Studies on New Synthetic Substrates for Cellulolytic Enzymes

A Thesis submitted for the degree of DOCTOR OF PHILOSOPHY,

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by

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STATEMENT

This project was carried out in the Chemistry Department of the University of Glasgow, with the guidance of Professor B. Capon. There is no part being submitted concurrently for another degree.

October 1974 - September 1977

Signed

(John W. Thomson)

Acknowledgments

I thank Professor B. Capon for his guidance and encouragement throughout the course of this work.

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To Ena

CONTENTS

ABSTRACT

The introduction consists of a review of the occurrence of cellulose, of the organisms responsible for cellulose degradation and of the cellulolytic enzymes.

A new method has been developed for the large scale fractionation of peracetylated cello-oligosaccharides using a large silica column and a solvent system consisting of carbon tetrachlaride/chloroform in the ratio 70 : 30 with 1% methanol added.

The carbon-13 NMR spectra of the peracetylated cello-oligosaccharides were determined and are discussed in relation to the carbon-13 NMR spectrum of cellulose acetate.

3,4-Dinitrophenyl β-D-glucopyranoside and the 3,4-dinitrophenyl β-cello-oligosaccharides with D.P. from 2 to 4 were prepared. This involved coupling of the acetobromo-sugars with 3,4-dinitrophenol in the presence of anhydrous potassium carbonate and dry acetone. The resultant peracetylated aryl oligosaccharides were de-O-acetylated by the method of Zemplén.

Some modified 3,4-dinitrophenyl β-D-glucopyranosides were also prepared.

An affinity column specific for β-glucosidases was prepared by coupling 4-aminophenyl β-D-thioglucopyranoside with Affi-gel 10, a commercial affinity column support matrix with a 10Å spacer arm.

Two enzymes from a commercial cellulase from Trichoderma viride were purified. A β-1,4-glucan glucanohydrolase, designated cellulase EI, was obtained by ion exchange chromatography on DEAE-Sephadex A-25, passage through Sephadex G-75 (twice) and finally passage through the affinity column, and a cellobiase, designated cellulase EII, obtained by ion exchange chromatography on DEAE-Sephadex A-25, passage through Sephadex G-75 ard then Sephadex G-100.

Both enzymes behaved as single proteins as judged by SDS-gel electrophoresis and Sephadex G-75 and G-100 chromatography. The molecular weights of cellulase EI and cellulase EII were estimated to be 12,000 and 74,400 respectively.

The aryl cello-oligosaccharides were used as substrates for the cellulase enzymes. The values of k_{cat}/Km for cellulase EI-catalysed hydrolysis of 3,4-dinitrophenyl β-cellobioside, cellotrioside and cellotetraoside were determined to be 161, 334 and 423 $l.M^{-1}.s^{-1}$ respectively. These values are compared with the lysozyme-catalysed hydrolyses of 3,4-dinitrophenyl β-chitobioside, chitotrioside and chitotetraoside under similar conditions.

Induced hydrolyses of 3,4-dinitrophenyl β-D-glucopyranoside and p-nitrophenyl β-D-glucopyranoside were observed in the presence of cellotriose, cellotetraose or cellopentaose and cellulase EI. Modified 3,4-dinitrophenyl β-D-glucopyranosides were incubated with cellopentaose and cellulase EI and a rapid rate of induced hydrolysis was observed with the 6-deoxy-glucopyranoside and the xylopyranoside as well as the glucopyranoside. With 3,4-dinitrophenyl 6-O-methyl and 6-chloro-6-deoxy β-D-glucopyranosides a much reduced induced rate of hydrolysis was observed. With p-nitrophenyl 2-deoxy-β-D-glucopyranoside there was no induced hydrolysis. These results are discussed and compared with previously reported results for induced hydrolyses catalysed by lysozyme.

The reaction rates for cellulase EII-catalysed hydrolysis of 3,4-dinitrophenyl and p-nitrophenyl β-D-glucopyranosides were compared with the reaction rates for the corresponding modified glucopyranosides. Any changes to the glucose moiety resulted in a considerable loss of activity compared to the parent glucopyranoside. With p-nitrophenyl 2-deoxy-β-D-glucopyranoside no enzymic hydrolysis was observed.

These results are discussed and compared with the hydrolyses of other modified glucopyranosides by various β-glucosidases which have been previously reported.

The Michaelis-Menten constants for catalysed hydrolysis of 3,4-dinitrophenyl β-D-glucopyranoside were determined for cellulase EII. The value of Vmax was 1.44×10^{-8} M/l/s and the value of Km was 7.09×10^{-5} M/l. The value of k_{cat}/Km was 8830 $lM^{-1}s^{-1}$.

ABBREVIATIONS

The abbreviated nomenclature used throughout this thesis is as follows:

Name	Abbreviated Form
D-Glucose	G_1
Cellobiose	G_2
Cellotriose	G_3
Cellotetraose	G_4
Cellopentaose	G_5

The per-O-acetylated derivatives have the prefix Ac; e.g. AcG_2 is cellobiose octa-O-acetate.

Substituents at C-1 of the reducing sugar are also written as prefixes to the sugar;

e.g. $34DNPG_2$ is 3,4-dinitrophenyl β-cellobioside.

If the sugar has a substituent at C-1 and is also acetylated the Ac prefix comes first;

e.g. $Ac34DNPG_4$ is 3,4-dinitrophenyl trideca-O-acetyl-β-cellotetraoside.

The linkage between pyranose sugars is β unless otherwise stated.

INTRODUCTION

As an introduction to this thesis it is proposed to give a description of the environment encountered by cellulolytic organisms followed by an account of the organisms which live on cellulose based material. A review on the development of cellulolytic enzyme research will then be given.

Cellulose

Cellulose is the most abundant naturally occurring organic substance, being found as the main constituent of higher plants. Cellulose is present in wood, seed hairs, bast fibres, straw, stalks, marine plants and peat. Commercially, cellulose is used in the form of wood, cloth, rayon, film, plastics, lacquers, paper, Cellophane, rope and fillers. It was the destruction of these products by organisms that initiated a real surge in cellulase research.

Cellulose is found almost pure in cotton fibres (98% on a dry weight basis). Bast fibres such as flax (80-90% cellulose), ramie (ca 80%) and jute (60-70%) are also good sources of the polysaccharide. Wood only contains 40-50% cellulose but provides the most important commercial source of cellulose.

Plant cell walls contain three fundamental parts: primary wall, defined as the part of the wall produced during surface growth; secondary wall, produced after the surface growth has ceased; and between adjacent cells in the tissue there exists the middle lamella. The secondary wall displays three distinct regions designated S1, S2 and S3. These are shown in Figure 1. The outer layer, S1, is a thin transition layer. The middle layer, S2, forms the bulk of the secondary wall. The inner layer, S3, is also thin. The layers S1 to S3 are distinguished by the orientation of the cellulose polymer in each region. These orientations are represented by the shading of regions S1 to S3 in Figure 1.

Within each layer of the secondary wall, the cellulose and other cell wall constituents are aggregated into bundles called microfibrils. The microfibrils are distinct entities in that few cellulose molecules,

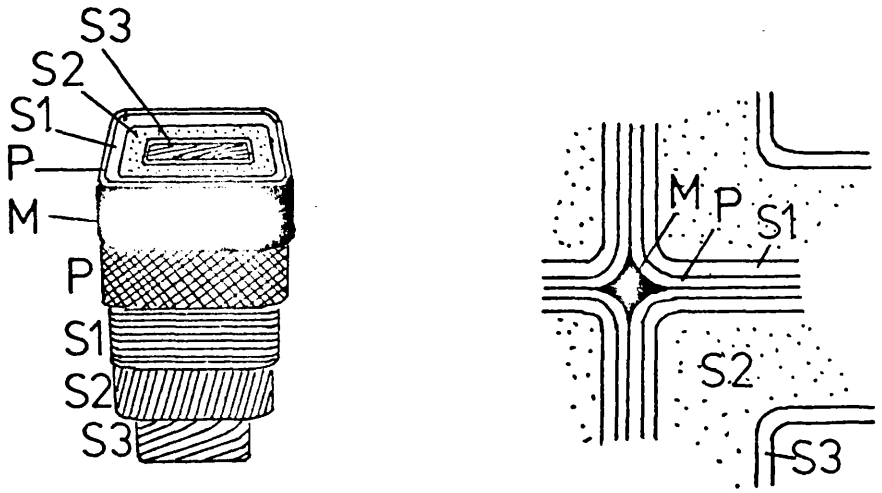


Figure 1

if any, ever cross over from one microfibril to another. These aggregates are shown diagrammatically in Figure 2. Within each microfibril, the linear molecules of cellulose are bound laterally by hydrogen bonds and Van der Waal forces into a linear, partially crytalline structure. As shown in Figure 2 the cellulose molecules are associated in various degrees of parallelism. Regions of a high degree of order are called crystallites or micelles; those in which the cellulose is more randomly oriented are called amorphous or paracrystalline regions.

Cellulose is a linear polymer of D-glucopyranose units linked by β-1,4-glycosidic bonds. The degree of polymerisation (DP) is believed to range from as low as 15 or less to as high as 7,000-10,000. A value of 15,000 for the degree of polymerisation was found for cellulose from unopened cotton balls (1). This corresponds to a molecular weight of 2.4×10^6 and a chain length of 7000nm. Figure 3 shows part of the cellulose molecule.

The highly ordered crystalline structure of cellulose permits the application of X-ray crystallography. Two main structural types of cellulose are found: cellulose I and cellulose II. Cellulose I is natural cellulose as found in cotton and wood. Cellulose II is

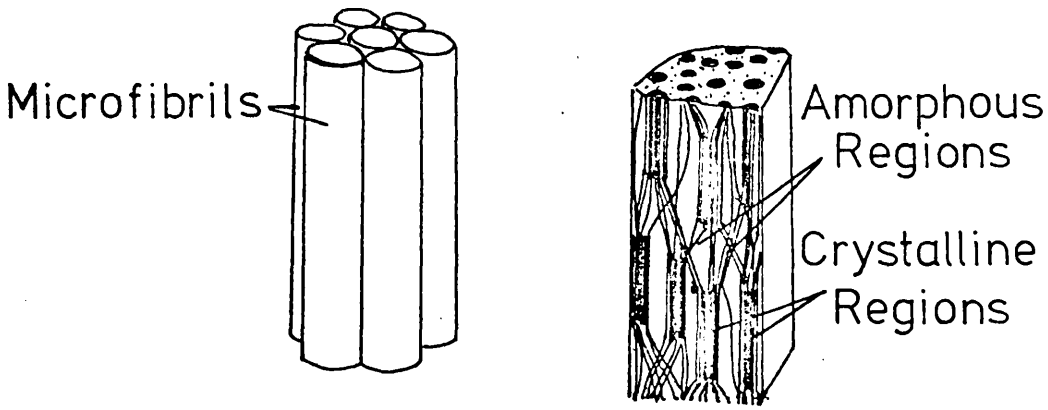


Figure 2

formed on recrystallisation of the polymer. Jones (2) and Aspinall (3) discuss more fully the crystal structure of these types of cellulose. A feature of both crystal types is the repeat distance of 10.3Å which corresponds to that of a cellobiose unit. This feature is also found in crystalline chitin, a polysaccharide with β-1,4 linked 2-acetamido-2-deoxy-D-glucopyranose residues.

Other constituents of wood are the hemicelluloses. These are relatively short polymers with DP of about 200. The units which

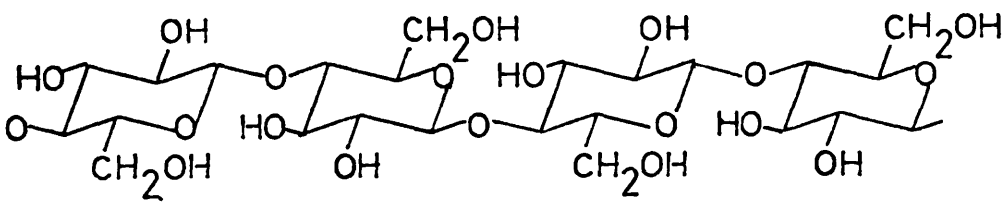


Figure 3

make up the polymer are usually monosaccharide units other than glucose. The more common sugars are xylose, galactose, mannose and arabinose as well as uronic acids of glucose and galactose. These monosaccharide units and derivatives are linked together by mainly

β-1,3, β-1,6 and β-1,4 glycoside bonds. A detailed survey of the chemistry of wood hemicelluloses has been made by Timell (4).

Lignin is another important constituent of cell walls. It is mostly found in the region of the lamella and primary wall. Lignin is a complex three-dimensional polymer formed from phenyl propane type units. Three molecules which are found in the degradation products of lignin are coniferyl alcohol, sinapic alcohol and p-cumaric alcohol. These are shown in Figure 4. It is thought that there are covalent bonds between the hemicelluloses and lignin.

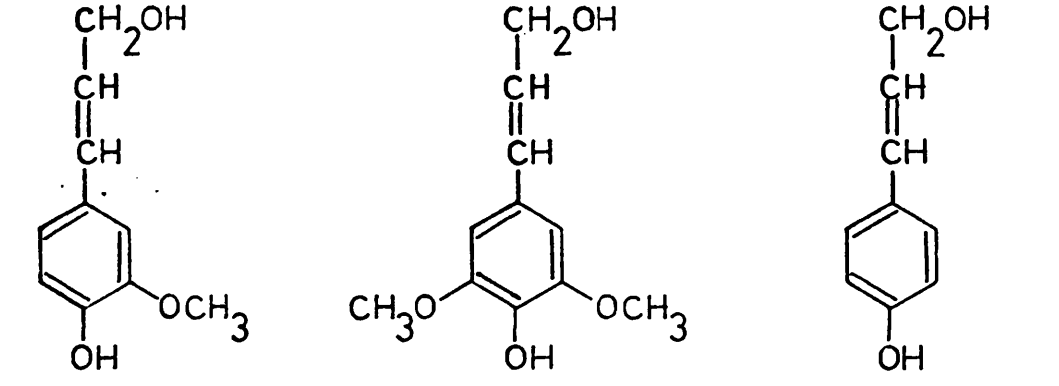


Figure 4

Other minor constituents are waxes, fats, essential oils, tannins, resins and fatty acids, terpenes, alkaloids, starch, gums, amino acids, proteins and nucleic acids.

Cellulolytic Organisms

It has been estimated that cellulose degradation returns 85 billion tonnes of carbon as carbon dioxide to the atmosphere each year. It has also been stressed that if degradation ceased while photosynthesis continued unabated, life as we know it would stagnate for lack of atmospheric carbon dioxide in under 20 years (5). In

this way degradation of cellulose is an indispensable process for the maintainance of the carbon balance in nature.

Most cellulolytic organisms are found among fungi and bacteria. Some protozoa are also cellulolytic.

Fungi

Accepting that the bulk source of cellulose is locked up in plants and wood, it is not surprising that many cellulolytic organisms live just on the surface or in the soil on dead or dying vegetation.

Fungi are by definition thallophyte plants that lack chlorophyll. Having no chlorophyll, they are unable to fix their own carbon and are therefore heterotrophic. Their main carbon source is the simple sugars which may serve as the only source for the majority of fungi. One exception is Leptomitus lacteus which grows on acetates and fatty acids..

Fungi can be saprophytic or parasitic. Saprophytic fungi either colonise dead plant and animal remains or they absorb organic materials which have exuded or leaked from living or dead organisms. Many parasitic fungi can also live as saprophytes either on the host which they have killed or on other dead organisms. Two interesting parasitic fungi are Dactylella drechsleri and Arthrobotrys dactyloides which trap nematode worms by holding the worms with sticky knobs and trapping them in constricting rings respectively. Saprophytic fungi are the most important cellulose degraders.

Fungal cell walls contain 80-90% polysaccharide with the remainder being protein and lipid. The three commonest building blocks of the former are D-glucose in glucans, N-acetyl glucosamine in chitin and D-mannose in mannans. The majority of fungi contain chitin and glucans in their walls. Chitin makes up 3-60% of dry

TABLE 1

Some Microbial Genera Capable of Utilising Cellulose

Fungi	
Alternaria	Polyporus
Aspergillus	Rhizoctonia
Chaetomium	Rhizopus
Coprinus	Trametes
Fomes	Trichoderma
Fusarium	Trichothecium
Myrothecium	Verticillium
Penicillium	Zygorhynchus
Bacteria	
Achromobacter	Clostridium
Angiococcus	Cytophaga
Bacillus	Polyangium
Cellfalcicula	Pseudomonas
Cellulomonas	Sorangium
Cellvibrio	Sporocytophaga
Actinomycetes	
Micromonospora	Streptomyces
Nocardia	Streptosporangium
Protozoa	
Hartmanella	Schizopyremus

weight of the walls. The glucans are non-cellulosic and contain in the main β-1,3 and β-1,6 linked glucose units. They are thus not degraded by cellulases. Mannans, associated with glucans, are characteristic of yeast cell walls.

