates of the suspected compounds xylotetraose and xylopentaose, xylose was the sole product of sulphuric acid hydrolysis (Plate 9).

Identification of xylooligosaccharides by means of  $R_x$  values.

Confirmation of the suspected D.P. of the unknown oligosaccharides was obtained from paper chromatography. Xylobiose and xylotriose whose identities had been independently established (see section 6.3.5) together with xylose were used as chromatographic standards. Xylobiose and xylotriose, generated by trifluoroacetic acid hydrolysis, were identified directly by comparison of their  $R_x$  values with those of the standards. Confirmation of the D.P. of the larger oligosaccharides was obtained from a plot of suspected D.P. vs.  $\log(1 - R_x/R_x)$  (Fig. 43).

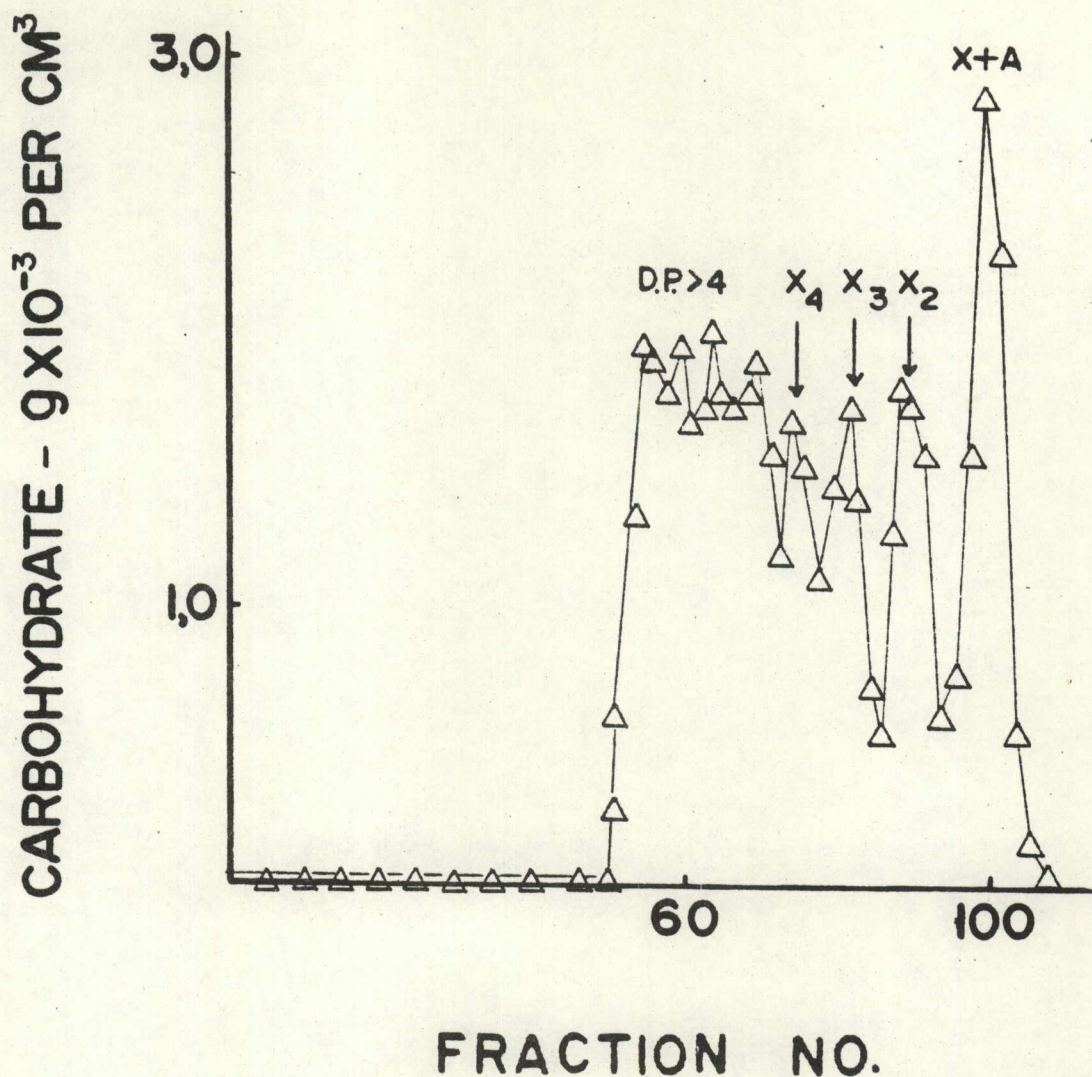


Figure 42. Chromatography of xylooligosaccharides on Sephadex G-15. Xylooligosaccharides were prepared by trifluoroacetic acid hydrolysis of purified Sigma xylan. The oligosaccharide mixture, containing 0,16 g of carbohydrate in water ( $1,7 \text{ cm}^3$ ) was applied to a column ( $124 \times 1,6 \text{ cm}$ ) of Sephadex G-15 pre-equilibrated in water at room temperature. Solutes were eluted from the column with water at a flow rate of  $12,5 \text{ cm}^3$  per hour. The fraction size was  $1,8 \text{ cm}^3$ . Carbohydrate in the fractions was assayed by the method of DuBois *et al.* (66).  $X_2$ ,  $X_3$ , and  $X_4$  represent xylobiose, xylotriose and xylo-tetraose. X and A represent xylose and arabinose.