Since cellulose is a large insoluble molecule it cannot be utilised as a carbon source until it has been broken down. Digestive enzymes synthesised inside the cells of the organism are released from the cells and hydrolyse the cellulose. The simple sugar products are then absorbed by the cells. Such digestion outside of the cells is called extracellular digestion.

Table 1 lists some of the genera of fungi capable of utilising cellulose.

Bacteria

Most bacteria are heterotrophic, being either saprophytes or parasites. Bacterial cell walls are composed of amino acids and amino sugars (and their derivatives, particularly muramic acid).

Bacteria are outstanding cellulose decomposers in more or less anaerobic, closed environments, as in intestines of herbivores, in the rumen of cattle and in the digestive juices of invertebrates.

Herbivores, such as the horse, have a large caecum at the posterior end of the digestive tract which contain large numbers of bacteria and protozoa capable of cellulose degradation.

Ruminants, like the cow, have a more efficient system utilising microbial digestion. Vast numbers of bacteria and protozoa live in the first and second chambers of the stomach, the rumen and the reticulum, where microbial fermentation takes place. Slowly the products of microbial action and the microbes themselves move on into the true stomach and intestine, where more usual types of digestion and absorption takes place. Since microbial digestion is in the

anterior portion of the digestive tract rather than in a posterior caecum, ruminants derive maximal benefit from the microbial action.

Many classes of molluscs digest cellulose probably by enzymes both from the gut of the mollusc and from microbes present in the gut. The snail Helix pomatia and some genera of gastropods and bivalves produce their own cellulases.

A variety of insects, notably the termites, feed on wood which they could not use were it not for intestinal microbes that can ferment the cellulose. A few species of wood eating beetles do, however, secrete cellulase enzymes and such beetles do not have to rely on intestinal microbes.

Protozoa

These are simple organisms which are mostly heterotrophic although some possess chlorophyll. A genus, Calonympha, found in the gut of termites participates in the digestion of cellulose. Many are found in conjunction with bacteria in the digestive tracts of mammals.

Several protozoa in pure culture are capable of cellulose breakdown, for example, species of Hartmanella and Schizopyrenus.

References 6, 7, 8, 9 and 10 are useful for an introduction to cellulolytic micro-organisms.

Cellulolytic Enzymes

In 1912, Pringsheim (11) performed one of the first experiments to determine the nature of the enzymes responsible for the degradation of cellulose. Using cellulolytic bacteria he showed that the organism degraded cellulose to glucose at ambient temperatures. When the incubation was carried out at 67°C he found that the end product of the degradation was cellobiose. This was explained by

the presence of two enzymes with distinct properties. Cellulose was degraded to cellobiose by an enzyme which was still active at 67°C and a 'cellobiase' whose activity was lost at 67°C, which hydrolysed cellobiose to glucose. This was also the first time that the degradation of cellulose was thought of as multi-enzymic.

The next important experiments to be undertaken were those of Grassmann et al (12,13,14) in 1931-1933. They took a commercial cellulase from Aspergillus oryzae and fractionated it on a 'clay' column into a 'polysaccharase' (cellulase) and an 'oligosaccharase' (cellobiose). The cellulase was active towards cellodextrin and cellohexaose but showed very little activity towards cellotetraose and even less towards cellotriose. No hydrolysis was observed with cellobiose as substrate.

Comparing the concentration of the cello-oligosaccharides with the initial activity of the cellobiase there is very little difference in initial rates of hydrolysis of glycosidic bonds. These results are an extension of Pringsheim's 21 years earlier.

Very little work was done on cellulolytic enzymes for the next 17 years. A β-1,4 glucanhydrolase was isolated from Aspergillus niger by several workers (15,16,17,18). The first cell-free filtrates of the fungus Myrothecium verrucaria which showed cellulolytic activity were obtained by Saunders, Siu and Genest (19).

In 1950, Reese, Siu and Levinson (20) published their now classic paper on 'The Biological Degradation of Soluble Cellulose Derivatives and its Relationship to the Mechanism of Cellulose Hydrolysis'. Their results led them to the assumption that the degradation of native cellulose, that is, the crystalline micelles found in nature, to glucose consists of at least two systems.

Their first step, designated as C_1, occurs preliminary to hydrolysis of the straight chain by Cx enzymes. Cellobiase enzymes were not considered important in the cell-free filtrates. The nature of enzyme component C_1 was unspecified. Their diagram summarises their postulates.

Native cellulose $\xrightarrow{C_1}$ shorter linear polyanhydroglucose chains $\xrightarrow{C_x}$ soluble, small molecules, capable of diffusion into the cell

(shorter linear polyanhydroglucose chains → soluble, small molecules, capable of diffusion into the cell: Noncellulolytic organisms)

(Native cellulose → soluble, small molecules, capable of diffusion into the cell: Cellulolytic organisms)

They also noted that cellulolytic enzymes are produced by the organism in response to the presence of a β-1,4 glucosidic linkage.

This report by Reese then set the pattern of cellulase research for the next few years. The question asked at that time was "Are there several cellulases, or is there only one type?". It is strange that this question was asked since the work of Pringsheim and Grassmann had shown there to be at least two enzymes involved in the degradation of cellulose.

Research workers then set out to isolate the cellulase fractions from the extracellular medium of cellulolytic organisms. In keeping with new methods of enzyme separation, new methods of determining the activities of the cellulase fractions were introduced.

In 1950, Reese (20,21) used soluble sodium carboxymethyl cellulose to determine Cx activity by the decrease in viscosity of the substrate as well as the increase in reducing power of the sugar solution. They found that for Cx enzymes a decrease in viscosity before the increase in reducing sugar was evident.

Another method developed for the determination of Cx activity was the action of the enzyme on cotton swollen by treatment with

phosphoric acid. Walseth (22) determined the decrease in the degree of polymerisation of the cotton cellulose whereas the formation of reducing groups from the enzymic hydrolysis was the method used by Gilligan and Reese (23), Myers and Northcote (24) and Whitaker (25).

Various methods were employed to determine C_1 activity proposed by Reese. Native cellulose in the form of cotton was used as substrate. Morphological changes were determined by microscopic observations (26), electron microscopic observations (27) and by measuring the tensile strength and alkali-swelling of the cotton (23). The weight decrease (19,28) and also the amount of reducing sugar produced (29) from dewaxed cotton were used to determine C_1 activity.

All the methods described give an overall indication of activity but give no specific information on which bonds are being hydrolysed.

Whitaker was one of the first workers in the field to determine the substrate specificity of a cellulase enzyme. Grassmann used β-1,4 oligoglucosides as enzyme substrates in 1933. It was 1954 before Whitaker (30) used them again with a purified cellulase from Myrothecium verrucaria. These types of substrates were extended by use of methyl β -1,4 oligoglucosides (31). A cellodextrin of average DP 24 was used as substrate by Whitaker (32). On hydrolysis by the enzyme the formation of reducing groups as well as the ratio of oligosaccharides produced was noted.

Spectrophotometric substrates were very rarely used to determine activity. Aryl glucosides were common for determination of glucosidase activity. As early as 1952 Hisizawa and Wakabayashi (33) had prepared p-nitrophenyl β-cellobioside for use in determining the mode of action of a cellulase from Irpex lacteus. p-Nitrophenyl and methyl β-cellotetraoside were also used as substrates for the

cellulase but the major hydrolytic process was the splitting of the central glycoside bond to give cellobiose and the aryl cellobioside (34).

In the early 1950's two of the major contributors to the field were Reese and Whitaker. Reese's approach was to look at the enzyme systems from various cellulolytic organisms. Whitaker, on the other hand, concentrated on one system. He closely examined the nature and properties of a cellulase from Myrothecium verrucaria.

According to Reese a true cellulolytic enzyme system is one in which native cellulose is degraded to glucose. This invoked the presence of the C_1 component since some organisms could only utilize modified cellulose. Between 1950 and 1954 Reese and his associates presented a series of papers on cellulolytic systems (20,21,35,36, 37,38,39,23). They used a variety of organisms as a source of cellulase enzymes. Some fungal species were Aspergillus luchuensis, Aspergillus terreus, Fusarium roseum, Myrothecium verrucaria and Trichoderma viride. Some bacterial species were Cellvibrio vulgaris and Sporocytophaga myxococcoides. They found that the cellulases from different organisms differed in their relative activities towards CMC and towards cotton (36). When the crude enzyme filtrates were separated by paper chromatography, several Cx components were frequently found (38). Other media used by Reese to fractionate cellulolytic components were cellulose columns and calcium phosphate gel columns. They found that the calcium phosphate gel columns gave the best separation.

Extracellular enzymes from Trichoderma viride were fractionated on a calcium phosphate gel column to give 3 main peaks labelled A,, B_1 and B_2, and CD which differed from each other in their rate of movement on the column, in their relative activities on certain

cellulosic substrates, in their mode of action, and in their behaviour in the presence of cellobiose. In addition there was a marked synergic effect shown when fractions were recombined.

Fractionation of the filtrate from Myrothecium verrucaria produced at least 6 peaks with cellulolytic activity.

Other workers were also examining the nature of the cellulase system. Walseth (22) was looking at the occurrence of cellulases in enzyme preparations from micro-organisms and the influence of fine structure of cellulose on the action of cellulases. Jermyn (40) in his work on fungal cellulases noted the complexity of enzymes from Aspergillus oryzae that split β-glucosidic linkages and their partial separation. Kooiman et al (41) observed the presence of higher oligomers in the degradation of cellulose by a cellulase from Myrothecium verrucaria and the presence of a cellobiase was also noted. Whistler and Smart (42) removed the cellobiase from a commercial cellulase preparation and as a result, cellobiose was the main product of hydrolysis by the purified cellulase preparation (cf. Pringsheim, 1912). Hash and King (43) also demonstrated an oligosaccharide intermediate in the enzymic hydrolysis products of cellulose.

The work of Reese and others brought Reese in 1955 to the conclusion that the cellulase system was very complex indeed (44). He still sought a C_1 component to modify native cellulose to linear cellulose chains. There was no limit to the number of Cx components which might be present to hydrolyse these linear chains primarily to cellobiose and similarly a variety of cellobiases or β-glucosidases might be present to hydrolyse cellobiose to glucose.

As stated earlier Whitaker studied the cellulase from Myrothecium verrucaria in great detail. His first concentrated

effort on the cellulase was in the period 1953-1957. The reports of the work are contained in references 25,45,30,46,47,48,32,49. He concluded that the degradation of native cellulose to glucose was performed by a single enzyme. This enzyme was of molecular weight 63,000 and was cigar shaped, measuring 200Å by 33Å. The hydrolytic action on cellulose was random. The cellulase's action on cello-oligosaccharides showed that the rate of hydrolysis increased with increasing degree of polymerisation up to a maximum of DP 5 or 6. The hydrolysis products as shown by their optical rotation are released with retention of configuration.

Since the results of Reese and Whitaker appeared contradictory each have given possible explanation as to the discrepancy of the cellulolytic system of Myrothecium verrucaria.

Reese (23) said "Several explanations are possible. First, the conditions under which the organism is grown determine the relative amounts of various components found in the medium. ..... Perhaps Whitaker's growth conditions lead to a relative enrichment in one component.. Second, diverse methods for measuring activity are necessary to detect the different components. Whitaker used two substrates (solid celluloses) that may have been too much alike to detect the differences that we have observed. ..... Third, enzymatic activity is a more sensitive and reliable determinant of homogeneity than are physicochemical methods (used by Whitaker)." Today electrophoretic and ultracentrifugal data are taken as strong indications of homogeneity.

Whitaker (50) suggested 5 reasons as to why variations in properties could orginate. He said " 1) By the formation of different types of cellulases. 2) By the formation of enzymes which differ only in a few fine details of primary structure. In this

case, the differences in amino acid sequence would have to give substantial differences in net charge or shape. 3) By exposure to conditions which produce enzymes differing in their secondary and tertiary structure. ..... 4) By the formation of stable complexes with other metabolic products secreted into the culture medium. ..... 5) By proteolysis without loss in activity. ....."

Kooiman (51) could find no direct evidence for a C_1 component in a cellulolytic system. Shortly after this Reese (52) modified his C_1 - Cx theory to a 'multiple Cx theory'. This statement appears to have gone unnoticed since subsequent workers still referred to a C_1 - Cx system..

By now it was clear that there was more than one enzyme responsible for the degradation of cellulose. It was also evident that for any meaningful results to be obtained the cellulases would have to be pure. Any contamination by a β-glucosidase would give a completely false picture of the mode of action of a cellulase.

A purified cellulase from Irpex lacteus was obtained in crystalline form by Nisizawa (53). No further increase in activity could be obtained on subsequent recrystallisations and the cellulase was considered to be pure. The preparation was active towards CMC and with p-nitrophenyl β-cellobioside preferentially hydrolysed the aryl glycosidic bond.

Toyama (54) obtained a crystalline cellulase from Trichoderma koningii which did not hydrolyse cellobiose.

Further separation and purification of cellulases from cellulolytic organisms were carried out with similar results to those already discovered. A purified cellulase from Poria vaillantii (a wood rotting fungus) did not hydrolyse cellobiose and gave only cellobiose as product on hydrolysis of cellulose (55,56).

It was apparent at the time that many enzymes had names which only gave a partial description of their potential as catalysts. Cellulases were no exception. Some workers had noticed that purified cellulase preparations were able to hydrolyse β-1,4 linked D-xylose residues. This property was observed by Grassmann (14), Bishop and Whitaker (57), Thomas (58), Kooiman (51) and Sörensen(59). It had been observed by Myers and Northcote (24) that a cellulase from the snail Helix pomatia did not hydrolyse β-1,4 xylan. It must be pertinent to ask if some cellulases hydrolyse xylan or if some xylanases hydrolyse cellulose?

It was noted that there was a distinction between an aryl β-glucosidase and a cellobiase. Hash and King (60) obtained an aryl β-glucosidase from cultures of Myrothecium verrucaria. The enzyme was not a cellobiase. A similar enzyme from the mycelium of Strachybotrys atra was obtained by Youatt (61).

At this time the terms endo- and exo-cellulases began to be used. These terms arose as a result of the action of various purified cellulases on carboxymethyl cellulose. Rapid decrease in viscosity associated with little increase in the amount of reducing power is indicative of random cleavage of the polymer, which in turn indicates hydrolysis by endo-cellulases. Increase in reducing power associated with little decrease in viscosity indicates hydrolysis of residues from the end of the polymer chain. This hydrolysis is a result of exo-cellulases.

Almost all the known techniques for enzyme purification up to 1963 had been employed in the case of cellulolytic enzymes. However, gel filtration with Sephadex preparations of different types offered a means of separating enzymes with little risk of deactivation and was first used for cellulolytic enzymes by Pettersson et al. (62)

and Pettersson and Porath (63). They fractionated cellulases from the fungus Polyporus versicolor. On assaying the fractions towards CMC and p-nitrophenyl β-D-glucopyranoside they found that the β-glucosidase activity was associated with the high molecular weight component and the CMC-ase activity with the lower molecular weight components. A similar result was found with cellulases from Aspergillus niger fractionated on Sephadex G-100 (64).

These new techniques of gel filtration and also ion-exchange chromatography sparked off a new wave of cellulolytic research.

In 1963 Li and King (65) fractionated industrial concentrates from Aspergillus niger culture filtrates. The filtrate was fractionated on Sephadex G-25, DEAE-Sephadex A-25 and alkali-swollen column chromatography to give 8 highly purified fractions with distinctive properties.

At this time Whitaker was renewing an interest in the cellulase from Myrothecium verrucaria. A new purification procedure gave an enzyme with a molecular weight of 49,000 (67). This cellulase was characterised by its activity towards methyl β-cello-oligosaccharides (31). His results are summarised in the following table.

Substrate	Enzyme conc. ($\times 10^{-8}$M)	Vmax ($\times 10^{-6}$M/l/min)	Km ($\times 10^{-4}$M)
Methyl cellobioside	68	< 1.1	2.5 - 5.0
Methyl cellotrioside	68	11±0.9	8.5±1.3
Methyl cellotetraoside	6.8	39.2±2.2	4.1±0.5
Methyl cellopentaoside	1.9	> 30	< 3

As can be seen Km decreases and Vmax increases as the DP of the substrate is increased from 3 to 5. The initial hydrolysis products of methyl β-cellopentaoside indicated that the interior but not the terminal linkages were hydrolysed by the enzyme.