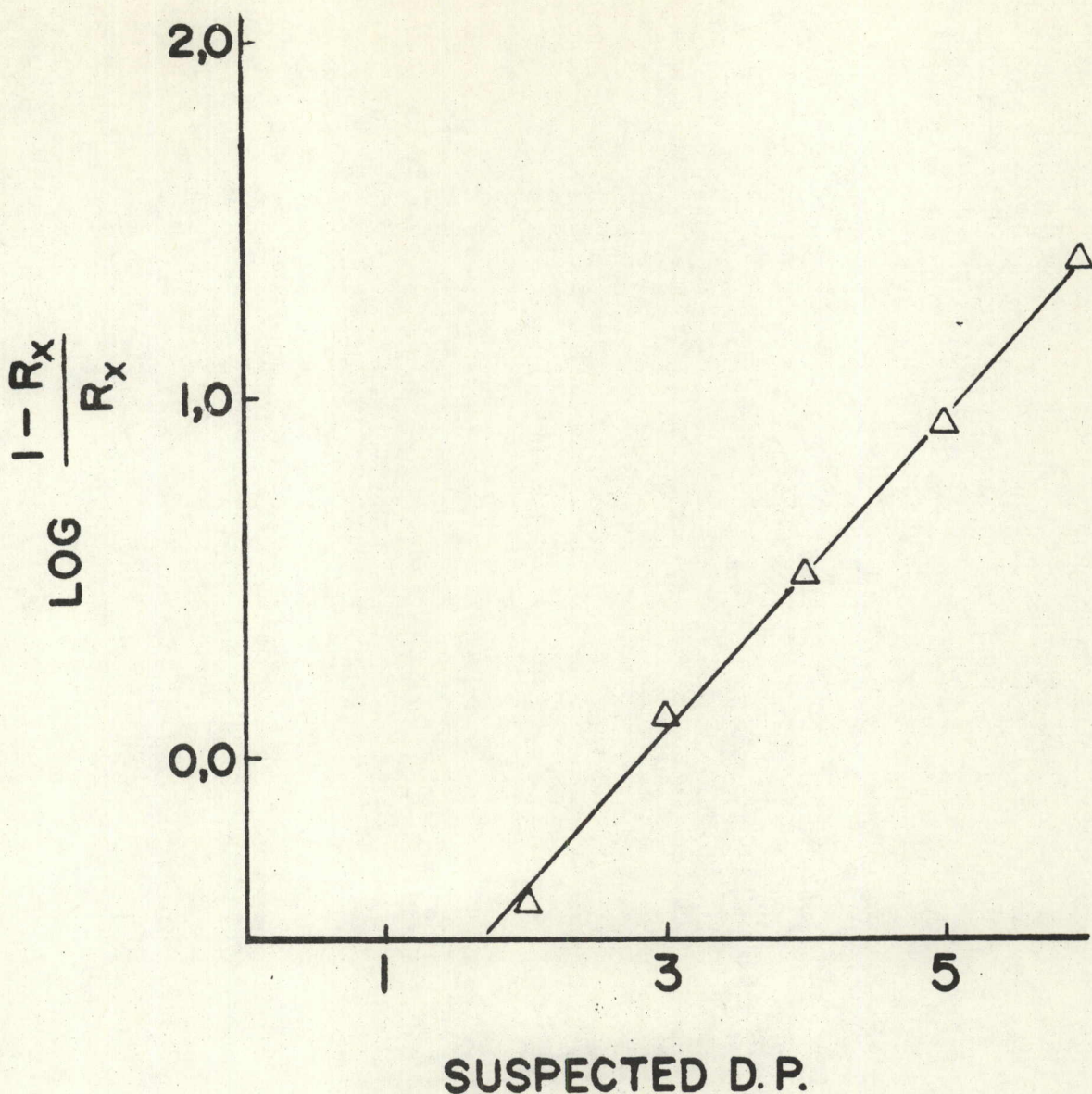


Figure 43. Identification of xylooligosaccharides by paper chromatography. Xylooligosaccharides generated by hydrolysis with trifluoroacetic acid were chromatographed on Whatman No.1 paper. Development was with ethyl acetate pyridine water (10:4:3). The straight line relationship between suspected D.P. and  $\log \frac{1-R_x}{R_x}$  is confirmation of the correctness of the assumed D.P..

Direct determination of the degree of polymerisation. In order further to confirm the identities of the larger oligosaccharides, a more direct determination of D.P. was undertaken. Each oligosaccharide was reduced at C<sub>1</sub> with sodium borohydride and identical masses of both reduced and non-reduced oligosaccharides were individually hydrolysed with sulphuric acid. From a comparison of the quantities of reducing sugar in the hydrolysates of corresponding pairs of reduced and unreduced oligosaccharides, the D.P. was inferred. The relevant data are presented in Table 7.

#### 6.3.3 Degradation of various xylan substrates

Ul was incubated with several different xylan preparations, including both crude and purified Sigma xylan, and the products of enzyme action subsequently chromatographed on paper. Two products (a) and (b) (Plate 8) were generated from all the xylans tested. A third product, denoted by (c) in Plate 8, was detected following degradation of only glucuronoxylans. Trace amounts of xylose and arabinose were also products of Ul action.

#### 6.3.4 Degradation of linear xylooligosaccharides

Ul degraded linear xylooligosaccharides of D.P.  $\geq 3$  in each case to a mixture of the products (a) and (b), earlier seen to be degradation products of all the xylan substrates tested.

TABLE 7. DETERMINATION OF THE D.P. OF XYLOOLIGOSACCHARIDES BY BOROHYDRIDE REDUCTION.<sup>a</sup>

SUSPECTED D.P.	REDUCING SUGAR AFTER HYDROLYSIS (g x 10 <sup>-6</sup> )		NUMBER OF DETERMINATIONS	A/B	DEGREE OF POLYMERISATION
	REDUCED (A)	NON-REDUCED (B)		( $\lambda$ )	( $\frac{1}{1-\lambda}$ )
4	67,75	89,75	2	0,755	4,08
4	29,25	38,25	1	0,765	4,26
5	55,75	69,5	2	0,802	5,05
6	52,0	61,75	1	0,842	6,33

<sup>a</sup>Xylooligosaccharides of D.P.  $\geq 4$  were hydrolysed in sulphuric acid before and after reduction with sodium borohydride (see section 2.16.1) and the D.P. in each case confirmed from the measured ratio of reducing sugar in reduced and unreduced samples. Reduction with borohydride renders the xylose residue at the reducing end of the oligosaccharide unreactive to the Nelson-Somogyi reagents. Thus, following hydrolysis, the measured concentration of xylose in the non-reduced sample will be greater than that in its reduced counterpart by the factor  $n/n-1$ , where  $n$  is the D.P.. Where more than a single determination was made, the mass of reducing sugar, given in the first two columns, is an average value.  $A/B = n-1/n = \lambda$ , degree of polymerisation =  $1/1-\lambda$ .

### 6.3.5 Identification of degradation products

The products (a) and (b) were isolated from reaction mixtures of Ul and purified Sigma xylan by chromatography on Sephadex G-15 and further purified by preparative paper chromatography. The identities of (a) and (b) were deduced from the three separate lines of investigation described below.

Determination of molecular mass. The molecular masses of the products were determined from measurements of their respective elution volumes on a column of Bio-Gel P-22, previously calibrated with known carbohydrate standards (Fig. 44). Each product chromatographed as a single symmetrical peak on the Bio-Gel column (Figs. 45 and 46), the elution volumes of (a) and (b) corresponding to compounds with molecular masses of 320 and 410 respectively.

Hydrolysis by sulphuric acid. The products (a) and (b) were separately hydrolysed in sulphuric acid and the hydrolysates subsequently analysed by paper chromatography. In each case, only a single compound appeared on the chromatogram with an  $R_f$  value equal to that of xylose.

Chromatography with known standards. Both (a) and (b) were chromatographed on paper alongside known carbohydrate standards including xylobiose and xylotriose. The  $R_x$  value of (a) corresponded to that of xylobiose and the  $R_x$  value of (b) to that of xylotriose.

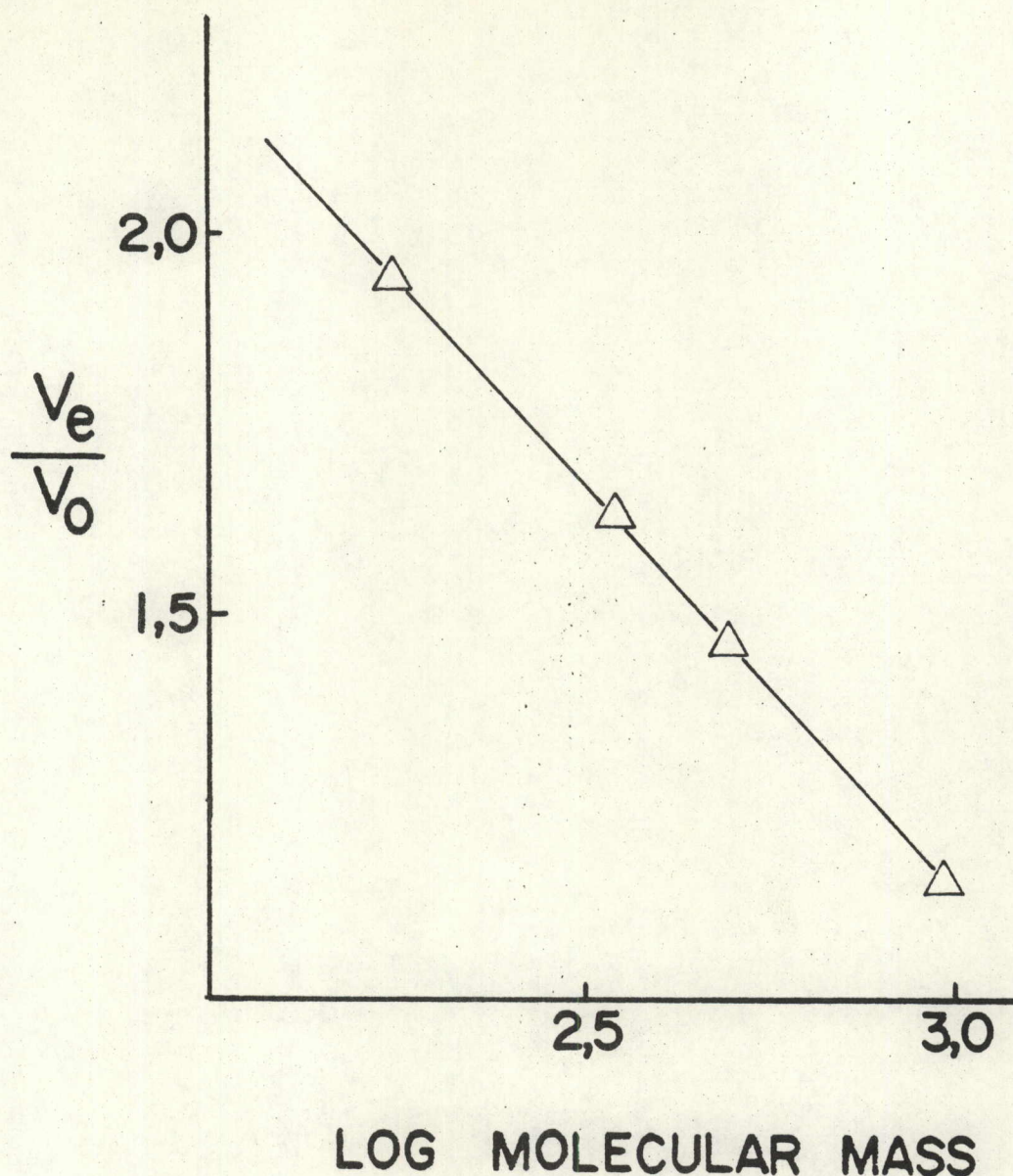


Figure 44. Calibration of the Bio-Gel P-2 column (redrawn from the data of Dr. S. C. Churms). The following carbohydrates were used to calibrate the column (molecular masses are given in brackets after the carbohydrate): (galactose  $\rightarrow$  galacturonic acid  $\rightarrow$  fructose)<sub>2</sub> (986), raffinose (504), biouronic acid (356) and glucose (180). Both the pre-equilibration of the resin and the subsequent elution of solutes from the column were with 1,0 mol dm<sup>-3</sup> sodium chloride at room temperature. Carbohydrate, (ca. 1,5 x 10<sup>-3</sup> g of each component), dissolved in 1,0 mol dm<sup>-3</sup> sodium chloride, was applied to the column (55 x 2,5 cm) and subsequently eluted at a flow rate of 40 cm<sup>3</sup> per hour. The fraction size was 2,0 cm<sup>3</sup>. Fractions were assayed for carbohydrate by the method of DuBois et al. (66).

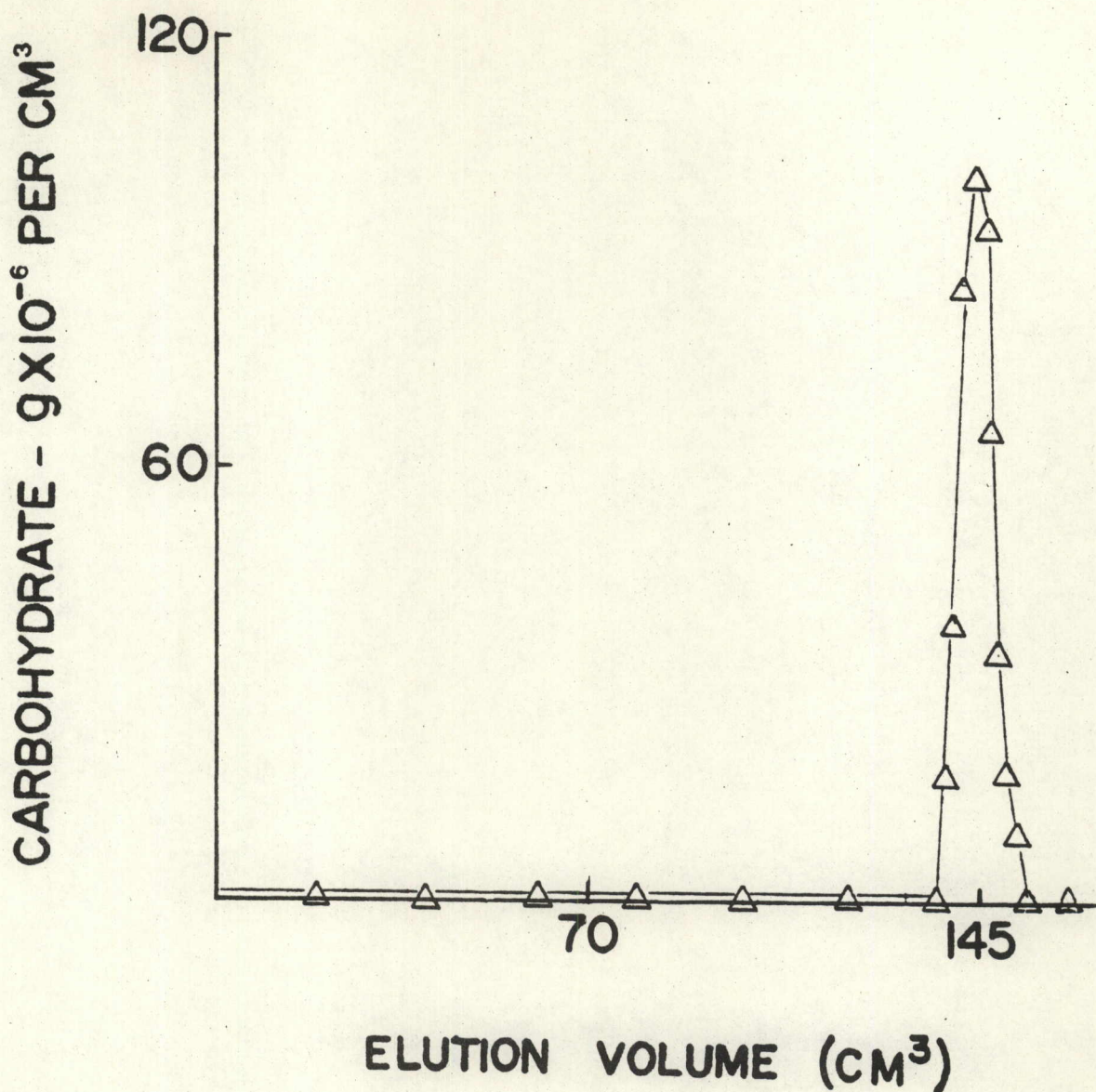


Figure 45. Chromatography of the xylooligosaccharide (a) on the Bio-Gel P-2 column. The procedure was as described in Figure 44. The molecular mass of (a) was 310.