The postulate of Reese could not be put to the test at this time. Although cellulolytic organisms such as Myrothecium verrucaria and Stachybotrys atra digested native cellulose with ease, it was found that cell-free culture filtrates showed high activity only towards soluble, swollen and partially degraded celluloses (68,69) which were known to be hydrolysed by Reese's Cx enzymes. A hopeful sign came from the discovery that filtrates from Myrothecium verrucaria, suitably manipulated, could produce 30% solubilisation of cotton (70). However, the discovery in 1964-1966 that culture filtrates from certain strains of Trichoderma viride and Trichoderma koningii (71,72,73,74,75,76,77) were capable of extensive hydrolysis of native cellulose made available to workers in the field the full range of enzymes in Reese's postulated C_1 - Cx system.

Halliwell (76,77) obtained complete solubilisation of cotton fibres with quantitative conversion to glucose within 19 days by a culture filtrate from Trichoderma koningii. Mandels and Reese (71) found similar results with cell-free preparations from Trichoderma viride. Reese had noted the exceptional ability of Trichoderma viride to degrade cellulose as early as 1954 (23). After repeated chromatograms on DEAE-dextrans, Mandels and Reese (71) succeeded in separating components having C_1, Cx and β-glucosidase activity. The β-glucosidase appeared very early and completely separated from C_1 and Cx. They assumed C_1 "to act in a way to permit an increased moisture uptake, hydrating the cellulose and pushing apart the closely packed chains," to make the linkages accessible for the action of the hydrolytic β-1,4-endo-glucanase.

Two of the most important papers which have directed cellulase research from the mid 1960's to the present day were those of Li, Flora and King (73) and Selby and Maitland (78,79).

Until this time, in no instance had it been possible to start with a crude cellulase system capable of complete hydrolysis of crystalline cellulose, separate the several components, establish their individual modes of action, and recombine the purified components so as to reproduce the activity of the original crude material. Only in this way, could no vital member of the complete enzyme system have gone unnoticed.

This experiment was first performed by Li, Flora and King (73). The starting enzyme was obtained from wheat bran-sawdust cultures of Trichoderma viride. The crude enzyme was passed through an Avicel column to separate the C_1 component from the Cx and β-glucosidase components. The details of the purification of the C_1 component was not included in the publication. King, (80) however, gave an approximate molecular weight of 60,000 for the C_1 component. The primary product (97%) of the degradation of native cellulose by the C_1 component was cellobiose. The Cx component and the β-glucosidase were fractionated on an alkali swollen cellulose column. These components were further purified to give an endo-cellulase, molecular weight 52,000 and an exo-cellulase, molecular weight 76,000.

The following table shows the percentage activity of cellulose hydrolysis by purified components alone and in combination over the crude.

Enzyme	Percentage activity of crude
C_1	86
endo-Cellulase	6
exo-Cellulase	0
C_1 + endo-Cellulase	91
C_1 + exo-Cellulase	105
C_1 + endo-Cellulase + exo -Cellulase	102

Selby and Maitland (78,79) performed a similar experiment on culture filtrates from Trichoderma viride. They found that the components essential for attack on cotton were a carboxymethyl cellulase, a cellobiase and a third (C_1) component which, unlike the C_1 componemt of Li, Flora and King, had no action on CMC, cellobiose or cotton. These three components, which together could completely convert cotton into water-soluble products, lost this ability when separated and regained it quantitatively when recombined in their original proportions. The following table shows the activities of the components of Trichoderma viride cellulase alone and in combinations.

	Recovery of activity (%) towards		
Component	Cotton	CMC	Cellobiose
C_1	1.2	0	0
CMC-ase	1	96	0
Cellobiase	1	0	96
CMC-ase + cellobiase	2	96	96
C_1 + CMC-ase	35	96	0
C_1 + cellobiase	20	0	96
C_1 + CMCase + cellobiase	101	96	96

The C_1 component was a glycoprotein with carbohydrate:protein in the approximate ratio 1:1. Its molecular weight was approximately 61,000. It appears that both sets of workers isolated the same component and that the difference in behaviour towards native cellulose can be explained by the synergic effect of C_1 and Cx components. According to Selby and Maitland the C_1 component, when highly purified, is no longer able to solubilise cotton and that the ability of the C_1 component of Li, Flora and King to solubilise

native cellulose is due to a Cx impurity.

The work of Selby and Maitland has been criticised by Wood (81) in that their activity towards CMC is a viscometric assay which would not detect the presence of an exo-cellulase component. Where Selby and Maitland failed in their characterisation of the C_1 component was in the limited number of substrates used. Wood and McCrae (81) and Wood (82) isolated a C_1 component from culture filtrates of Trichoderma koningii. The C_1 enzyme showed little activity towards highly ordered forms of cellulose. It could still produce reducing sugars from a solution of CMC (to a very limited extent), but which could not decrease the viscosity of the CMC solution. However, the C_1 component readily degraded phosphoric acid-swollen cellulose, the principal product (97%) being cellobiose. Cellotetraose and cellohexaose were hydrolysed almost exclusively to cellobiose. Cellotriose and cellopentaose were hydrolysed to cellobiose and glucose. On this evidence Wood called the C_1 component a β-1,4-glucan cellobiohydrolase. The synergic effect of components from Trichoderma koningii was evident as can be seen from the following table.

Enzyme	Percentage recovery of cellulase activity
C_1	4
Cx + β-glucosidase	3
C_1 + Cx + β-glucosidase	96
Original culture filtrate	100

Halliwell and Griffin (83) also isolated a C_1 component from cultures of Trichoderma koningii. The enzyme was shown to act as a β-1,4-glucan cellobiohydrolase on both simple and complex forms of native cellulose.

Wood has shown that some strains of Fusarium solani, when grown under certain conditions, produced culture filtrates that could degrade cotton with the same facility shown by filtrates of Trichoderma viride and Trichoderma koningii. Separation of the culture filtrates produced 3 components essential for the degradation of cellulose (84). These were identified as a C_1 component, a Cx component and a β-glucosidase. The relative cellulase activities of the C_1, Cx and β-glucosidase components of Fusarium solani, alone and in combination is shown in the table below.

Enzyme	Percentage recovery of cellulase activity
C_1	3
Cx	6
β-glucosidase	1
C_1 + Cx	50
C_1 + β-glucosidase	13
Cx + β-glucosidase	14
C_1 + Cx + β-glucosidase	81
Original culture filtrate	100

Selby (85) obtained a mutant strain of Penicillium funiculosum which was found to produce a powerful cellulase, and this has also been resolved into a C_1 component, a CMCase and a cellobiase. The C_1 component had low activity towards cellulose but was increased more than 20-fold on recombination with the Cx and the cellobiase.

Other species which produce a true cellulase system in culture filtrates are Irpex lacteus (86,87), Fusarium moniliforme (88), Pseudomones fluorescens (89), Sporotrichum pulverulentum (90), Cellvibrio gilvus (91) and Geotrichum candidum (92). Some of these cellulase components have been purified and investigated extensively.

Trichoderma viride has been the most extensively investigated cellulolytic organism and since this present work involves the fractionation of enzymes from a commercial cellulase from Trichoderma viride a list of the enzymes isolated from this organism with some details of their physical and enzymic properties is given on pages 26 to 38. Pages 39 to 42 give some details of cellulolytic enzymes isolated from other species in recent years. Other species which have been shown to contain cellulolytic enzymes include Chryosporium lignorum (111,112), species of Flavobacterium (113), Fomes annosus (111), the tomato plant Lycpersicum esculentum (114,115), Penicillium notatum (116), germinating kidney beans, Phaseolus vulgaris (117,118), the termite Trinervitermes trinervoides (119) and ungerminated seeds of rye (120).

Although the enzymes which are responsible for the degradation of native cellulose to glucose are well characterised, the exact pathway of hydrolysis of cellulose is still not clear. Several workers have proposed mechanisms for the degradation of native cellulose.

Selby (121) said 'It is reasonable to assume that the regular array of molecular chains will be disturbed at intervals by the occurrence of chain ends, as shown diagrammatically in Figure 5.

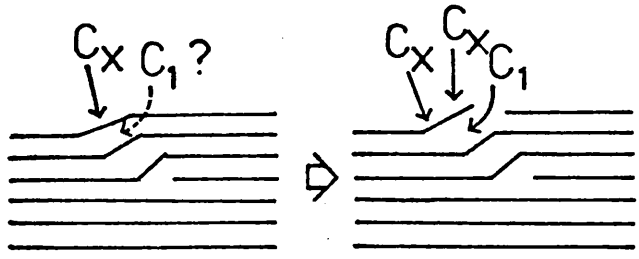


Figure 5

The accompanying disturbance in hydrogen bonding between chains in the vicinity of the chain end may be insufficient to enable Cx, acting alone, to split off soluble sugars, but when both components

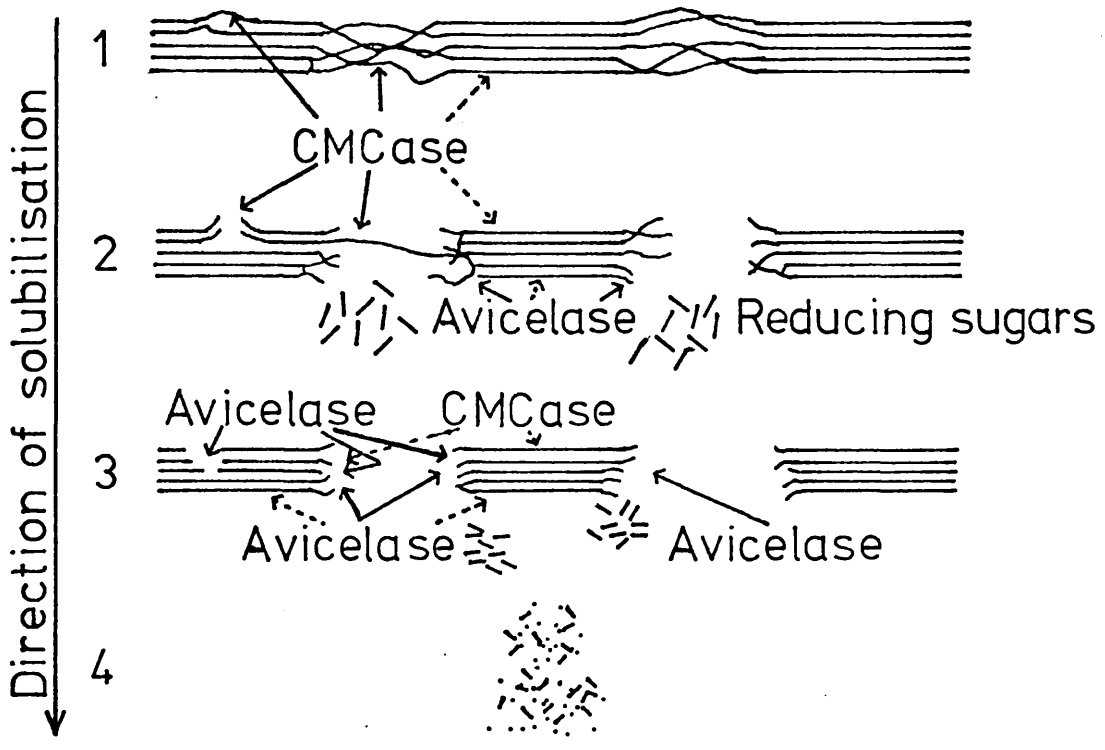


Figure 6

are present, a single bond-rupture by Cx might allow the hydrogen bonding to be further disturbed by C_1 with consequent loosening of a short length of surface chain, which might then be susceptible to more extensive attack by Cx.'.

Nisizawa (101) said 'In view of the substrate specificities of Avicelase and CMCase, their synergistic action for the solubilisation of cotton fibre may be explained by the illustrations shown in Figure 6. As is clear from the figure, it appears that Avicelase and CMCase may correspond to C_1 and Cx, which initiates the degradation of native cellulose, then C_1 can saccharify actively the cellulose fragments produced by Cx.'.

Today there is still no clear ratification as to the mechanism for the degradation of native cellulose. In a recent paper Wood (110) said 'The hypothesis that the attack on highly ordered cellulose is initiated by a chain-separating enzyme is attractive, but difficult to prove or disprove. It has some support from the observation that

the cellulose substrates that are refractory to enzymes classified as Cx are readily attacked after they have been rendered more accessible by ball-milling, swelling, or reprecipitation from solvents. As C_1 enzymes are not required for the hydrolysis of these highly hydrated substrates, the argument that these treatments have simulated C_1-action clearly has some appeal. However, until a chain-disaggregating, prehydrolytic factor has been isolated, this hypothesis must give way, for the present, to the more plausible argument that hydrolysis of "crystalline" cellulose must be described in terms of endo- and exo-glucanase activities.'.

Whatever is discovered by future work on the mechanism of the degradation of cellulose, the statement by Li, Flora and King (73) that 'The system as a whole is therefore not merely multienzymic by happenstance, but necessarily so for both maximum rate of attack on native cellulose and for maximum completeness of conversion to glucose.' will always be relevant.

Species: Trichoderma viride Source: Onozuka SS

Enzyme: β-1,4-glucan cellobiohydrolase EC 3.2.1.74

Isolated by Pettersson et al. (93,94).

Physical properties: M.W. 42,000. Carbohydrate content: 9.2%.

Amino acid composition:

Tryptophan	6
Lycine	10
Histidine	4
Arginine	6
Aspartic acid + asparagine	44
Threonine	39
Serine	41
Glutamic acid + glutamine	32
Proline	21
Glycine	45
Alanine	22
Half-cystine	16
Valine	17
Methionine	4
Isoleucine	8
Leucine	21
Tyrosine	19
Phenylalanine	9
Total	364

Iso-electric point: pH 3.79 (10°C).

Enzymic properties: Crystalline cellulose (Avicel), phosphoric acid-swollen Avicel and cellotetraose were degraded by the enzyme and in each case the principal product was cellobiose. The enzyme was capable of about 80% degradation of microcrystalline cellulose within 72 hours provided the products (mainly cellobiose) were

continuously removed. No activity was found towards carboxymethyl cellulose, nor was there any β-glucosidase activity. Optimum pH: 4.8. Enzyme completely denatured after 3-minute incubation at 78°C.

Species: Trichoderma viride Source: Meicelase P.

Enzyme: β-1,4-glucan cellobiohydrolase

Isolated by Gum Jr. and Brown Jr. (95).

Physical properties: M.W. 48,400. Carbohydrate content: 11.3%.

Amino acid composition:

Tryptophan	13
Lycine	10
Histidine	4
Arginine	6
Aspartic acid + asparagine	47
Threonine	42
Serine	43
Glutamic acid + glutamine	27
Proline	21
Glycine	46
Alanine	26
Half-cystine	16
Valine	18
Methionine	2
Isoleucine	7
Leucine	21
Tyrosine	17
Phenylalanine	11
Total	377

Enzymic properties: The enzyme degraded both crystalline cellulose and phosphoric acid-swollen cellulose to cellobiose.

Species: Trichoderma viride Source: Comm. Mycol. Inst., Kew, Surrey. Strain 92 027.

Enzyme: 'C_1' component.

Isolated by Selby and Maitland (78,79).

Physical properties: M.W. 61,000. Carbohydrate content: 50%.

Enzymic properties: The enzyme had no activity towards cotton, CMC or cellobiose. The enzyme was essential for the degradation of native cellulose in conjunction with either Cx or β-glucosidase components.

Species: Trichoderma viride Source: Wheat bran-sawdust cultures as described by Toyama (104).

Enzyme: 'C_1' component.

Isolated by Li, Flora and King (73).

Physical properties: M.W. 60,000.

Enzymic properties: The enzyme was active towards native cellulose, the main product being cellobiose.

Species: Trichoderma viride Source: Onozuka.

Enzyme: 'Avicelase'

Isolated by Nisizawa et al. (101).

Physical properties: M.W. 53,000. Iso-electric point: pH 4.2.

Enzymic properties: The enzyme was active towards crystalline cellulose giving cellobiose as sole product. The enzyme also had some activity towards CMC, the ratio of Avicelase to CMCase activity was 18:1.

Species: Trichoderma viride Source: Meicelase.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Okada (96,97).

Physical properties: M.W. 30,000. Carbohydrate content: 12-14%.