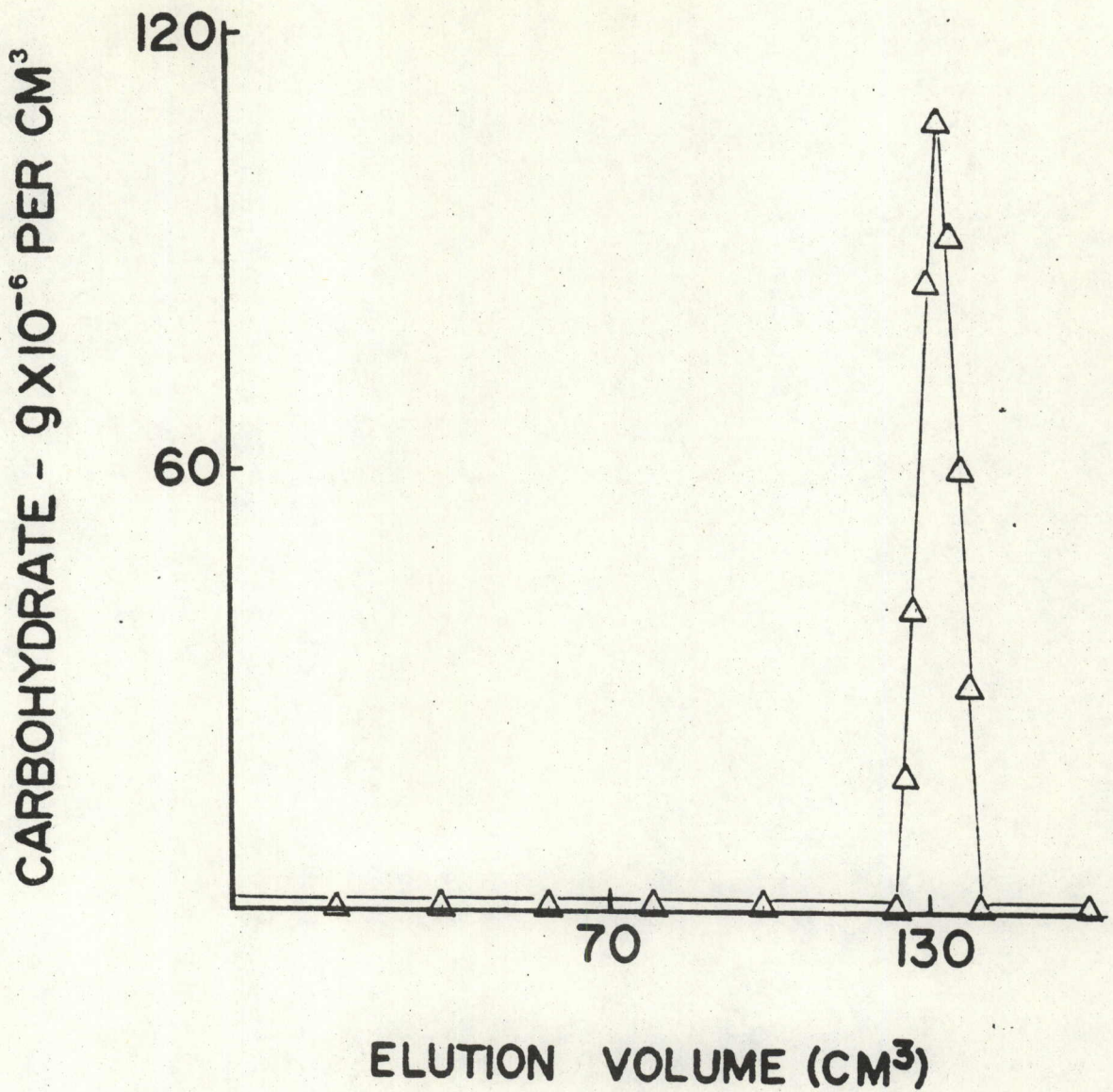


Figure 46. Chromatography of the xylooligosaccharide (b) on the Bio-Gel P-2 column. The procedure was as described in Figure 44. The molecular mass of (b) was 420.

#### 6.4 Discussion

U1 was homogeneous by SDS gel electrophoresis, moreover, the enzyme appeared as a single symmetrical peak during preparative electrophoresis and U1 therefore appears to conform with the level of purity reported for similar enzymes.

The purification factor for U1 was 4,8, a value which might seem surprisingly small particularly as U1 was a relatively minor component of the complex mixture of proteins observed in S. rolfsii culture filtrates. It is possible that the observed instability of U1 resulted in losses in enzyme activity during handling and storage, thus contributing to the low purification factor. It is not helpful in this context to compare the specific activity of U1 with the reported specific activities of other xylanases to ascertain whether the specific activity of U1 is abnormally low. In the first place, U1 probably attacks only soluble xylan (see below) which appears to be an abnormal property of xylanases. In the second place, different temperatures, pH values and substrate concentrations have been used by different workers to determine specific activity data for other xylanases. Therefore, direct comparison of the data for U1 with published data for other xylanases is not possible.

A further possible cause of the low purification factor observed may be suggested. It was noticed that several xylanase fractions were obtained during purification of S. rolfsii enzymes, i.e. the xylanases of S. rolfsii are clearly nesozymes (see Chapter 4). It seems,

therefore, quite likely that the observed low purification factor is at least partly a manifestation of the nesozyme nature of S. rolfsii xylanases. It is difficult to prove this last contention from the available data, since such proof would entail knowing, in advance, the activities and recoveries of each individual nesozyme component. When dealing with an unknown protein mixture, (such as the culture filtrate), this knowledge is unavailable. Further, by the arguments of section 4.7.3, the recovery of nesozyme activity from unknown mixtures normally cannot be measured. However, the nesozyme concept and its relevance to specific activity calculations is mathematically plausible, and can be used to rationalise the comparatively low purification factors obtained for nesozyme components.

Fungal xylanases, like cellulases, exhibit a wide range of physicochemical properties and most of the remarks already made about the difficulties of comparing some of the physicochemical properties of different cellulases (see section 4.7.1) also apply to xylanases. It nevertheless seems that the temperature optimum of U1 is unusually high at 70°C. Comparison with published data on fungal xylanases shows, in addition, that the pI of U1 is unusually low at 3.6. However, neither of these values is so extreme as to be without precedent and equally high temperature optima (137) and equally low pI values (138) have been recorded for fungal xylanases.

What is remarkable, is that U1 should be unstable at pH values below 6.0 particularly as, during growth of S. rolfsii in liquid culture, the pH of the medium falls below 3.0. It seems, therefore, as

if U1 was unstable in the growth medium. However, it is conceivable that the apparent association between U1 and U2 mentioned in section 6.1.3 might have acted to stabilise U1 at low pH values.

The pH stability of U1 is of importance when assessing the relative merit of the various steps used in the purification of U1. Thus, electrofocusing appears to be the least desirable purification step since U1 is clearly unstable at its pI (3,6). However, there seems to have been no way of realising this in advance.

The instability of U1 at its pH optimum posed a problem when designing suitable enzyme assay conditions for the purified enzyme. However, since degradation of purified Sigma xylan was linear for 10 minutes at pH 4,5 (Fig. 41) there seemed to be no need to assay at a pH value other than the optimum, particularly when comparatively short incubation periods were involved.

The insensitivity of the initial reaction rate of U1 catalysed xylan degradation to substrate concentrations from 0,91 to 13,6 g dm<sup>-3</sup> is an unusual finding. The most obvious explanation for the observed insensitivity is that U1 was saturated with substrate at all substrate concentrations tested. This in turn would imply a K<sub>m</sub> for U1 of considerably less than 0,91 g dm<sup>-3</sup>. This value is exceptionally low compared with published K<sub>m</sub> values for other xylanases acting on xylan substrates. An explanation other than substrate saturation was therefore sought.

It seemed possible that U1 acted in the first instance to remove arabinose from the xylan backbone before attacking the backbone itself. Adopting this postulate, it was reasoned that the insensitivity of the initial reaction to substrate concentration might reflect a high affinity (small  $K_m$ ) of the enzyme for arabinose residues rather than for the xylan backbone. However, there was no evidence of an initial burst of arabinose production and the idea that initial substrate conversion was largely arabinose release was subsequently abandoned.

An alternative explanation could be that U1 attacks only soluble xylan. Neither the crude nor the purified Sigma larchwood xylan was completely soluble under the assay conditions. It is therefore possible, over the substrate concentration range employed, that the xylan suspension represented a saturated solution with dissolved xylan in equilibrium with undissolved solid xylan. Under these conditions the initial xylan concentration in solution would be constant irrespective of the total mass of xylan present. Support for the idea that U1 might be capable of degrading only soluble xylan comes from observations on the mode of action of a xylanase from A. niger that was shown to degrade only soluble xylan (139).

#### 6.4.1 Mode of action of U1

One of the chief reasons for investigating the mode of attack of both cellulases and hemicellulases on different substrates is to throw light on the mechanism of catalysis. However, there is a more fundamental reason for studying the mode of action of hemicellulases in

particular, namely to identify unambiguously the enzyme(s) involved in substrate degradation. There is little difficulty in identifying cellulases as enzymes that attack cellulose (or in some cases CMC). In contrast, the ability of an enzyme to attack hemicellulose does not necessarily prove that the enzyme is a hemicellulase, since a number of specific exo-glycosidases also attack hemicellulose. In such cases, a detailed examination of the enzyme's mode of action is required to establish its true identity.

The results presented in this Chapter demonstrate that U1 is a xylanase and not a glycosidase. Thus, U1 liberated xylobiose and xylo-triose as the major products of larchwood arabinoxylan degradation. Since larchwood xylan does not normally contain xylose as a side chain constituent it is highly probably that attack by U1 was on the xylan backbone. U1 is therefore, by definition, a xylanase (see section 1.2). Additional support for this contention comes from the observation that U1 did not attack xylobiose, a substrate attacked by xylobiases but not by xylanases.

Xylobiose and xylo-triose were the major products of U1-catalysed xylooligosaccharide degradation, xylose itself being generated in only trace amounts. The absence of significant xylose production during xylooligosaccharide degradation (particularly in the case of xylo-triose and xylo-tetraose) suggests the operation of a catalytic mechanism involving transglycosylation. The observation that U1 degraded ONX to xylobiose and xylo-triose provides further evidence of U1-catalysed transglycosylation reactions. Xylanase -catalysed transglycosylation

with ONX apparently follows a complex series of reactions that are catalysed by xylooligosaccharides and the discovery that a xylanase could degrade ONX was described as novel (140). The implication was that few, if any, other xylanases showed this capability. However, the observation that U1 had a similar capability suggests that the phenomenon might be fairly wide-spread among xylanases. In the light of these findings, caution should be exercised in ascribing xylobiase activity to an enzyme capable of degrading ONX, without further investigating the enzyme's specificity.

In conclusion, U1 is best described as an arabinose-releasing xylanase. Several features make U1 an unusual xylanase. These features include an abnormally low pI, a high temperature optimum and the ability to degrade ONX. The observation that U1 catalysed extensive transglycosylation reactions adds to the unusual character of this enzyme.

## CHAPTER 7

GENERAL DISCUSSION

The studies reported in this thesis contribute to the enigma of cellulose and hemicellulose biodegradation. Details of the main research results have already been discussed and, in the present discussion, only selected findings will be reviewed, with an emphasis placed on their relevance to biotechnology.

The topic of cellulose and hemicellulose biodegradation is not new and already forms a substantial part of the global biotechnological effort. The reason is simple. Polysaccharides are both plentiful and, more importantly, renewable, and represent a large reserve of stored solar energy.

An obvious way to tap this energy would be to burn the polysaccharide and use the heat of combustion to drive a heat engine. However, as most of the world's engines run on a liquid hydrocarbon fuel, it is more reasonable to convert the polysaccharide to a liquid before combustion in a heat engine. As economic ways of converting polysaccharide to liquid hydrocarbons have not been found, efforts have been directed towards converting the polysaccharide into ethanol.

At present, the most intensive research is centred on the microbial production of efficient extracellular cellulases, the isola-

tion of these enzymes from culture filtrates and their subsequent use, in a separate reactor, as catalysts in the conversion of polysaccharides to simple sugars. The simple sugars are then fermented anaerobically to alcohol (and acetic acid).

Most cellulase production studies have involved batch culture and the best enzyme yields have thus far been obtained using batch procedures (1). Less information is available on continuous culture methods but present evidence indicates that they give yields of only 10 to 30% of that obtained by the best batch procedures. However, Wilke Yang and Stockar (141) have published a process description of cellulase production in continuous culture followed by biodegradation of newsprint. In these studies, a two stage fermentation process was envisaged in which biomass production was initially boosted on glucose, followed by enzyme induction on cellulose. This yielded an enzyme productivity of 1,8 i.u. dm<sup>-3</sup> per hour which could be further increased to values of 5,69 and 7,33 i.u. dm<sup>-3</sup> per hour by the inclusion of multiple induction stages on 1% cellulose.

As an alternative to this multistage process, the direct or single step conversion of cellulose to ethanol has been investigated, using either a single organism, e.g. Clostridium thermocellum (142), or a coculture of two bacteria (143). Some success has attended these investigations as the coculture was able to convert 30% of a cellulose powder into ethanol whilst C. thermocellum converted a more modest 20%. A drawback of direct conversion by cocultures is the sensitivity of the organisms to ethanol inhibition (144). Thus, a coculture of the

biomass-degrading anaerobes C. thermocellum and C. thermosaccharolyticum are strongly inhibited by ethanol concentrations in excess of 3 to 4 % (144). In contrast, the yeast Saccharomyces cerevisiae, which could be used to ferment glucose in a multistage conversion process, tolerates at least 10 to 14% ethanol (144). It has been argued by the proponents of the single stage process that direct conversion is simpler than the multistage process. This is true, but the choice between alternatives will have to be made ultimately on economic grounds. Before this can be done, the two processes must be contrasted not only in terms of their ease of execution, but also in terms of ethanol yield, biomass production and enzyme recovery. Unfortunately, lack of sufficient published data precludes making any meaningful comparisons here.

It is certain however that, regardless of which bioconversion process is adopted, a knowledge of the properties of the individual enzymes involved would be useful in establishing optimal process conditions. This is particularly true of cellulose bioconversion processes, since efficient cellulose degradation requires the concerted action of several enzymes with distinct physicochemical properties and modes of action. Further, there is no reason to expect that any single organism produces the most efficient possible cellulase complex. This contention is supported by studies of the cellulase complex of T. reesei. Unlike most cellulolytic microorganisms, T. reesei releases, into the culture filtrate, all the necessary enzymes for in vitro cellulose saccharification. However, the T. reesei cellulase complex is deficient in cellobiase (145), a deficiency that might lead

to accumulation of cellobiose during saccharification with consequent inhibition of both exo- and endocellulase components. This inhibition, in turn, would reduce the efficiency of substrate conversion. The deficiency of cellobiase could be alleviated, by supplementing the T. reesei cellulase complex with exogenous cellobiase such as that produced by a mutant strain of A. niger or A. phoenicus. In addition, marked differences have been noted in the ability of different exo- and endocellulases (from the same organism) to act synergistically in degrading various cellulosic substrates. It seems, however, that little attempt has been made to produce a more efficient cellulase complex by suitable blends of cellulases from different organisms.

The foregoing arguments underline the importance of studying individual cellulases and provide part of the rationale for the studies in this thesis. It has not been established firmly, in the present study, whether or not S. rolfsii elaborates the full cellulase complex. S. rolfsii culture filtrates readily catalysed CMC degradation, but manifested only weak activity on crystalline cellulose. This observation argues against the presence of an exocellulase in the culture filtrate. However, cellulases often adsorb strongly to crystalline cellulose (1). It is therefore possible that some S. rolfsii cellulases, including one or more exocellulases, became adsorbed to undegraded cellulose during growth of the organism and, consequently, were not recovered. Without exocellulase, culture filtrates would be inactive against crystalline cellulose. In the absence of evidence indicating exocellulase activity in S. rolfsii culture filtrates, attention was focused on the endocellulases.

Endocellulases are indispensable components of the cellulosic complex, but their precise role in cellulose degradation is unclear and they therefore require closer study. In this thesis the properties of an endocellulase, F2, is reported. Whilst the physicochemical properties of F2 appeared to be in no way unusual, its mode of action exhibited some unusual features. In spite of the similarity of the reaction products, the degradation of cellooligosaccharides by F2 proceeds via different mechanisms depending on the D.P. of the oligosaccharide substrate. Thus, F2-catalysed cellotriose degradation is thought to proceed by a head-to-tail condensation of two cellotriose molecules, followed by hydrolysis of the condensation product. In contrast, there is evidence to suggest that F2-catalysed breakdown of cellotetraose and cellopentaose involves glucosyl transfer from the non-reducing end of the substrate molecule. Finally, cellohexaose appears to be directly hydrolysed without undergoing transglycosylation. The differences between the above mechanisms are more profound than those hitherto suggested which usually invoke a simple hydrolytic cleavage at different sites in an oligosaccharide.

To the author's knowledge, the evidence presented in this thesis for a head-to-tail condensation of cellotriose molecules is the strongest thus far in support of such a mechanism. A mechanism involving glucosyl transfer in the degradation of cellooligosaccharides, such as proposed here for cellotetraose and cellopentaose degradation, has been proposed elsewhere (38). However, this idea seems to have evoked little comment subsequent to its publication, and the findings of this thesis are of value as supportive evidence for the

mechanism. Until more is known about the detailed mechanism of cellulase action, the significance of transglycosylation in the mechanism of cellulose degradation cannot be assessed.

The artificial substrate, CMC, is conventionally used as a substrate for the assay of endocellulase activity. It possesses the advantages of being soluble in aqueous solvents and of being readily degraded by endocellulases. In addition, the extent of CMC degradation can be monitored by measuring either the generation of reducing sugars or the accompanying decrease in viscosity of the reaction mixture. Endocellulases may also be distinguished by their different modes of attack on CMC. It is therefore important to establish optimal conditions for CMCase assay and likewise to define the limitations of the assay procedure. This has been done in Chapter 4.