Enzymic properties: The enzyme hydrolysed cellulolytic substrates in the order CMC>filter paper>Avicel>cotton. The Michaelis-Menten constants with cello-oligosaccharides as substrate are shown below.

	Substrate			
	G_3	G_4	G_5	G_6
Km ($\times 10^4$M)	-	0.4	1.5	3.3
Vmax (units)	-	3.3	10.0	31.8

On hydrolysis the β-glycosidic linkage of the substrate was retained. On incubation with p-nitropheryl β-cellobioside or with cellobiose transglycosylation products were observed. Optimum pH: 4.5-5.0. Enzyme retained 27% of its activity after heating at 100°C for 10 minutes.

Species: Trichoderma viride Source: Meicelase.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Okada (96,97).

Physical properties: M.W. 43,000. Carbohydrate content: 12-14%.

Enzymic properties: The enzyme hydrolysed cellulolytic substrates in the order CMC>filter paper>Avicel>cotton. The Michaelis-Menten constants with cello-oligosaccharides as substrate are shown below.

	Substrate			
	G_3	G_4	G_5	G_6
Km ($\times 10^4$M)	16.3	4.2	2.8	4.6
Vmax (units)	5.4	9.3	11.3	22.9

On hydrolysis the β-glycoside linkage of the substrate was retained. On incubation with p-nitrophenyl β-cellobioside or with cellobiose transglycosylation products were observed. Optimum pH: 4.5-5.0. Enzyme retained 41% of its activity after heating at 100°C for 10 minutes.

Species: Trichoderma viride Source: Meicelase.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Okada (98).

Physical properties: M.W. 45,000.

Enzymic properties: The enzyme hydrolysed CMC in a less random random fashion than the two previous endo-cellulases isolated by Okada. The Michaelis-Menten constants with cello-oligosaccharides as substrate are shown below.

	Substrate			
	G_3	G_4	G_5	G_6
Km ($\times 10^4$M)	29.4	6.7	5.6	4.5
Vmax (units)	8.6	14.8	44.3	55.3

On hydrolysis the β-glycosidic linkage of the substrate was retained. The enzyme preferentially attacked the aglycone linkage of p-nitrophenyl β-cellobioside. It also catalysed the rapid synthesis of cellotetraose from cellobiose. Optimum pH: 4.5-5.0. Optimum temperature: 50°C. Enzyme retained 40% of its original activity after heating at 100°C for 10 minutes.

Species: Trichoderma viride Source: Onozuka SS

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Pettersson et al. (99).

Physical properties: M.W. 12,500. Carbohydrate content: 21%.

Iso-electric point: pH 4.60 (10°C).

Enzymic properties: The enzyme hydrolysed CMC and was active in releasing free fibres from filter paper. The enzyme had little effect on native cellulose.

Species: Trichoderma viride Source: Onoszuka SS.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Pettersson et al. (99).

Physical properties: M.W. 50,000. Carbohydrate content: 12%.

Iso-electric point: pH 3.39.

Enzymic properties: The enzyme hydrolysed CMC and was active in releasing free fibres from filter paper. The enzyme had little effect on native cellulose.

Species: Trichoderma viride Source: Comm. Mycol. Inst., Kew, Surrey. Strain 92 027.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Selby and Maitland (78,79).

Physical properties: M.W. 12,600.

Enzymic properties: The enzyme was active towards CMC.

Species: Trichoderma viride Source: Wheat bran-sawdust cultures as described by Toyama (104).

Enzyme: β-1,4-glucan glucanohydrolase

Isolated by Li, Flora and King (73).

Physical properties: M.W. 52,000.

Amino acid composition:	
Tryptophan	-
Lycine	12
Histidine	9
Arginine	15
Aspartic acid	74
Threonine	44
Serine	40
Glutamic acid	46
Proline	21
Glycine	62
Alanine	46
Half-cystine	0-1
Valine	30
Methionine	2
Isoleucine	30
Leucine	36
Tyrosine	17
Phenylalanine	18

Enzymic properties: The enzyme was active towards CMC. The Michaelis-Menten constant Km with cello-oligosaccharides as substrate are shown below.

	Substrate				
	G_2	G_3	G_4	G_5	G_6
Km ($\times 10^4$M)	190	31	28	7.0	1.0

The location of the glycosyl bonds most susceptible to attack was determined by using reduced cellopentaose (4-O-tetra-β-1,4-glucosyl-D-sorbitol) as substrate. The relative frequencies of attack at the 4 glycosyl bonds starting from the non-reducing end were 1 : 3.2 : 28.8 : 2.2.

Optimum pH: With amorphous cellulose, broad plateau 4.0-5.0.
With CMC, sharp optimum at 5.3.

An Arrhenius plot from 10° to 57°C with amorphous cellulose gave a linear relationship with a calculated activation energy of 5.1Kcal $mole^{-1}$. The Arrhenius plot with CMC indicated an activation energy of 6.4Kcal $mole^{-1}$ from 37° to 60°C. Below 37°C there was an abrupt change to 16.7Kcal $mole^{-1}$. This reflects a phase change from a solution to a gel.

Species: Trichoderma viride Source: Onozuka P.

Enzyme: β-1,4-glucan cellobiohydrolase and
β-1,4-glucan glucohydrolase

Isolated by Shikata and Kisizawa (100).

Physical properties: M.W. 53,000. Carbohydrate content: 4.8%.

Enzymic properties: The enzyme caused an increase in reducing power of CMC without noticeable decrease in viscosity. The main product of hydrolysis was cellobiose and the reation stopped at nearly 25% hydrolysis of CMC. The enzyme is an exo-type. With cellobiose or reduced cellotriose the enzyme resembled a β-glucosidase. The enzyme also attacked xylan removing xylobiose residues one by one. Hydrolysis of p-nitrophenyl β-cellobioside proceeded stepwise indicating that the enzyme was acting as a β-glucosidase. In contrast to other β-1,4-glucan cellobiohydrolases the enzyme had no activity towards various kinds of insoluble cellulose. Optimum pH: 4.5-5.0. The enzyme was stable up to 60°C after which the activity was rapidly lost.

Species: Trichoderma viride Source: Onozuka.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Nisizawa *et al.* (101).

Physical properties: M.W. 44,000.

Enzymic properties: The enzyme hydrolysed CMC and Avicel very slightly. The ratio of Avicelase to CMCase activity was less than 0.1. The hydrolysis of CMC was random.

Species: *Trichoderma viride* Source: Worthington Biochemical Co.

Enzyme: β-1,4-glucan cellobiohydrolase

Isolated by Maguire (154).

Physical properties: M.W. 44,000.

Enzymic properties: The enzyme did not hydrolyse CMC or *p*-nitrophenyl β-D-glucopyranoside. Reaction with cellulose fibre gave mainly cellobiose as product (96-97%). The kinetics of reaction of the enzyme with the insoluble substrate were carried out and it was shown that the initial rate of reaction varied directly with the surface area of the cellulose when the enzyme concentration is large. The association constant for adsorption was calculated to be $1.19 \times 10^{4} M^{-1}$. Cellobiose was found to inhibit the reaction.

Optimum pH: 5.2. The energy of activation of the overall reaction between 5 and 60°C was 5.3 Kcal.mole^{-1}.

Species: *Trichoderma viride* Source: Enzyme Development Corp., New York, N.Y. Lot No. WR1432.

Enzymes: 3 Cellobiases

Isolated by Gong *et al.* (155).

Physical properties: M.W. 76,000.

Enzymic properties : Three chromatographically distinct cellobiases had similar kinetic properties. They hydrolysed cellobiose to glucose via a noncompetitive mechanism and were subject to significant product inhibition.

Species: Trichoderma viride Source: Onozuka SS.

Enzyme: β-Glucosidase EC 3.2.1.21

Isolated by Berghem and Pettersson (102).

Physical properties: M.W. 47,000. No carbohydrate present.

Amino acid composition:

Tryptophan	10
Lycine	14
Histidine	6
Arginine	16
Aspartic acid + asparagine	58
Threonine	33
Serine	38
Glutamic acid + glutamine	31
Proline	26
Glycine	50
Alanine	47
Half-cystine	6
Valine	38
Methionine	6
Isoleucine	22
Leucine	33
Tyrosine	16
Phenylalanine	12
Total	462

Iso-electric point: pH 5.74 (10°C).

Enzymic properties: No activity was found towards Avicel or CMC.

The Michaelis-Menten constants for the reactions with cellobiose, cellotetraose and p-nitrophenyl β-D-glucopyranoside ($PNPG_1$) are shown below.

	Substrate		
	G_2	G_4	$PNPG_1$
Km ($x10^4$M)	15	3.5	2.8
Vmax ($mol.min^{-1}.mg^{-1}$)	33	19	32,200

The enzyme activity was inhibited at high concentrations of cellobiose and cellotetraose.

Species: Trichoderma viride Source: Wheat bran-sawdust cultures as described by Toyama(104).

Enzyme: β-Glucosidase EC 3.2.1.21

Isolated by Li, Flora and King (73).

Physical properties: M.W. 76,600. Carbohydrate content: 0-2%.

Amino acid composition:

Tryptophan	-
Lycine	14
Histidine	7
Arginine	16
Aspartic acid	65
Threonine	38
Serine	46
Glutamic acid	35
Proline	34
Glycine	55
Alanine	50
Half-cystine	3
Valine	25
Methionine	5
Isoleucine	21
Leucine	32
Tyrosine	18
Phenylalanine	19

Enzymic properties: All of 13 β-glucosides were hydrolysed including 6 aryl β-glucosides, β-1,2, β-1,3, β-1,4 and β-1,6 disaccharides of glucose, cellohexaose, cellobionic acid, CMC and β-1,3-glucan. No α-glucosyl bonds were hydrolysed, nor were β-1,2 and β-1,6-glucans. The Michaelis-Menten constant, Km with cello-oligosaccharides as substrates is shown below.

	G_2	G_3	G_4	G_5	G_6
Km ($x10^4$M)	22	1.8	0.65	0.60	1.6

The hydrolysis of the substrates released glucose predominantly in the α-configuration. With reduced cellopentaose it was shown that the enzyme removed successive glucosyl residues from the non-reducing end of the polymer chain. Catalytic numbers for p-nitropheryl β-D-glucoside, cellotriose, cellobiose and CMC were 1320, 676, 397 and 301 moles/min/micromole of enzyme, respectively.

Optimum pH: 4.9. Arrhenius plots indicated activation energies of 6.54Kcal $mole^{-1}$ for cellotetraose and 7.20Kcal $mole^{-1}$ for carboxymethyl cellulose. After 3 minutes incubation the recovery of activity was 17%.

Species: Trichoderma viride Source: Worthington Biochemical Co.

Enzyme: Cellobiase EC 3.2.1.21

Isolated by Maguire (153).

Physical properties: M.W. 45,000.

Enzymic properties: The enzyme hydrolysed cellobiose and p-nitrophenyl β-D-glucopyranoside. The Michaelis-Menten parameters are given in the following table.

	Cellobiose	p-nitrophenyl glucoside
Km ($x10^{-3}$M)	2.68	0.334
Vmax ($x10^{-6}Ms^{-1}$)	1.24	0.843

Species: Triohoderma viride Source: Meicelase.

Enzyme: β -1,4-xylan xylanohydrolase EC 3.2.1.8

Isolated by Hashimoto, Muramatsu and Funatsu (103).

Physical properties: M.W. not given. The enzyme was crystallised and the crystals show a thin rhombic shape.

Enzymic proerties: The enzyme hydrolysed soluble xylan and glycol xylan. Insoluble xylan was not hydrolysed.

Optimum pH: 3.9.

Optimum temperature: 50°C. The enzyme was stable up to 40°C after which the activity rapidly fell off.

Species: Irpex lacteus (Polyporus tulipiferae). Source: Own cultures

Enzyme: 'Avicelase'

Isolated by Kanda, Wakabayashi and Nisizawa (105).

Physical properties: M.W. 56,000. Carbohydrate content: 12.2%.

The amino acid composition is listed.

Enzymic properties: The enzyme hydrolysed insoluble cellulose, CMC and cello-oligosaccharides. The main product in all cases was cellobiose. But with p-nitrophenyl β-cellobioside equal amounts of glucose and cellobiose were produced.

Optimum pH: 4.0-5.0. Optimum temperature: 50°C.

Species: Irpex lacteus (Polyporus tulipiferae). Source: Own cultures

Enzyme: 'Avicelase'

Isolated by Kanda, Wakabayashi and Nisizawa (105,106).

Physical properties: M.W. 7,700.

Enzymic properties: The enzyme hydrolysed insoluble cellulose and CMC.

Species: Irpex lacteus (Polyporus tulipiferae). Source: Own cultures

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

β-1,4-xylan xylanohydrolase EC 3.2.1.8

Isolated by Kanda, Wakabayashi and Nisizawa (105,106).

Physical properties: M.W. 35,600.

Enzymic properties: The enzyme hydrolysed both carboxymethyl cellulose and xylan. Kinetic experiments showed competitive inhibition between CMC and xylan and it appeared that the same active site of the protein catalyses the hydrolysis of CMC and xylan. The enzyme, when mixed with either of the above Avicelases showed remarkable synergic action.

Optimum pH: 4.0-5.0. Optimum temperature: 40°C.

Species: Fusarium moniliforme Source: Own cultures.

Enzyme: Cellulase

Isolated by Matsumoto et al. (88).

Physical properties: M.W. 25,000. Carbohydrate content: 26%.

Enzymic properties: The enzyme hydrolysed cellulolytic substrates in the order filter paper>>CMC>Avicel.

Optimum pH: 4.5. Optimum temperature: 60°C. Half the activity was lost on heating for 30 minutes at 80°C.

Species: Sporotrichum pulverulentum Source: Royal College of Forestry, Stockholm.

Enzymes: Five β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Eriksson and Pettersson (107).

Physical properties:

M. W.	Carbohydrate content	Iso-electric pt.
32,300	10.5%	pH 5.32
36,700	0%	pH 4.72
28,300	7.8%	pH 4.40
37,500	4.7%	pH 4.65
37,000	2.2%	pH 4.20

The amino acid composition for each enzyme is listed.

Enzymic properties: The activities of the five enzymes towards CMC are in the ratio 4 : 1 : 1 : 1 : 1 respectively.

Species: Sporotrichum pulverulentum Source: Royal College of Forestry, Stockholm.

Enzyme: β-1,4-glucan cellobiohydrolase

Isolated by Eriksson and Pettersson (90,108).

Physical properties: M.W. 48,600. Carbohydrate content: 0%.

The amino acid composition is listed.

Enzymic properties: The enzyme mainly produces cellobiose by an end-wise attack at the non-reducing end of cellulose and higher oligosaccharides. A strong synergistic response was observed between the five endo-1,4-glucanases and the exo-1,4-glucanase when these enzymes were allowed to degrade de-waxed cotton and Avicel. No synergism was observed if phosphoric acid-swollen was used. The exo-1,4-β-glucanase released its products in the α-configuration.

Species: Geotrichum candidum (strain 3S) Source: Own cultures

Enzyme: C_1 component

Isolated by Rodionava et al. (92).

Physical properties: M.W. 64,600.

Enzymic properties: The enzyme had no activity on cotton fibre but hydrolysed filter paper.

Optimum pH: acetate buffer, 4.0; citrate/phosphate buffer, 5.0.

Species: Geotrichum candidum (strain 3S) Source: Own cultures.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Rodionova et al. (92).

Physical properties: M.W. 51,700.

Enzymic properties: The enzyme hydrolysed CMC.

Optimum pH: acetate buffer, 4.0; citrate/phosphate buffer, 3.5. The separate C_1 and Cx enzymes did not act on cotton fibre even after prolonged incubation of high concentrations of the enzyme with the substrate after 20 days. With the joint use of C_1, Cx and a cellobiase there was an almost total cleavage of cotton fibre to glucose in 5-6 days.

Species: Aspergillus niger Source: Sigma Chemical Co.

Enzyme: β-1,4-glucan glucanohydrolase EC 3.2.1.4

Isolated by Hurst et al. (109).

Physical properties: M.W. 26,000. Carbohydrate content: 0%.

The amino acid composition is listed.

Enzymic properties: The enzyme was active towards CMC but not towards p-nitrophenyl β-D-glucoside or cellobiose. Kinetic studies gave pK values between 4.2 and 5.3 for groups involved in the enzyme-substrate complex.

Optimum pH: 3.8-4.0. Optimum temperature: 45°C.

Species: Fusarium solani Source: F. solani I.M.I. 95994

Enzyme: C_1 component.

Isolated by Wood and McCrae (110).

Physical properties: M.W. 41,000.