With the benefit of hindsight, the pitfalls in the assay of CMCases seem obvious but the full significance of these problems appears not to have been realised by other workers. The suggestions made regarding substrate limitation and the non-stoichiometry of the CMCcase assay have less fundamental impact than the nesozyme concept. Provided CMC hydrolysis is not allowed to proceed for too long, an accurate measurement of relative reaction rate will be obtained and linear dilution curves can be constructed from the data. It is even possible to calculate an absolute value for the  $K_m$  from relative velocity measurements, although  $V_{max}$  can only be known to the extent of an arbitrary multiple of its true value.

However, as CMCases are nesozymes, even where linear dilution curves are obtained, the recovery and purification data calculated on measurements of enzyme activity cannot be applied meaningfully. Therefore, the validity of the quantitative aspects of much of the published recovery and purification data for nesozymes is questionable. The pitfalls associated with non-stoichiometric assays and with nesozymes are relevant not only in the assay of cellulases but also in the assay of hemicellulases, amylases, and proteases. As isozymes are a special class of nesozyyme, the remarks made about assaying nesozymes will apply equally well to systems containing two or more isozymes.

Considerable effort has been expended on attempting to improve the efficiency of enzyme-catalysed cellulose degradation. Greatest success in this regard has been achieved through the production of "hyper-cellulase-producing" mutants of T. viride such as T. reesei QM9414 (90). Further efforts have been directed mainly at optimising conditions for both enzyme production by the chosen organism and for the subsequent degradation process (1). However, since the mechanism of cellulase action is still imperfectly understood, efforts to improve the efficiency of cellulose biodegradation at present are largely empirical.

A knowledge of the mechanism of cellulase action could reveal which enzyme characteristics are most important for efficient cellulose conversion. Such knowledge might in turn lead to a reduction of the degree of trial-and-error associated with an empirical approach. The important topic of the mechanism of cellulase action is addressed in

Chapter 5, where it is suggested that the rate of cellulase catalysed cellulose degradation is limited either by product inhibition or by the rate at which cellulases diffuse over the cellulose surface. It is comparatively easy to alleviate the effects of product inhibition simply by removing the products as they accumulate. In contrast, if the rate of enzyme diffusion is limiting, it is not obvious how to increase the rate of substrate conversion. However, many cellulases are glycoproteins and the carbohydrate moiety of the protein may play a role in substrate binding (146) and enzyme mobility on the cellulose surface. Modification of the carbohydrate moiety of cellulases (e.g. by glycosidases) should be attempted to determine the possibility of enhancing catalytic rates. Further investigation into the mechanism of cellulase action is required before any definite conclusions can be drawn.

Whereas cellulose saccharification is the subject of intensive research, hemicellulose saccharification has, until now, received scant attention. There is a growing awareness of the importance of xylan as a chemical feedstock and, at present, several studies are aimed at developing aspects of xylan bioconversion, particularly xylose fermentation (147-149). Because xylan bioconversion is still comparatively underdeveloped, it is difficult to assess the broader implications of xylanase research. Nevertheless, the remarks already made regarding the importance of studying the mode of action of individual cellulases apply equally well to xylanases. The results of Chapter 6 bring to light some unusual features in the mode of action of the xylanase, U1. These include the ability to catalyse extensive

transglycosylation of xylooligosaccharides (and o-nitrophenyl- $\beta$ -D-xylo-  
side) and an apparent specificity of the enzyme for soluble xylan. As  
in the case of cellulases, the importance of transglycosylation in  
substrate degradation is not known. However, the specificity of U1 for  
soluble xylan indicates that U1 might play a role similar to  
xylosidases in catalysing the further breakdown of soluble xylooligo-  
saccharides formed from an initial attack by other S. rolfsei xylanases  
on solid xylan.

Although much of the groundwork in the study of both cellulases  
and xylanases has been laid by other workers, new challenges still  
confront the investigator. Some of these challenges have arisen from  
the findings of this thesis. It is hoped that a stimulus has been  
provided to other workers seeking a further contribution in this field.

SUMMARY

Published work from other laboratories has shown that S. rolfssii produces at least 3 physicochemically distinct endocellulases and one xylanase. However the enzymes were not rigorously purified or characterised and this paucity of detail, together with the potential industrial importance of cellulases and xylanases, prompted the present enquiry.

Initial efforts were directed toward finding a suitable carbon source for large scale production of cellulases and xylanases by S. rolfssii. Microcrystalline cellulose, filter paper, CMC, Eragrostis tef hemicellulose and bagasse were tested as carbon sources and, as growth on microcrystalline cellulose showed no significant lag phase and resulted in the highest levels of enzyme activity, a microcrystalline cellulose (Avicel) was selected for large scale enzyme production.

Following 10 to 12 days growth in aerated liquid culture, culture filtrates were freeze-dried and proteins were precipitated by adding 2,5 volumes of acetone. The acetone powder was the starting material for the further purification of a carboxymethylcellulase (endocellulase) and a xylanase.

The carboxymethylcellulase (1,4 - (1,3:1,4) $\beta$ -D-glucan 4-glucanohydrolase, EC 3.2.1.4), F2, was isolated in a 3-step procedure

involving adsorption on cellulose, molecular exclusion chromatography on Bio-Gel P-150 and preparative flat-bed electrofocusing. The final preparation was homogeneous on SDS-polyacrylamide gels but could be resolved into three components with pI values between 4,3 and 4,5 by electrofocusing. All three components appeared to be glycoproteins. The molecular mass of the enzyme was between  $5,4 \times 10^4$  and  $5,7 \times 10^4$ , its pH optimum was ca. 4,0 and it was stable for 20 min at temperatures below  $60^\circ\text{C}$ . A  $K_m$  of  $2,06 \text{ g dm}^{-3}$  was determined, using carboxymethylcellulose 4 H1F as substrate. The purified enzyme degraded carboxymethylcellulose and phosphoric acid swollen cellulose (Walseth cellulose) as well as short chain cellooligosaccharides of D.P.  $\geq 3$ .

Studies on the degradation of cellooligosaccharides revealed unusual features in the mode of action of F2. Oligosaccharides of D.P. 4 and 5 appeared to be degraded via a mechanism involving transfer of a glucosyl unit derived from the non-reducing end of the substrate. A similar mechanism has been reported only once before but has received little attention subsequently. In contrast, cellotriase degradation is preceded by a head-to-tail condensation of two cellotriase molecules, a mechanism not previously reported.

During purification of F2 it was realised that several pitfalls are associated with the assay of CMCCase activity. For example, the dilution curves of CMCases are often curvilinear and this complicates the calculation of both specific activity and recovery. It is shown that non-linear dilution curves could result from non-linear progress curves. It is further shown that CMCCase progress curves rapidly become

non-linear owing, primarily, to the effects of substrate depletion during reaction. Another difficulty arises because CMCases often occur as families of physicochemically distinct enzymes that nevertheless have the same nominal activity. In this thesis, the name nesozyme has been suggested to describe such families of enzymes. Even when linear dilution curves are obtained, the usual calculation of recovery and purification cannot be made with nesozymes.

Examination of the cellulase literature revealed that substrate limitation could not always account for the usual non-linear progress and dilution curves manifested by cellulases. Such curves often obey a relationship known as Schutz's law. Although Schutz's law was first formulated almost a century ago, no coherent explanation of the law has been proposed. An explanation of this law is presented in terms of product inhibition where Michaelis-Menten kinetics apply and diffusion limited catalysis where Michaelis-Menten kinetics do not apply. Verifiable suggestions are offered as to why cellulases, in particular, should obey this law.

In addition to the isolation of a carboxymethylcellulase and the studies of cellulase kinetics, a xylanase (1,4- $\beta$ -D-xylan 4-xylanohydro-lase, EC 3.2.1.8), U1, was purified and characterised. Purification involved adsorption on cellulose, ion exchange chromatography on DEAE-Sephadex, column electrofocusing and preparative electrophoresis. The final preparation was homogeneous by SDS gel electrophoresis. The molecular mass of U1 was  $5,3 \times 10^4$ , its pI was 3,6 and its pH optimum was between 4 and 5. The enzyme was relatively stable above pH 6. The

temperature optimum of U1 was 70°C when measured over a 10 or 20 minute reaction period, although the enzyme was relatively unstable, at pH 4,5, on exposure to temperatures above 30°C.