Enzymic properties: The purified enzyme showed little capacity for hydrolysing Avicel or cotton fibre, but phosphoric acid-swollen cellulose and soluble cello-oligosaccharides such as cellotetraose and cellohexaose, were readily hydrolysed; cellobiose was the principal product in each case. The hydrolysis of CMC was minimal and ceased after the removal of a few unsubstituted residues from the end of the chain. These facts indicate that the C_1 component is a β-1,4-glucan cellobiohydrolase.

Synergic action was found on combination of the enzyme with Cx^x components in the solubilisation of cotton fibre.

PREPARATIVE EXPERIMENTAL

General

Melting points were recorded on a Kofler-Reichert hot stage melting point apparatus and all are uncorrected.

IR Spectra were run on a Perkin Elmer 151 spectrophotometer.

UV Spectra were recorded on a Unicam SP800 spectrophotometer.

PMR Spectra were run on either a Varian T60 spectrometer, Varian HA 100MHz spectrometer or a Varian XL-100 NMR spectrometer with VFT-100 Fourier transform accessory.

Carbon-13 NMR spectra were recorded on the Varian XL-100 NMR spectrometer with the VFT-100 Fourier transform accessory.

Optical rotations were measured on a Perkin Elmer 141 Polarimeter.

Elemental analyses were determined by Mrs. W. Harkness, University of Glasgow. The figures quoted are percentage weights.

Preparation of the Peracetylated Oligomers of β-1,4-Linked Glucose

Penta-O-acetyl-α-D-glucopyranose (AcG_1)

Anhydrous glucose was converted to the α-penta acetate by the standard method of adding glucose slowly to a stirred solution of acetic anhydride with perchloric acid as catalyst (122). This gave the α-acetate in 90% yield, with mp 110-112°C (lit.(122)112-113°C). The α-configuration was confirmed by nmr which showed that the anomeric proton at δ = 6.3ppm was a doublet with splitting 4Hz.

Octa-O-acetyl-α — cellobiose (AcG_2)

Cellobiose (Koch-Light Laboratories) was acetylated by the above method to give the α-acetate in 85% yield, with mp 228°C (lit.(123)229°C). nmr ($CDCl_3$), δ=6.3(d, J=4Hz, 1H), 5.6-3.4(m, 13H), 2.3-2.0(8 CH_3 s, 24H). $[\alpha]_D$= +39.8° (lit.(123)+41°). The nmr and optical rotation confirms the presence of the α-configuration.

Per-O-acetates of cellotriose, -tetraose and -pentaose ($AcG_{3,4,5}$)

The mixture of acetylated cellodextrins was prepared by a modification of the methods of Dickey and Wolfrom(124), Wolfrom and Dacons(125)and Miller *et al* (126).

Whatman Cellulose Powder (120g) was slowly added to a stirred solution of acetic anhydride (430ml), acetic acid (430ml) and concentrated sulphuric acid (45ml). The reaction was carried out in an ice bath to ensure that the temperature did not rise above 40°C. (Any further increase in temperature leads to caramellisation). The reaction was then maintained at 30°C. After 48 hours the mixture was monitored every 2 hours by tlc until the predominant oligomer was the tetramer. This took a further 6 hours. The reaction was stopped at this point by pouring the solution into 10 litres of vigorously stirred ice water. The precipitated oligomers were then filtered off, washed with sodium hydrogen carbonate solution and water, and dried. Yield 200g.

Tlc of this material showed that it contained oligomers with D.P. 1 to 9. By dissolving this in a minimum volume of refluxing methanol cellobiose octa-acetate selectively crystallised out, enriching the mother liquor in higher oligomers. This also provided a further source of the dimer. The filtered solution of oligomers was poured into 5 litres of saturated brine and left for a few hours for the precipitate to coagulate. This was then filtered off and well washed to ensure the mixture was free of salt. The mixture of cello-oligomers was air dried and stored in a vacuum dessicator. Yield 105g, 49%. This provided a stock for chromatographic separation.

The separation of the cellodextrins is normally carried out on the free sugars using carbon-celite or gel filtration columns. For the purposes of further synthesis it would be advantageous to obtain a supply of acetylated cello-oligomers directly from the peracetylated mixture (rather than deacetylation, separation and subsequent acetylation). Ballardie and Capon(127)have successfully obtained the oligosaccharide peracetates of chitin in preparative quantities using a large silica column. The conditions which Ballardie and Capon used for the separation of the chitin oligosaccharides (10% methanol/chloroform) only succeeded in washing the cellodextrin acetates straight through the column. This difference in polarity can only be due to the N-acetyl group on carbon 2 on each of the sugar residues of acetylated chitin oligomers, compared with the O-acetyl group of the glucose based oligomers. After much trial and error the following system was developed.

The adsorbent used was Mallinckrodt Silicic Acid, 100 mesh, activated for 12 hours at 200°C. 1.8kg were used, and 5g of the cellodextrin acetate mixture could be separated. The column (210cm x 4.2cm) was packed wet with a slurry of the adsorbent and carbon

tetrachloride. The mixture of acetylated oligomers was applied in a minimum volume of 30:70 chloroform/carbon tetrachloride. The column was eluted with 30:70 chloroform/carbon tetrachloride with 1% methanol added. The proportion of methanol was found to be critical since too much methanol caused the oligomers to come off the column together and too little caused them to adhere to the column. 30ml fractions were collected using an L.K.B. Ultrovac fraction collector over a period of 10-14 days. The separation of the acetates was monitored by tlc analysis of every tenth tube on 20cm x 20cm silica gel plates. Solvent 2% methanol in 30:70 chloroform/carbon tetrachloride. The fractions were shown by development with ceric sulphate. Homogeneous fractions were pooled and the solvent removed on a rotary evaporator. Cellobiose octa-acetate could be recrystallised from methanol but was normally discarded. Cellotriose, cellotetraose and cellopentaose acetates were recrystallised from ethanol. The following table shows a typical distribution when the column is separating well.

Fractions (30ml)	Compound	Typical weight
221-261	AcG_2	0.3g
291-321	AcG_3	1.3g
381-461	AcG_4	1.6g
471-501	AcG_5	0.8g
511-551	AcG_6	0.5g

The fractionated peracetylated cellodextrins were further characterised through their 100MHz nmr spectra, optical rotation and melting points. Their properties are shown in Table 2.

PROPERTIES OF PERACETYL CELLO-OLIGOSACCHARIDES.

	^{1}H-Nmr/ppm (J/Hz)			Optical Rotation ($\|\alpha\|_D$)	Melting Point/$^\circ$C
	Anomeric Proton	Ring Protons	Acetate Methyls	(lit. value)	(lit. value)
AcG_2	6.24(4) (1H)	5.6-3.6 (13H)	2.3-2.0 (24H)	$+39.8^\circ$ ($+41^\circ$)	228 (229)
AcG_3	6.24(4) (1H)	5.6-3.5 (20H)	2.3-2.0 (33H)	$+21.1^\circ$ ($+22^\circ$)	223-225 (223-225)
AcG_4	6.24(4) (1H)	5.6-3.5 (27H)	2.3-2.0 (42H)	$+10.6^\circ$ ($+13^\circ$)	230-232 (235-236)
AcG_5	6.23(4)	5.6-3.5	2.3-2.0	$+7^\circ$ ($+4.7^\circ$)	235-238 (240)

TABLE 2.

Carbon-13 NMR of the Acetylated Cello-oligosaccharides

To further characterise the cello-oligosaccharides, their C-13 NMR spectra were recorded with some interesting results.

The structures of the acetylated oligosaccharides are shown in Figure 7 . The structures are drawn with the anomeric acetoxy group in the α-configuration as determined by proton NMR.

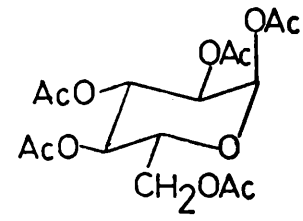
The chemical shifts of the carbon atoms of glucose penta-acetate and cellobiose octa-acetate were assigned by comparison with the results of Gagnaire, Taravel and Vignon(128). The chemical shifts are given in Table 3 . As pointed out by Gagnaire the chemical shifts of carbons 6 and 6' in cellobiose octa-acetate cannot be specifically assigned to any one atom. The C-13 NMR spectra of hepta-O-acetyl-4-nitrophenyl β-cellobioside was recorded in the hope that the chemical shift of C-6 on the reducing ring would be altered. As can be seen from the accompanying data on the cellobioside this did not occur.

To assign the spectrum of AcG_3 it is assumed that the carbon atoms of the glucose rings at the reducing end and the non-reducing end have similar chemical shifts to those of AcG_2 and that the new signals which arise come from the internal glucose ring.

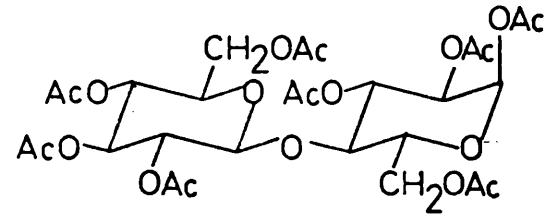
The atoms of C-1, C-4, and C-6 for each ring are readily assigned (the ambiguity for the outer C-6's remains, although the C-6 signal of the internal ring is unique). Carbons C-2, C-3 and C-5 of the reducing ring are easily picked out as is C-2 of the non-reducing ring. The value of 72.09ppm for C-2 of the internal ring and 71.89ppm for C-5 of the non-reducing ring may be interchanged as may be the values of 72.80ppm for C-3 of both the non-reducing ring and the internal ring and 72.92ppm for C-5 of the internal ring.

Structure of Acetylated Cello-oligosaccharides

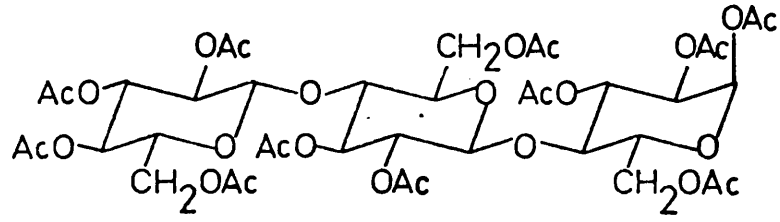
AcG_1



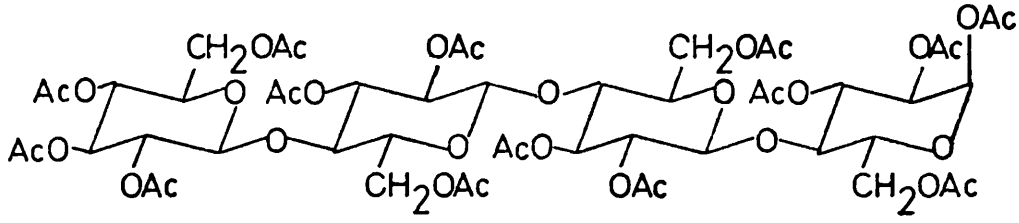
AcG_2



AcG_3



AcG_4



AcG_5

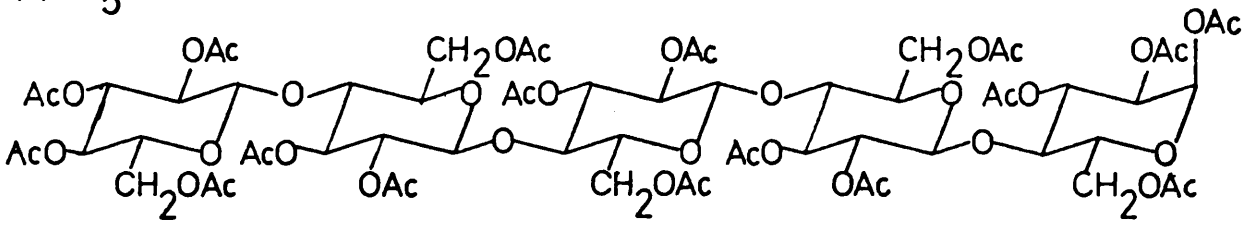


Figure 7

^{13}C - NMR of Acetylated Cello-oligosaccharides

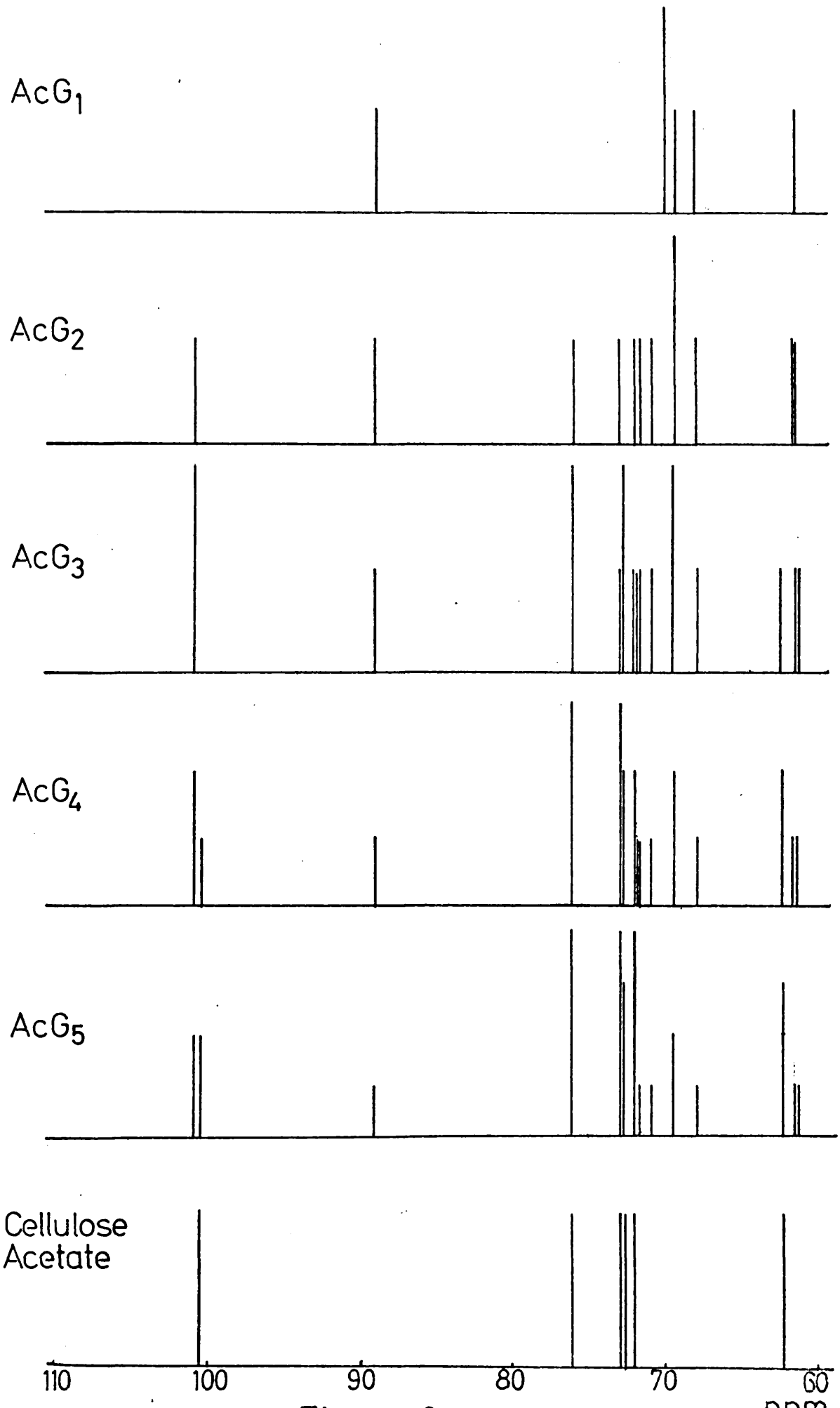


Figure 8

TABLE 3

CHEMICAL SHIFTS FOR ^{13}C-NMR SPECTRA OF ACETYLATED CELLO-OLIGOSACCHARIDES

AcG_1					C1 89.08
					C2 69.25
					C3 69.86
					C4 67.96
					C5 69.86
					C6 61.51
AcG_2				C1 100.89	C1 88.96
				C2 71.69	C2 69.38
				C3 72.99	C3 69.38
				C4 67.88	C4 76.05
				C5 71.99	C5 70.80
				C6 61.65*	C6 61.50*
AcG_3			C1 100.81	C1 100.81	C1 88.98
			C2 71.68	C2 72.09	C2 69.45
			C3 72.80	C3 72.80	C3 69.45
			C4 67.85	C4 76.17	C4 76.17
			C5 71.89	C5 72.92	C5 70.80
			C6 61.57*	C6 62.26	C6 61.32*
AcG_4		C1 100.81	C1 100.54	C1 100.81	C1 88.98
		C2 71.66	C2 72.05	C2 72.05	C2 69.42
		C3 72.87	C3 72.71	C3 72.71	C3 69.42
		C4 67.84	C4 76.15	C4 76.15	C4 76.15
		C5 71.87	C5 72.87	C5 72.87	C5 70.79
		C6 61.58*	C6 62.15	C6 62.15	C6 61.33*
AcG_5	C1 100.81	C1 100.53	C1 100.53	C1 100.81	C1 88.96
	C2 71.66	C2 71.95	C2 71.95	C2 71.95	C2 69.40
	C3 72.86	C3 72.66	C3 72.66	C3 72.66	C3 69.40
	C4 67.83	C4 76.13	C4 76.13	C4 76.13	C4 76.13
	C5 71.95	C5 72.86	C5 72.86	C5 72.86	C5 70.80
	C6 61.56*	C6 62.12	C6 62.12	C6 62.12	C6 61.34*

Cellulose Acetate : C1 100.45; C2 72.00; C3 72.60; C4 76.10; C5 72.95; C6 62.15.