The purified enzyme degraded both arabinoxylan and glucuronoxylan, apparently showing greatest affinity for soluble forms of these xylans. The major products of U1-catalysed xylan degradation were, in all cases, xylobiose and xylotriose, together with traces of xylose and arabinose. An additional unidentified product was formed during degradation of glucuronoxylan. The enzyme also catalysed the degradation of linear xylooligosaccharides of D.P.  $\geq 3$ , in each case yielding xylobiose and xylotriose together with traces of xylose. Xylobiose and xylotriose were also products of enzyme attack on o-nitrophenyl- $\beta$ -D-xyloside (ONX). The nature of the products released during degradation of linear xylooligosaccharides and ONX indicates the operation of a degradation mechanism involving transglycosylation.

Individual cellulases and xylanases often differ greatly in their physicochemical properties and modes of action. In addition, the experimental conditions used to characterise these enzymes differ from laboratory to laboratory. These circumstances make it difficult to compare the properties of F2 and U1 with the properties of similar enzymes published elsewhere. Aspects of the kinetic studies reported in this thesis are seen as being of fundamental relevance to the study of cellulases and xylanases.

## APPENDIX 1

DOMINANCE OF THE  $x^2$  TERM IN THE LOGARITHMIC EXPANSION

A necessary condition for the  $x^2$  term to dominate in equation {8} of Chapter 5 is

$$C_2 (x^2/2a_0^2) > \lambda (C_1 (x/a_0) + C_2 R_2) \quad \{14\}$$

where

$$C_1 = K_p (K_a + a_0)$$

$$C_2 = K_a (K_p + a_0)$$

and  $\lambda > 1$  is a constant which expresses the extent of the dominance (e.g.  $\lambda = 10$  implies that the  $x^2$  term is 10 times the remaining terms). Inequality {14} is difficult to deal with directly, however, it will be satisfied if both

$$C_2 (x^2/2a_0^2) > 2\lambda C_1 (x/a_0) \quad \{15\}$$

and

$$x^2/2a_0^2 > 2\lambda R_2 \quad \{16\}$$

Inequality {15} places a lower bound, say  $\ell$ , on  $x$ . Further, using the inequality

$$R_2 \leq \left(\frac{x}{a_0}\right)^3 / 3 \left(1 - \frac{x}{a_0}\right)^3$$

it is easily shown that inequality {16} will hold for all  $x$  in some interval  $[0, m)$ . Given a reasonable value for  $\lambda$  (say  $\lambda = 10$ ), it is possible to choose the constants  $a_0$ ,  $K_a$  and  $K_p$  such that  $\ell < m$ . Hence

Schutz's law (equation {4}) should be a good approximation of  $x$  in the interval  $(\ell, m)$ .

In the non-competitive case a similar argument establishes lower and upper bounds for  $x$  in the situation where the  $x^2$  term will dominate in equation {10} of Chapter 5. Note too that inequality {14} implies  $K_p < K_a$ , whereas the corresponding inequality for the non-competitive case does not imply this.

PLATE LEGENDS

Plate 1. SDS gel electrophoresis of D3 and other selected fractions obtained by electrofocusing D3 in a sucrose gradient over the pH range 2,5 to 5,0. A: fractions 30 and 31 (E3 of Fig. 12), B: fraction D3 before electrofocusing, C: molecular mass markers (BDH) in the range  $1,43 \times 10^4$  to  $8,6 \times 10^4$ . The symbol + indicates a protein marker of molecular mass  $5,72 \times 10^4$ . D and E: fractions 27 to 29 and 22 to 24 of Fig. 12.

Plate 2. Analytical electrophoresis of CMCCase fractions P3 and F2. A: fraction P3 ( $2,7 \times 10^{-5}$  g) and B: fraction F2 ( $1,4 \times 10^{-5}$  g) after electrophoresis in 7,5 % polyacrylamide gels containing 0,1 % SDS. C: fraction F2 ( $1,9 \times 10^{-5}$  g) after non-SDS gel electrophoresis in a 7,5 % polyacrylamide gel at pH 8,9. Diffuse stained regions located at the position of the bromophenol blue marker are visible at the bottom of gels B and C. The origin of this charged, low molecular mass material is unknown. However, it only appeared on gels during electrophoresis of purified F2 and therefore may have arisen during flat bed electrofocusing.

Plate 3. Analytical electrofocusing of F2. Fraction F2 was focused in a 4,9 % polyacrylamide gel slab over the pH range 3,5 to 9,5. F2 (3 bands) is flanked on either side by pI markers used to determine the pI of F2 components.

Plate 4. Separation of the products of F2 action on cellotetraitol by TLC. Reaction times in minutes were: a,0; b,10; c,20; d,30; e,45; f,60. Reduced and non-reduced standards are denoted by SH and S respectively. Oligosaccharides are designated by the symbol G followed by a subscript indicating the D.P. The symbol H signifies reduction at C<sub>1</sub>, e.g. cellotriitol = G<sub>3</sub>H. the position of the G<sub>3</sub>H spot is indicated by +.

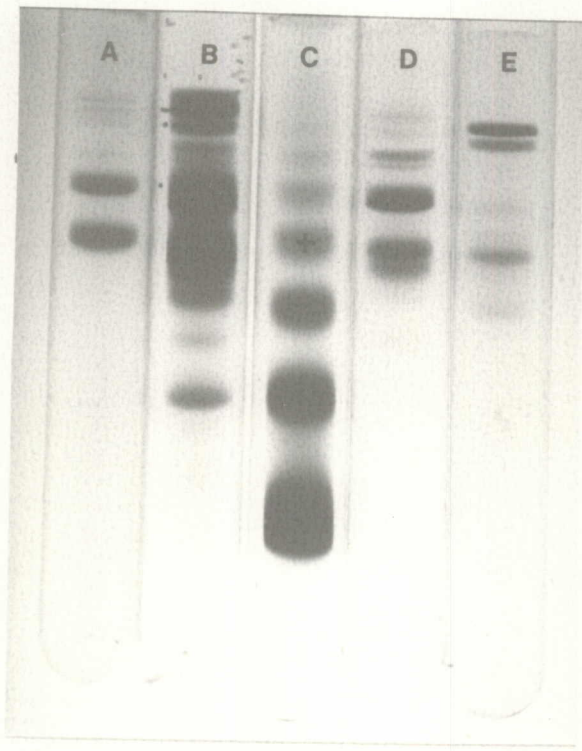
Plate 5. Thin layer chromatography of the products of F2 action on A: cellopentaitol and B: cellohexaose. Symbols have the same meanings as in Plate 4.

Plate 6. SDS gel electrophoresis of selected fractions obtained during the purification of U1. Fractions I1 and I2 were obtained by electrofocusing fraction D3 in a sucrose gradient over the pH range 2,5 to 5,0 (Fig. 37). Fraction D3 (middle gel) was obtained by chromatography of fraction A2 on DEAE-Sephadex (Fig. 36). The respective masses in g of I1, D3 and I2 loaded on the gels were  $3,0 \times 10^{-5}$ ,  $3,6 \times 10^{-5}$  and  $3,8 \times 10^{-5}$ .

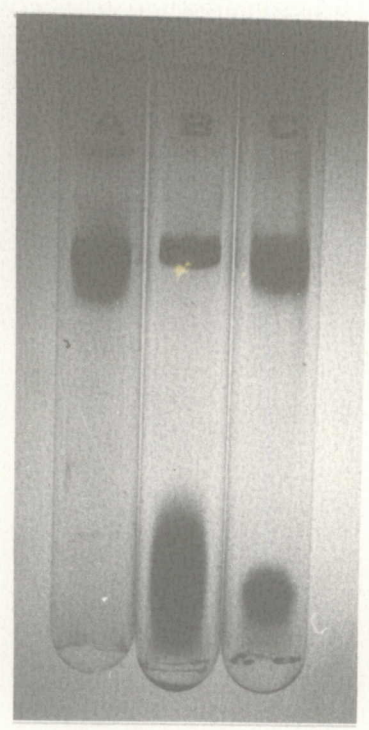
Plate 7. SDS gel electrophoresis of the purified xylanase U1. The mass of U1 loaded on the gel was  $1,5 \times 10^{-5}$ g.