*Chemical shifts may be interchanged.

Structure of Ac4NPG$_2$

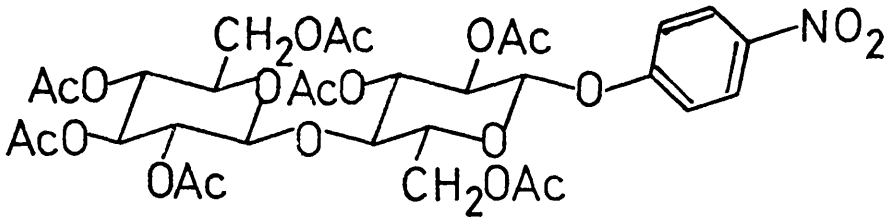


Figure 9

C-13 NMR Spectrum of Ac4NPG$_2$

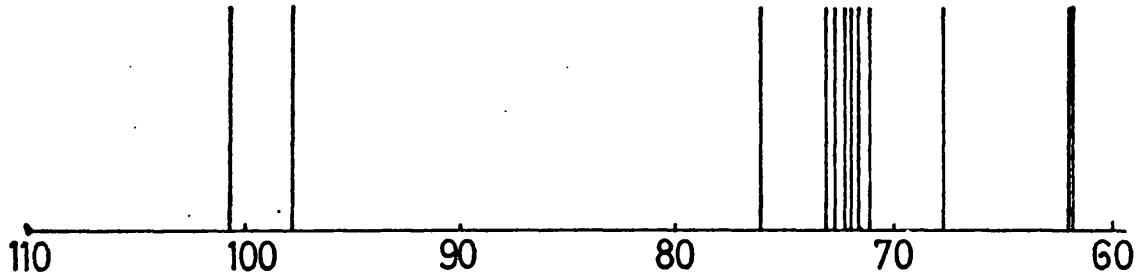


Figure 10

CHEMICAL SHIFTS FOR ^{13}C-NMR SPECTRA OF HEPTAACETYL -

4-NITROPHENYL β-CELLOBIOSIDE

C1	100.81	C1	97.84
C2	71.62	C2	72.29
C3	72.85	C3	71.11
C4	67.83	C4	76.20
C5	71.98	C5	73.22
C6	61.85*	C6	61.66*

* Chemical shifts may be interchanged.

TABLE 4

The β-anomeric carbons of AcG_3 have the same chemical shift value of 100.81ppm. On addition of a glucose residue to form AcG_4 a new anomeric resonance at 100.54ppm. On addition of a further glucose residue to form AcG_5 the C-13 NMR spectrum shows 2 β-anomeric carbon resonances at 100.81ppm and 2 at 100.53 ppm as well as the α-anomeric carbon resonance at 88.96ppm. The chemical shift at 100.53ppm is typical of the anomeric carbon of a 'normal' internal ring of cellulose acetate. (A 'normal' ring is one in the middle of the long chain polymer). Since one resonance at 100.81ppm is attributable to the anomeric carbon of the glucose ring at the non-reducing end of the cello-oligosaccharide chain; the other must belong to a ring which is special compared to that of the 'normal' internal ring of cellulose acetate.

It cannot definitely be said whether this 'special' glucose ring should be considered to be inserted between the reducing ring and the internal ring or the non-reducing ring and the internal ring of AcG_3 but a good guess is that this 'special' ring is the one next to the reducing end.

This 'special' internal ring is only characterised by its differing chemical shift of the anomeric carbon.

If the 'special' ring is adjacent to the reducing end, the anomeric carbon would be 5 atoms away from the carbon at which there is a structural difference from the 'normal' ring. (A 'normal' ring is one in the middle of a long chain polymer). If the 'special' ring were adjacent to the non-reducing end of the chain the anomeric carbon would be 8 atoms away from the carbon at which a structural difference from a 'normal' ring occurs. Therefore the reducing end would be expected to make the larger contribution to any deviation. On this basis the chemical shifts were assigned to the carbon atom of AcG_4

as shown in Table 3 . The assigments for C-1, C-4 and C-6 of each ring are readily made (again there is ambiguity for the outer C-6's). C-2, C-3 and C-5 of the reducing ring and C-2 of the non-reducing ring are easily picked out. Other assignments are made on the basis of the first assumption (that the outer rings retain their characteristic chemical shifts) and that the internal rings are beginning to be typical of cellulose acetate.

On addition of a further glucose residue to form AcG_5, the C-13 NMR spectrum does not become more complicated. Assuming that the outer rings retain their chemical shift identity, the 'special' ring is adjacent to the reducing ring, and that the oligosaccharide tends towards the polymer cellulose acetate; the assigrments shown in the table result.

Taking a broader look at the C-13 NMR spectra of the acetylated cello-oligosaccharides it can be seen that the C-13 NMR spectrum of AcG_2 is not representative of the spectrum of cellulose acetate. The spectrum of AcG_3 still does not explain the spectrum of the polymer although the chemical shift for C-6 of cellulose acetate is represented by the chemical shift of the internal C-6. It is not until the tetrameric compound is studied that all the chemical shifts of the carbons of cellulose acetate are explained.

From the above results the following underlined glucose units represent the internal glucose residues responsible for the C-13 NMR spectrum of cellulose acetate.

Glu - Glu - Glu - Glu - Glu - Glu - Glu - Glu - Glu - Glu

Preparation of the Oligosaccharides of β-1,4-Linked Glucose

α-D-Glucopyranose (G_1)

D-glucose was obtained from B.D.H. and was of 'Analar' grade. It was used without further purification.

α-Cellobiose (G_2)

Cellobiose was obtained from Koch-Light Laboratories and was used without further purification.

α-Cellotriose (G_3)

Peracetyl-α-cellotriose (0.5g) was dispersed in anhydrous methanol (5ml) and sodium methoxide (1.5ml, 0.5g Na in 100ml) added. The slurry was shaken for 40 minutes by which time the mixture had become clear. Amberlite IR 120 (H^+) ion exchange resin was added until the sodium had been removed. The resin was filtered off and the methanol removed on a rotary evaporator to give an amorphous white powder which was recrystallised from 1:1 ethanol/water.
Yield 0.22g, 88%. mp 207-213°C (lit. (129) 206-209°C; (130) 224°C.
IR No acetates present. ν = 3600-3100cm^{-1}, O-H. (KBr).
$[\alpha]_D$ = +30.5° (water, 0.28g/100ml). (lit. (130) +32° → +23°).

α-Cellotetraose (G_4)

Peracetyl-α-cellotetraose (0.5g) was treated with sodium methoxide in methanol as for the trimer. The oligosaccharide dissolved and then the de-O-acetylated material began to crystallise out. The mixture was shaken for a further 30minutes, cooled to 0°C and filtered to afford the tetramer. Yield 0.20g, 77%.
mp 245-251°C (lit. (129) 252°C; (131) 256-257°C).
IR No acetates present. ν = 3600-3100cm^{-1}, O-H. (KBr).
$[\alpha]_D$ = +14.4° (water, 0.19g/100ml). (lit. (129) +16°).

α-Cellopentaose (G_5)

Peracetyl-α-cellopentaose (0.3g) was treated with sodium

methoxide in methanol as before. As with the tetramer the de-O-acetylated material crystallised out. The mixture was shaken for a further 30 minutes, cooled to 0°C and the cellopentaose filtered off. Yield 0.12g, 75%. mp 262°C (lit. (129) 266-268°; (132) 268.5°C)

IR No acetates present. ν = 3600-3100cm^{-1}, O-H. (KBr).

$[\alpha]_D$ = +11.7° (water, 0.11g/100ml). (lit. (129) +11°; (132) +12.4°).

Preparation of 3,4-Dinitrophenyl-β-D- Glycosides of G_1, G_2, G_3 and G_4

It is well known that the reaction of nucleophiles with acetobromo-glucose gives a product with a 1,2-trans-relationship between the acetoxy group on carbon 2 and the added nucleophile on carbon 1. The use of the acetobromo-sugars was therefore employed in the synthesis of the aryl β-D-glycosides.

3,4-dinitrophenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (Ac34DNPG_1)

Acetobromo-glucose was prepared pure and in high yield by the method of Haynes and Newth(133) mp 87-88°C (lit.(134)87°C).

Acetobromo-glucose (3.5g), anhydrous potassium carbonate (2.5g) and 3,4-dinitrophenol (1.5g) were stirred together for 5 days in 50ml dry acetone. The solution was then poured into 100ml of ice-water and the glucopyranoside extracted with 4 x 100ml chloroform. The solution was dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotary evaporator. The resultant yellow syrup readily solidified and was recrystallised from methanol. Yield 2.9g, 66%. mp 169-171°C.

Analysis: Calculated for $C_{20}H_{22}N_2O_{14}$: C,46.70; H,4.31; N,5.45.
Found: C,46.50; H,4.52; N,5.25.

IR ν =1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.00 (d,J = 8.5Hz, 1H), 7.42 (d,J = 2.5Hz,1H), 7.26 (q,J = 8.5Hz, 2.5Hz, 1H), 5.40-5.05 (m,4H), 4.30-3.90 (m,3H), 2.20-2.05 (4s,12H).

UV λ max = 282nm, log ε = 3.78 (methanol).

$[\alpha]_D$ =-42.3° (chloroform; c, 0.46g/100ml).

3,4-dinitrophenyl β-D-glucopyranoside ($34DNPG_1$)

The acetate groups were removed by the method of Zemplén (135). $Ac34DNPG_1$ (1g) was dispersed in methanol (10ml) and sodium methoxide (2.5ml, 0.7g Na in 100ml methanol) added. The slurry was shaken for 30 minutes, by which time the solution was clear. Amberlite IR 120 (H^+) ion exchange resin was added to remove the sodium ions. The solution was filtered and the solvent removed on a rotary evaporator to afford the crude glucopyranoside. The product was recrystallised from a 1:4 solution of methanol/water.

Yield 0.6g, 90%. mp 105-109°C.

Analysis: Calculated for $C_{12}H_{14}N_2O_{10}(H_2O)$: C, 39.56; H 4.43; N 7.69

Found: C 39.09; H 4.72; N 7.61.

IR No acetates present. ν =3600-3100, O-H; 1615cm^{-1}, aromatic. (KBr).

nmr (pyridine-d_5), δ =8.04(d, J=8.5Hz,1H), 7.79 (d, J=2.5Hz,1H), 7.48 (q, J=8.5Hz, 2.5Hz, 1H), 5.78 (d, J=8Hz,1H), 4.68-4.05 (m,6H). (pyridine-d_5/D_2O) additional singlet at δ =5.6 (4H).

UV λ max = 282nm, log ε = 3.8 (water, pH=5).

$[\alpha]_D$ = -67° (water; c, 0.1g/100ml).

3,4-dinitrophenyl hepta-O-acetyl-β — cellobioside ($Ac34DNPG_2$)

Cellobiose octa-acetate was converted to the hepta-acetyl bromide in 92% yield by the method of Haynes and Newth (133). mp 189-191°C (lit.(136) 182°C).

Acetobromo - cellobiose (3.5g), potassium carbonate (2g) and 3,4-dinitrophenol (1g) were stirred together in 100ml of dry acetone for 5 days at room temperature. The mixture was then treated as for $Ac34DNPG_1$ to give pale yellow crystals from 50:50 methanol/ethanol. Yield 2.1g, 52.3% mp 240-242°C.

Analysis: Calculated for $C_{32}H_{38}N_2O_{22}$: C, 47.89; H, 4.77; N, 3.49.

Found : C, 47.95; H, 4.97; N, 3.30.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol)

nmr ($CDCl_3$), δ = 8.02 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.26 (q, J=8.5, 2.5Hz, 1H), 5.42-3.60 (m, 14H), 2.24-1.92 (7s, 21H).

UV λ max = 282nm, log ε = 3.75 (methanol).

$[\alpha]_D$ = -37°, (chloroform; c, 0.24g/100ml).

3,4-dinitrophenyl β — cellobioside ($34DNPG_2$)

De-O-acetylation of $Ac34DNPG_2$ was accomplished under Zemplén conditions.

$Ac34DNPG_2$ (1g) was deacetylated to give 0.58g of the aryl cellobioside, recrystallised from 1:4 methanol/water. mp 187-192°C.

Analysis : Calculated for $C_{18}H_{24}N_2O_{15}(H_2O)$: C, 41.07; H, 4.98; N, 5.32.

Found : C, 41.00; H, 5.01; N, 5.32.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic.(KBr)

nmr (pyridine-d_5), δ = 8.00 (d, J=8.5Hz, 1H), 7.75 (d, J=2.5Hz, 1H), 7.42 (q, J=8.5Hz, 2.5Hz, 1H), 6.70 (s, 7 O-H), 5.74 (d, J=8Hz, 1H), 5.16 (d, J=8Hz, 1H), 4.70-3.85 (m, 12H).

UV λ max = 284nm, log ε = 3.8 (water, pH=5).

$[\alpha]_D$ = -166.3°. (water; c, 0.154g/100ml).

3,4-dinitrophenyl deca-acetyl-β — cellotrioside ($Ac34DNPG_3$)

Peracetyl cellotriose (0.4g) was added to dichloromethane (5ml) and 40% hydrogen bromide in acetic acid (2.5ml) and left at room temperature for 2 hours. The solution was then poured into 10ml of ice water, the bromide extracted with chloroform and washed with sodium hydrogen carbonate solution. After drying over magnesium sulphate the solvent was removed to give a pale yellow syrup which solidified on trituration with ether. Yield 0.35g, 85%.

The acetobromo-cellotriose was immediately reacted with potassium

carbonate (0.5g) and 3,4-dinitrophenol (0.2g) in 25ml dry acetone. After 5 days the cellotrioside was worked up as for $Ac34DNPG_1$ to give a pale yellow syrup. This syrup would not crystallise. Tlc analysis of the product showed that the aryl glucoside and cellobioside were present as impurities. The product was then separated on 1mm, 20cm x 20cm silica gel plates using 2% methanol in 30:70 chloroform/carbon tetrachloride as eluent. Three bands were shown to be present by uv. The lower band was washed from the silica with chloroform and evaporated to give a clear syrup which was easily recrystallised from ethanol. It was shown to be the peracetate of the aryl cellotrioside. Yield 0.15g, 40%. mp 135-139°C.

Analysis : Calculated for $C_{44}H_{54}N_2O_{30}$: C, 48.44; H, 4.99; N, 2.57.

Found : C, 48.25; H, 5.03; N, 2.28.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.02 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.26 (q, J=8.5Hz, 2.5Hz, 1H), 5.45-3.60 (m, 21H), 2.26-1.90 (10s, 30H).

UV λ max = 282nm, log ε =3.8 (methanol).

$[\alpha]_D$ = -22.2° (chloroform; c, 0.216g/100ml).

3,4-dinitrophenyl β - cellotrioside ($34DNPG_3$)

$Ac34DNPG_3$ was de-O-acetylated by the method of Zemplén. $Ac34DNPG_3$ (0.1g) was deacetylated to give a pale yellow syrupy product. Water (1ml) was added and the solution passed through a column of Sephadex G-15 (1.6cm x 20cm). The effluent was monitored its uv absorption at 282nm. The homogeneous fraction was freeze dried to give a white amorphous powder. Yield 54mg, 88%. mp 165-170°C(dec.)

Analysis : Calculated for $C_{24}H_{34}N_2O_{20}$: C, 43.00; H, 5.11; N, 4.18.

Found : C, 43.22; H, 5.37; N, 4.20.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic. (KBr).

nmr (D_2O), δ = 8.16 (d, J=8.5Hz, 1H), 7.62 (d, J=2.5Hz, 1H), 7.44 (q, J=8.5Hz, 2.5Hz, 1H), 5.28 (d, J=7Hz, 1H), 4.54 (d, J=8Hz, 1H), 4.46 (d, J=8Hz, 1H), 4.10-3.16 (m, 18H).

H-O-D signal at 4.78δ.

UV λmax = 282nm, log ε = 3.75 (water, pH=5).

$[\alpha]_D$ = -23.4° (water; c, 0.095g/100ml).

3,4-dinitrophenyl trideca-O-acetyl-β - cellotetraoside ($Ac34DNPG_4$)

Since the reaction of the peracetyl cellotriose with hydrogen bromide led to partial degradation, it was expected that that of the peracetyl cellotetraose would do so as well. This was found to be so.

Peracetyl cellotetraose (0.5g) was dissolved in a mixture of dichloromethane (3ml) and 40% hydrogen bromide in glacial acetic acid (2.5ml). The solution was left at room temperature for 2 hours. It was treated as for the cellotriose acetate to give a clear syrup which solidified on trituration with ether. Yield 0.44g, 86%.

The acetobromo-cellotetraose was immediately added to a stirred mixture of potassium carbonate (0.5g) and 3,4-dinitrophenol (0.2g) in 25ml dry acetone. The reaction was left for 5 days at room temperature and then worked up as for $Ac34DNPG_1$ to give a pale yellow syrup. As with $Ac34DNPG_3$ it would not crystallise and tlc analysis showed that the glucoside, cellobioside and cellotrioside were present as impurities. Unfortunately preparative separation on 1mm thick silica gel plates did not resolve the tetramer from the trimer. It was necessary to use a column (2cm x 40cm) packed with dry silica gel. The sample was applied to the top of the column in a minimum volume of 30:70 chloroform/carbon tetrachloride. The column was eluted with 2% methanol in 30:70 chloroform/carbon tetrachloride. Fractions (25ml) were collected and their contents analysed by tlc. The $Ac34DNPG_4$ fractions were pooled and the

solvent removed on a rotary evaporator to give a clear syrup which was easily recrystallised from ethanol. Yield 0.25g, 53%. mp 140-146°C.

Analysis: Calculated for $C_{56}H_{70}N_2O_{38}$: C, 48.77; H, 5.12; N, 2.03.

Found : C, 48.40; H, 5.10; N, 2.50.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.00 (d, J=8.5Hz), 7.42 (d, J=2.5Hz), 7.26 (q, J=8.5Hz, 2.5Hz), 5.44-3.58 (m), 2.26-1.92 (CH_3 singlets).

UV λ max = 284nm, logε = 3.8 (methanol).

$[\alpha]_D$ = -19.2° (chloroform; c, 0.24g/100ml).

The carbon-13 nmr spectrum of Ac34DNPG$_4$ was recorded. The resonances of the carbon atoms in each ring are tabulated below. The assignments were made on the basis of the carbon-13 studies of the acetylated cello-oligosaccharides and heptaacetyl-4-nitrophenyl β-cellobioside.

CHEMICAL SHIFTS FOR ^{13}C-NMR SPECTRUM OF Ac34DNPG$_4$

C1	100.78	C1	100.47	C1	100.47	C1	97.84
C2	71.63	C2	72.06	C2	72.06	C2	73.12
C3	72.40	C3	72.67	C3	72.67	C3	71.03
C4	67.79	C4	75.97	C4	75.97	C4	76.58
C5	71.78	C5	72.89	C5	72.89	C5	73.60
C6	61.96	C6	61.96	C6	61.96	C6	61.55

The values going from left to right refer to the glucose rings going from the non-reducing end of oligosaccharide to the aryl group.

3,4-dinitrophenyl β-cellotetraoside (34DNPG$_4$)

Ac34DNPG$_4$ was de-O-acetylated by the method of Zemplén. Ac34DNPG$_4$ (0.2g) was de-O-acetylated and the crude glycoside passed through the Sephadex G-15 column as for 34DNPG$_3$. The eluate containing the aryl cellotetraoside was freeze dried to give a white amorphous powder. Yield 104mg, 86%. mp 183-187°C (dec.).

Analysis: Calculated for $C_{30}H_{44}N_2O_{25}$: C, 43.27; H, 5.33; N, 3.37.

Found : C, 43.12; H, 5.57; N, 3.26.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic. (KBr).

nmr (D_2O), δ = 8.16 (d, J=8.5Hz, 1H), 7.61 (d, J=2.5Hz, 1H),

7.44 (q, J=8.5Hz, 2.5Hz, 1H), 5.28 (d, J=7Hz, 1H), 4.50 (m, 3H

4.10-3.12 (m, 24H). H-O-D signal at 4.78δ.

UV λmax = 283nm, logε = 3.75 (water, pH=5).

$[\alpha]_D$ = -14.4° (water; c, 0.16g/100ml).

3,4-dinitrophenyl β-chitotrioside

This compound was prepared by M. Cuthbert according to the procedures described in reference 127.

3,4-dinitrophenyl β-chitotetraoside

This compound was also prepared by M. Cuthbert according to the procedures described in reference 127.

Preparation of Modified 3,4-Dinitrophenyl β-D-Glucopyranosides

3,4-dinitrophenyl tri-O-acetyl-6-deoxy-β-D-glucopyranoside

6-Deoxy-D-glucose (Koch-Light Laboratories; 0.2g) was acetylated with acetic anhydride and perchloric acid in the same manner as for glucose. The resultant clear syrup was treated with 40% hydrogen bromide in acetic acid (1ml). This was left at room temperature for 3 hours and the acetobromo-sugar worked up as for acetobromo-glucose to give a pale yellow syrup.

To the acetobromo-6-deoxy-glucose in 10ml dry acetone was added 3,4-dinitrophenol (0.2g) and potassium carbonate (0.2g) and the mixture stirred for 5 days at room temperature. The mixture was poured into 50ml ice-water and the deoxy sugar extracted with 4 x 50ml chloroform, dried over anhydrous magnesium sulphate and the solvent removed on a rotary evaporator to give a syrup which was triturated with ether to cause solidification. The solid was recrystallised from methanol. Yield 0.18g, 32%. mp 176-178°C.

Analysis: Calculated for $C_{18}H_{20}N_2O_{12}$: C, 47.38; H, 4.42; N, 6.14.

Found: C, 47.5; H, 4.41; N, 6.44.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr($CDCl_3$), δ = 8.00 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.24 (q, J=8.5Hz, 2.5Hz, 1H), 5.40-5.10 (m, H), 3.88-3.46 (m, H), 2.22-2.02 (3s, 9H), 1.26 (d, J=6Hz, 3H).

UV λ max = 284nm, log ε = 3.76 (methanol).

$[\alpha]_D$ = -83.5° (chloroform; c, 0.07g/100ml).

3,4-dinitrophenyl 6-deoxy β-D-glucopyranoside

The aryl tri-O-acetyl 6-deoxy-glucopyranoside was de-O-acetylated by the method of Zemplén. De-O-acetylation of the 6-deoxy glucoside (0.1g) followed by recrystallisation from 1:1 ethanol/water afforded 3,4-dinitrophenyl 6-deoxy-β-D-glucopyranoside. Yield 0.07g, 93%. mp 95-98°C.

Analysis : Calculated for $C_{12}H_{14}N_2O_9(H_2O)$: C, 41.39; H, 4.63; N, 8.04.

Found: C, 41.62; H, 4.75; N, 8.19.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic. (KBr).

nmr (pyridine-d_5), δ = 8.04 (d, J=8.5Hz, 1H), 7.80 (d, J=2.5Hz, 1H), 7.48 (q, J=8.5Hz, 2.5Hz, 1H), 5.76 (d, J=8Hz, 1H), 4.72-3.96 (m, 4H), 1.28 (d, J=6Hz, 3H). (pyridine-d_5/D_2O), δ = 5.50 (s, H-O-D, 3H).

UV λ max = 282nm, log ε = 3.85 (water, pH=5).

3,4-dinitrophenyl tri-O-acetyl-6-chloro-6-deoxy-β-D-glucopyranoside

3,4-dinitrophenyl tri-O-acetyl-6-chloro-6-deoxy-β-D-glucopyranoside

6-Chloro-6-deoxy-D-glucose was supplied by Professor B. Capon(137). 6-Chloro-6-deoxy-D-glucose (0.2g) was acetylated and treated with 40% hydrogen bromide in acetic acid as for 6-deoxy-D-glucose to give acetobromo-6-chloro-6-deoxy-glucose as a clear syrup. Yield 0.32g, 82%.

The acetobromo-sugar was added to a stirred mixture of 3,4-dinitrophenol (0.2g), potassium carbonate (0.2g) and dry acetone (10ml). After stirring for 5 days, the product was isolated using the same methods as for the 6-deoxy glucopyranoside. The material was re-crystallised from methanol to give small, pale yellow crystals. Yield 0.28g, 69%. mp 177-181°C.

Analysis : Calculated for $C_{18}H_{19}N_2O_{12}Cl$: C, 44.04; H, 3.90; N, 5.72; Cl, 7.23.

Found : C, 44.20; H, 3.88; N, 5.99; Cl, 7.24.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.02 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.24 (q, J=8.5Hz, 2.5Hz, 1H), 5.42-5.02 (m, 4H), 4.34-3.92 (m, 3H), 2.20-2.00 (3s, 9H).

UV λ max = 284nm, log ε = 3.82 (methanol).

$[\alpha]_D$ = -69.3° (chloroform; c, 0.15g/100ml).

3,4-dinitrophenyl 6-chloro-6-deoxy β-D-glucopyranoside

3,4-dinitrophenyl 6-chloro-6-deoxy β-D-glucopyranoside

[illegible] deacetylated using the method of Zemplén.

3,4-Dinitrophenyl tri-O-acetyl-6-chloro-6-deoxy-β-D-glucopyranoside (0.2g) was de-O-acetylated to give a pale yellow syrup which crystallised on trituration with ethanol. The glucoside was recrystallised from 1:1 ethanol/water. Yield 0.11g, 74%. mp 135-139°C.

Analysis : Calculated for $C_{12}H_{13}N_2O_9Cl(H_2O)$: C, 37.65; H, 4.23; N, 7.32; Cl, 9.26.

Found : C, 37.47; H, 4.53; N, 6.96; Cl, 9.68.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic.

nmr (pyridine-d_5), δ = 8.00 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.26 (q, J=8.5Hz, 2.5Hz, 1H), 5.74 (d, J=8Hz, 1H), 4.70-3.94 (m, 6H), (pyridine-d_5/D_2O), δ = 5.56 (s, H-O-D, 3H).

UV λ max = 283nm, log ε=3.78 (water, pH=5).

$[\alpha]_D$ = -40.9° (water; c, 0.22g/100ml).

3,4-dinitrophenyl tri-O-acetyl-6-O-methyl β-D-glucopyranoside

6-O-Methyl-D-glucose was supplied by Professor B. Capon (137). 6-O-Methyl D-glucose (0.5g) was converted to 3,4-dinitrophenyl tri-O-acetyl-6-O-methyl β-D-glucopyranoside by the usual sequence. (Acetylation, treatment with hydrogen bromide in acetic acid and then reacting the acetobromo-sugar with 3,4-dinitrophenol (0.4g) and potassium carbonate (0.4g) in 20ml dry acetone). The compound was recrystallised from methanol to give a white, fluffy crystalline mass. Yield 0.56g, 60.4%. mp 201-204°C.

Analysis : Calculated for $C_{19}H_{22}N_2O_{13}$: C, 46.91; H, 4.56; N, 5.78.

Found : C, 47.02; H, 4.54; N, 5.5.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.02 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.24 (q, J=8.5Hz, 2.5Hz, 1H), 5.62-5.10 (m, 4H), 4.30-3.58 (m, 3H),

3.40 (s, 3H), 2.18-2.04 (3s, 9H).

UV λ max = 284nm, log ε = 3.8 (methanol).

$[\alpha]_D$ = -42° (chloroform; c, 0.148g/100ml).

3,4-dinitrophenyl 6-O-methyl β-D-glucopyranoside

The Zemplén method of de-O-acetylation was used to prepare the aryl 6-O-methyl glucopyranoside. 3,4-Dinitrophenyl tri-O-acetyl-6-O-methyl-β-D-glucopyranoside (0.4g) was de-O-acetylated to give a white solid which was recrystallised from 1:1 ethanol/water.

Yield 0.24g, 81%. mp 148-150°C.

Analysis : Calculated for $C_{13}H_{16}N_2O_{10}(H_2O)$: C, 41.27; H, 4.79; N, 7.43.

Found : C, 41.57; H, 4.74; N, 6.99.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic.

nmr (pyridine-d_5), δ = 7.98 (d, J=8.5Hz, 1H), 7.42 (d, J=2.5Hz, 1H), 7.26 (q, J=8.5Hz, 2.5Hz, 1H), 5.76 (d, J=8Hz, 1H), 4.70-3.98 (m, 6H), 3.42 (s, 3H). (pyridine-d_5/D_2O), δ = 5.5 (s, H-O-D, 3H).

UV λ max = 282nm, log ε = 3.78 (water, pH=5).

$[\alpha]_D$ = -111.4° (water; c, 0.07g/100ml).

3,4-dinitrophenyl tri-O-acetyl-β-D-xylopyranoside

Xylose (10g) was acetylated by the same method as that used for glucose to give a colourless syrup which could not be induced to crystallise. Yield 20.5g, 96%.

Tetra-O-acetyl-α-D-xylose (10g) was treated with 40% hydrogen bromide in acetic acid (25ml) to give acetobromo-xylose after the usual work up. The acetobromo-xylose was obtained as a colourless syrup which crystallised on standing in the refridgerator under dry ether for 3 days. The compound was recrystallised from ether.

Yield 8.7g, 81%. mp 100-101°C (lit. (138) 101°C).

Acetobromo-xylose (2g), 3,4-dinitrophenol (1g) and potassium carbonate (1g) were stirred together in 20ml dry acetone for 5 days.

The acetone mixture was poured into 100ml ice-water and the xylopyranoside extracted with 4 x 50ml chloroform, dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotary evaporator to give a pale yellow solid. The product was recrystallised from methanol. Yield 1.6g, 61%. mp 181-183°C.

Analysis : Calculated for $C_{17}H_{18}N_2O_{12}$: C, 46.15; H, 4.10; N, 6.35.

Found : C, 46.22; H, 4.20; N, 6.60.

IR ν = 1755, C=O; 1615cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.00 (d, J=8.5Hz, 1H), 7.39 (d, J=2.5Hz, 1H), 7.29 (q, J=8.5Hz, 2.5Hz, 1H), 5.46-4.80 (m, 4H), 4.22 (q, J=12Hz,4Hz, 1H), 3.68 (q, J=12Hz, 6Hz, 1H), 2.20-2.08 (3s, 9H).

UV λ max = 282nm, log ε = 3.76 (methanol).

$[\alpha]_D$ = -116.4° (chloroform, 0.22g/100ml).

3,4-dinitrophenyl β-D-xylopyranoside

3,4-Dinitrophenyl tri-O-acetyl-β-D-xylopyranoside (1g) was de-O-acetylated by the method of Zemplén to give the crude aryl xylopyranoside which was recrystallised from methanol. Yield 0.52g, 73%. mp 142-144°C (phase change at 130-133°C).

Analysis : Calculated for $C_{11}H_{12}N_2O_9$: C, 41.77; H, 3.82; N, 8.88.

Found : C, 41.78; H, 3.87; N, 8.55.

IR No acetates present. ν = 3600-3100, O-H; 1615cm^{-1}, aromatic.

nmr (pyridine-d_5), δ = 8.00 (d, J=8.5Hz, 1H), 7.58 (d, J=2.5Hz, 1H), 7.34 (q, J=8.5Hz, 2.5Hz, 1H), 5.74 (d, J=8Hz, 1H), 4.60-3.62 (m, 5H). (pyridine/D_2O), δ = 5.60 (s, H-O-D).

UV λ max = 282nm, log ε = 3.82 (water, pH=5).

$[\alpha]_D$ = -70.5° (water; c, 0.15g/100ml).

3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside

A sample of this material was prepared by M. Cuthbert. It had the following characteristics:

mp 138-139°C

Analysis: Calculated for $C_8H_{17}N_3O_{10}$: C, 43.42; H, 4.42; N, 10.85.