Plate 8. Paper chromatogram showing the products of action of U1 on xylans from different sources. A: crude Sigma xylan, B: Sigma xylan purified by barium chloride precipitation, C: larchwood glucuronoxylan (a gift from Dr. M. Sinner), D: glucuronoxylan (a gift to Dr. R. F. H. Dekker from Dr. T. E. Timell), E: Eragrostis teff arabinoxylan and F: birchwood glucuronoxylan (both gifts from Dr. J. P. Joseleau). The xylan concentration in each case was  $5,0 \text{ g dm}^{-3}$  in  $0,1 \text{ mol dm}^{-3}$  sodium acetate buffer, pH 4,5, containing  $0,2 \text{ g dm}^{-3}$  sodium azide. Incubation was at  $53^\circ\text{C}$  for 24 hours. Xylooligosaccharide standards were chromatographed in the space between C and D. In order of decreasing mobility the standards are xylose (Xyl) xylobiose, xylotriose, xyloetraose and xylopentaose. Xylose and arabinose (Ara) were chromatographed in the space next to F.

Plate 9. Paper chromatogram showing the products of sulphuric acid catalysed xylooligosaccharide hydrolysis. Following hydrolysis in  $1,0 \text{ mol dm}^{-3}$  sulphuric acid, an amount of carbohydrate corresponding to between  $1,0 \times 10^{-4}$  and  $2,0 \times 10^{-4}$  g of the original sample was chromatographed. S: standard mixture of xylose and arabinose (Ara).



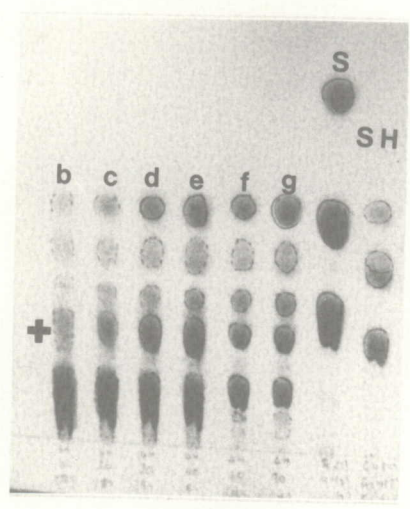
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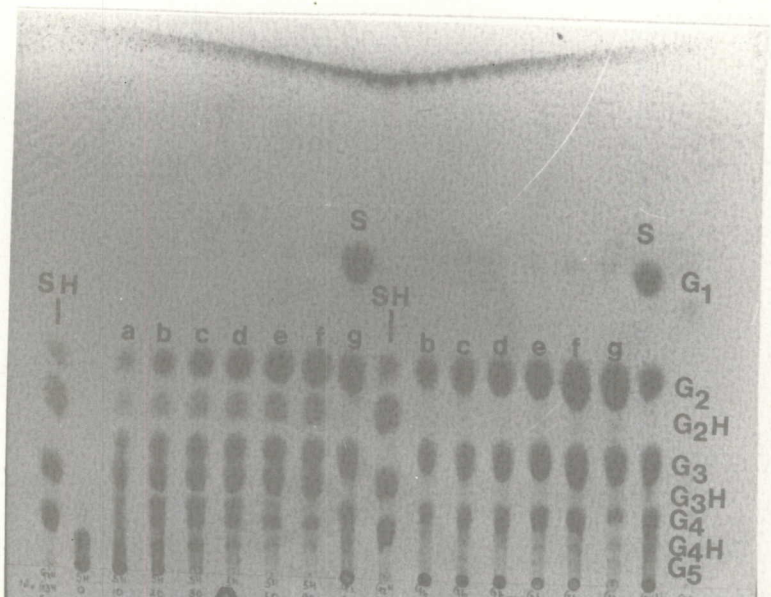
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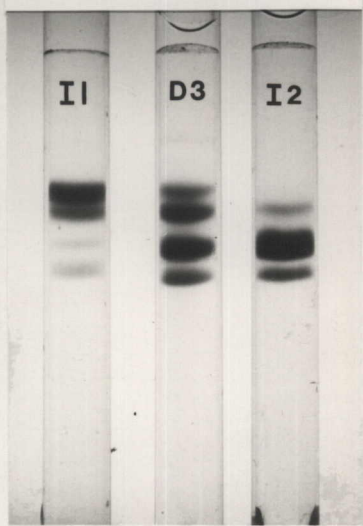


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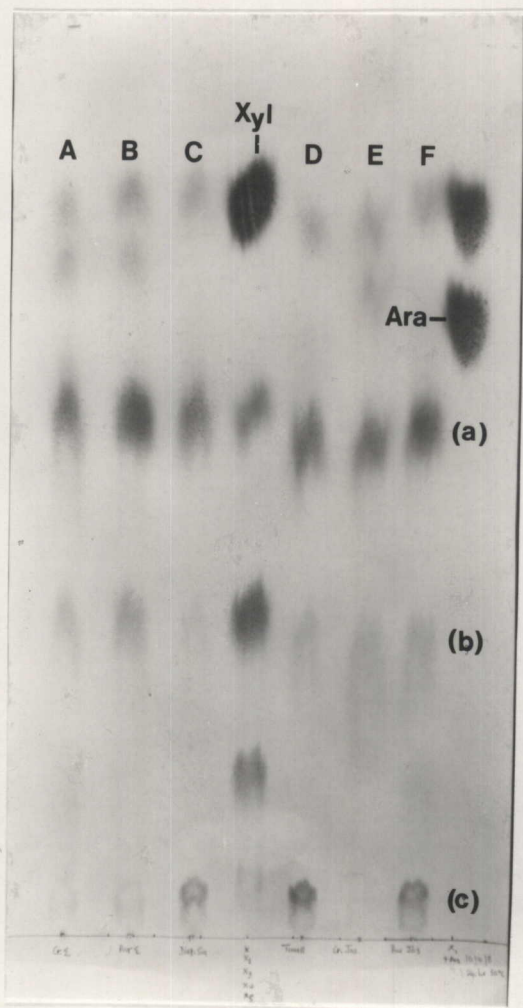




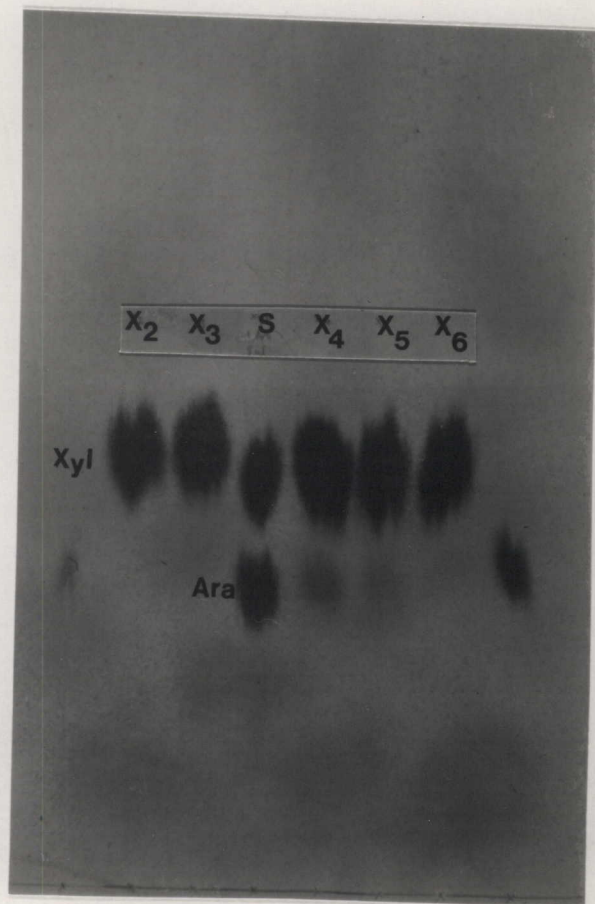
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