Found: C, 43.02; H, 4.59; N, 10.79.

4-nitrophenyl β-D-glucopyranoside

This compound was synthesized by the methods already described. Acetobromo-glucose (5g), 4-nitrophenol (2.5g) and potassium carbonate (2.5g) were stirred together in 50ml dry acetone. After 4 days the reaction mixture was poured into 200ml ice-water, extracted with 4 x 100ml chloroform, dried over magnesium sulphate, filtered and the solvent removed under reduced pressure. The material was recrystallised from ethanol. Yield 3.8g, 67%. mp 176°C (lit. (139) 174-176°C).

nmr ($CDCl_3$), δ = 8.14 (d, J=8Hz, 2H), 7.56 (d, J=8Hz, 2H), 5.40-4.66 (m, 4H), 4.28-4.14 (m, 2H), 3.94-3.68 (m, 1H), 2.20-2.00 (4s, 12H).

4-Nitrophenyl tetra-O-acetyl-β-D-glucopyranoside (1g) was de-O-acetylated by the method of Zemplén to give a white product. Yield 0.46g, 73%. mp 164-165°C. (lit. (139) 164-165°C).

nmr (d_5-pyridine), δ = 8.08 (d, J=8Hz, 2H), 7.74 (d, J=8Hz, 2H), 5.49 (d, J=8Hz, 1H), 4.64-3.94 (m, 6H).

(pyridine/D_2O), δ = 5.56 (s, H-O-D).

4-nitrophenyl 2-deoxy-β-D-glucopyranoside

A sample of this material was also prepared by M. Cuthbert. It had mp 165-166°C (lit. (140) 167-169°C).

Analysis: Calculated for $C_{12}H_{15}NO_7$: C, 50.53; H, 5.30; N, 4.91.

Found: C, 50.73; H, 5.63; N, 5.48.

Preparation of an Affinity Column Specific for β-Glucosidases

Cuatrecasas(141)has purified a β-galactosidase by affinity chromatography. The column was prepared by covalently linking a specific inhibitor to an agarose matrix. A spacer arm was required to affect binding of the enzyme to the column. Using the methods Shah and Bahl(142)for the preparation of the ligand and a commercial affinity chromatographic product, Affi-Gel 10, an affinity column specific for β-glucosidases was constructed.

4-nitrophenyl tetra-O-acetyl-β-D-thioglucopyranoside

The sodium salt of 4-nitrothiophenol was prepared by a combination of the methods described by Waldron and Reid(143)and Vogel (144).

4-Chloronitrobenzene (157g) was suspended in ethanol (150ml) and sodium disulphide (prepared from sodium sulphide and sulphur;110g) slowly added and left to stir for 5 minutes. The dark red solution was added to 2 litres of ice-water and the unreacted diaryl disulphide filtered off. The red solution was acidified to precipitate the impure mercaptan. This was filtered off immediately and dissolved in 500ml of a boiling 5% sodium hydroxide solution. The red solution was allowed to cool and a saturated solution of sodium hydroxide adde to precipitate the sodium salt. The thiophenolate salt precipitated as golden flakes which were filtered off and dried in a vacuum desiccator. Yield 110g, 63%.

Acetobromo-glucose (8g) and sodium 4-nitrothiophenolate (5g) were stirred together in 200ml of dry acetone for 4 days. The flask was flushed with nitrogen before being stoppered. After the 4 days the solution was poured into 300ml ice-water and the thioglucopyranoside extracted with 4 x 100ml chloroform,dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotary evaporator. The crude thioglucopyranoside was recrystallised from

methanol to give pale yellow needles. Yield 7.2g, 76%. mp 184-185°C.

Analysis: Calculated for $C_{20}H_{23}NO_{11}S$: C, 49.49; H, 4.78; N, 2.89; S, 6.59.

Found: C, 49.22; H, 4.77; N, 2.83; S, 6.81.

IR ν = 1750, C=O; 1600cm^{-1}, aromatic. (Nujol).

nmr ($CDCl_3$), δ = 8.14 (d, J=8.5Hz, 2H), 7.57 (d, J=8.5Hz, 2H), 5.38-4.76 (m, 4H), 4.28-4.15 (m, 2H), 3.94-3.70 (m, 1H), 2.20-2.00 (4s, 12H).

UV λ max = 313nm, logε = 3.97 (methanol).

$[\alpha]_D$ = -35.5° (chloroform, 1.2g/100ml).

4-nitrophenyl β-D-thioglucopyranoside

The peracetylated thioglucopyranoside (5g) was de-O-acetylated by the method of Zemplen as for Ac34DNPG$_1$ to give the crude product which was recrystallised from 1:4 methanol/water to give pale yellow needles. Yield 3.12g, 95.5%. mp 165-167°C.

Analysis: Calculated for $C_{12}H_{15}NO_7S(H_2O)$: C, 42.99; H, 5.11; N, 4.18; S, 9.55.

Found: C, 42.95; H, 5.18; N, 3.89; S, 10.57.

IR No acetates present. ν = 3600-3100, O-H; 1600cm^{-1}, aromatic.

nmr (pyridine-d_5), δ = 8.06 (d, J=8.5Hz, 2H), 7.76 (d, J=8.5Hz, 2H), 7.00-4.80 (broad singlet, 4 O-H), 5.48 (d, J=9Hz, 1H), 4.66-3.94 (m, 6H).

$[\alpha]$ λ max = 312nm, logε = 3.95 (1:1 methanol/water).

$[\alpha]_D$ = -113° (1:1 methanol/water, 0.42g/100ml).

4-aminophenyl β-D-thioglucopyranoside

4-Nitrophenyl β-D-thioglucpyranoside (1g) and 10% palladium/barium sulphate catalyst were stirred in methanol (150ml) under 1 atmosphere of hydrogen for 18 hours. Filtration through celite and removal of the methanol under reduced pressure afforded the crude amine. This was recrystallised from n-propanol to give pale gray crystals. Yield 0.38g, 42%. mp 144-145°C. (lit. (145) 147-148°C).

Coupling of Ligand to Affi-Gel 10

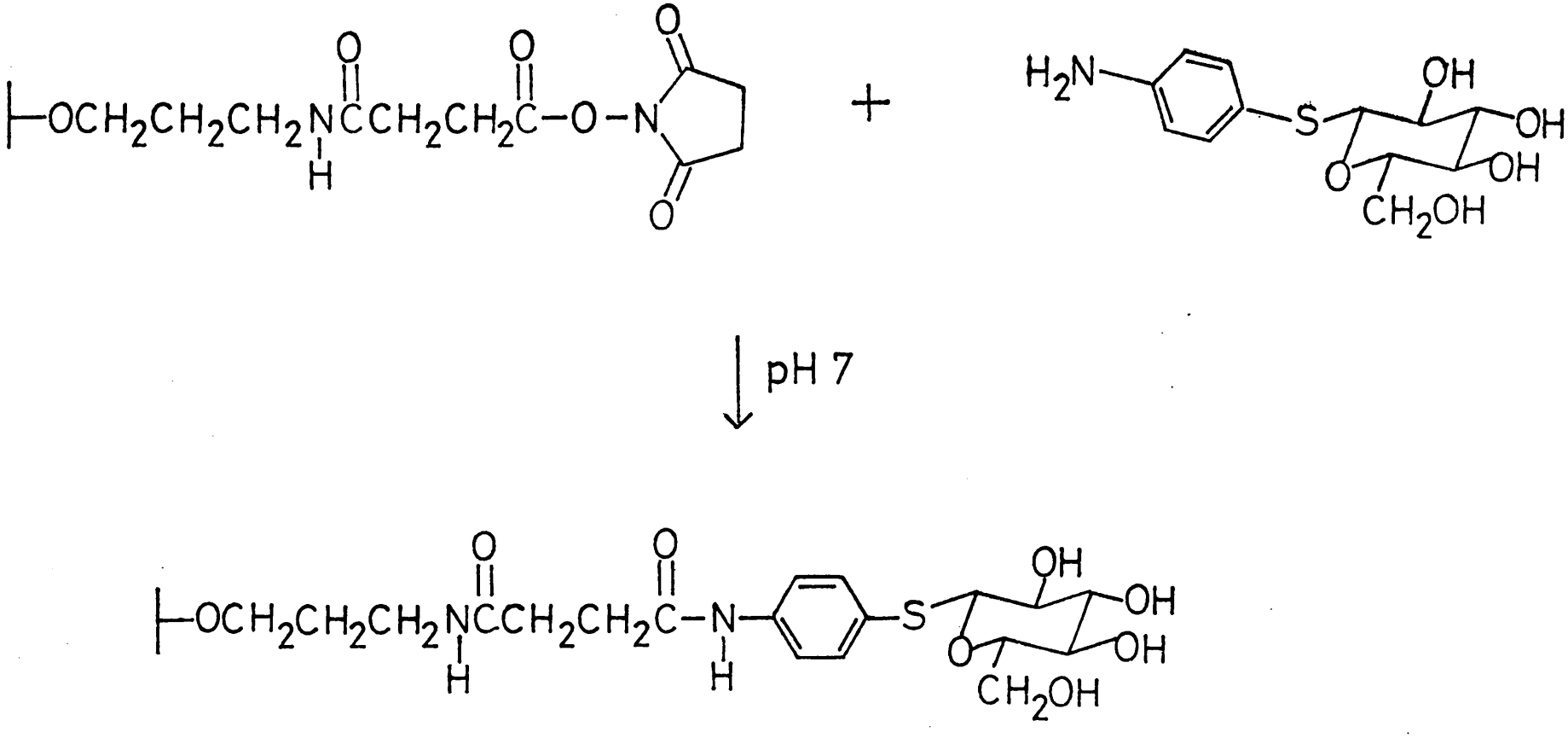


Figure 11

Analysis: Calculated for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87; S, 11.16.

Found: C, 50.36; H, 6.23; N, 4.88; S, 11.36.

IR ν = 3600-3100, O-H and N-H; 1600cm^{-1}, aromatic.

nmr (pyridine-d_5), δ = 7.78 (d, J=8.5Hz, 2H), 6.62 (d, J=8.5Hz, 2H),

Exchangeable protons at 7.20 and6.32 δ.

UV λmax = 252nm, log ε = 2.95 (water, pH=5).

$[\alpha]_D$ = -43° (1:1 methanol/water; 0.14g/100ml).[5].

Affinity column ml).

Affinity column

The support matrix for the β-thioglucopyranoside ligand was Affi-Gel 10 (Bio-Rad Laboratories). Affi-Gel 10 is an N-hydroxy-succinimide ester of succinylated aminoalkyl agarose support. The spacer arm is anchored to the matrix by an ether linkage. The structure of the ligand is shown in Figure 11.

From the data supplied with the gel it was calculated that the amount of bound ligand for each gram of gel would be 140 μmoles.

4-Aminophenyl β-D-thioglucopyranoside (60mg, 210 μM) was dissolved in 25ml 0.1M phosphate buffer, pH 7. This solution was added to Affi-Gel 10 (1g) and the gel shaken for 24 hours. The initial addition was carried out at 4°C. After coupling was complete the slurry was poured into a column (1.6cm x 20cm) and washed with 1M sodium chloride solution until the absorbance at 260nm reached baseline. This indicates removal of the N-hydroxysuccinimide released during coupling.

ENZYME PURIFICATION

The chromatographic columns used in the enzyme purification procedures were obtained from Pharmacia Fine Chemicals and were fitted with water jackets. Diagram 1 shows the column arrangement for the enzyme fractionations. The enzyme solution to be separated was poured into the syringe barrel. By means of the 4-way valve and the flow adaptor the solution was placed directly onto the column packing. The eluent was allowed to pass through the column by turning the valve back again.

The absorbance at 280nm of the effluent was continuously monitored by a Cecil CE 212 Variable Wavelength Ultraviolet Monitor. Fractions were collected and stored at $4^{\circ}C$ until required.

Eluent buffer was normally 0.06M acetate. This was prepared by addition of sodium hydroxide to a 0.06M acetic acid solution until the pH was 5. Acetate buffer used for assay and kinetic studies was such that the ionic strength was 0.1M.

Column Arrangement for Fractionation of Enzymes

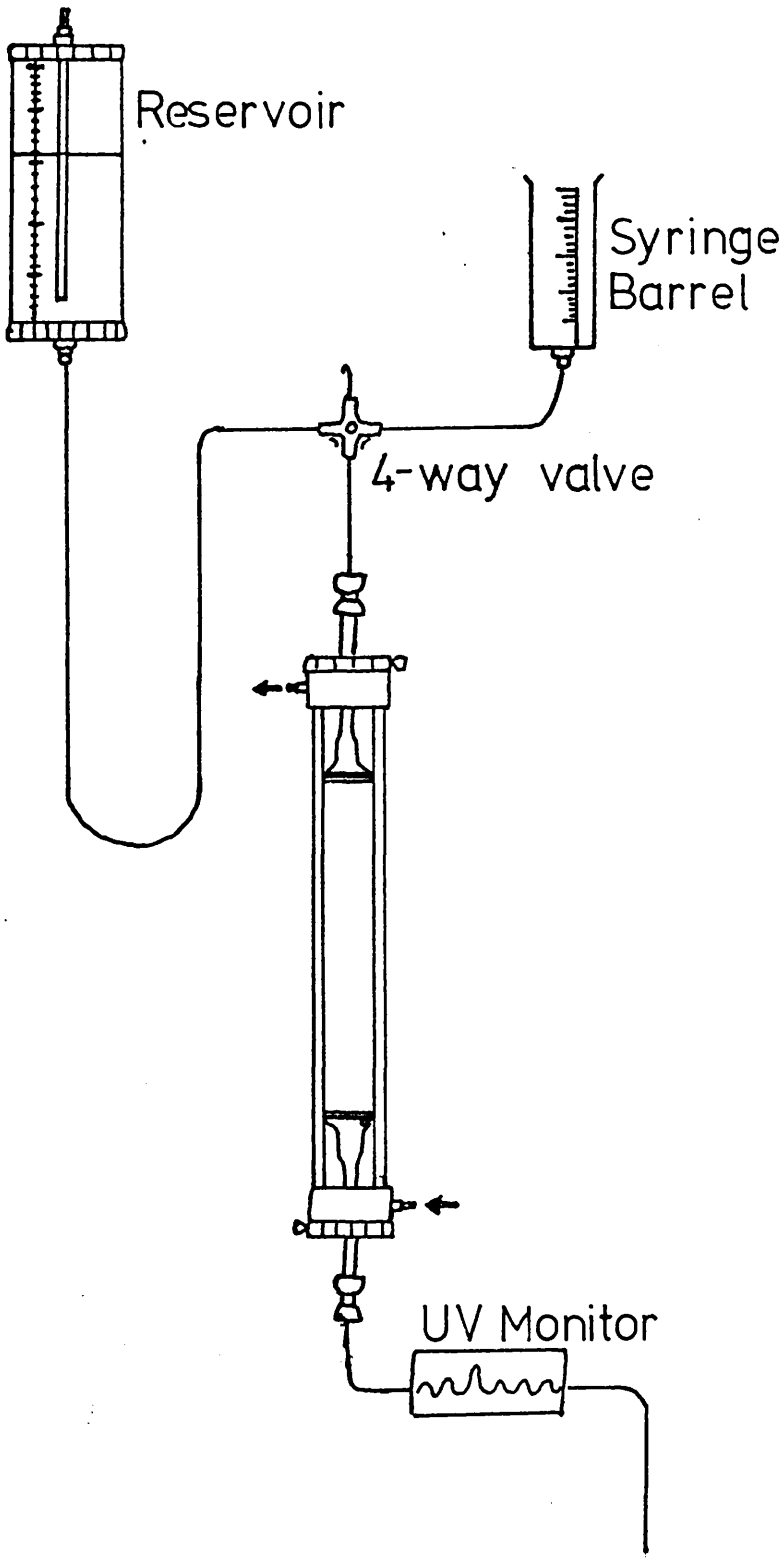


Diagram 1

Cellulase Enzyme Assays

Activity towards carboxymethylcellulose (CMC)

A solution of carboxymethlcellulose was prepared as follows. CMC (5.1g), low viscosity with degree of substitution 0.7-0.8 (BDH), was dissolved in 350ml of water at 90°C over a period of 2 hours. When the solution was cool 100ml of 0.5M citrate buffer (citric acid (4.375g) and sodium citrate (12.25g) in 100ml water) was added. Glucose (0.05g) was also added and the solution made up to 500ml. This gives 1% CMC, 0.1M citrate buffer pH 5 and 0.1mg/ml glucose. This glucose compensates for the destruction of reducing sugar.

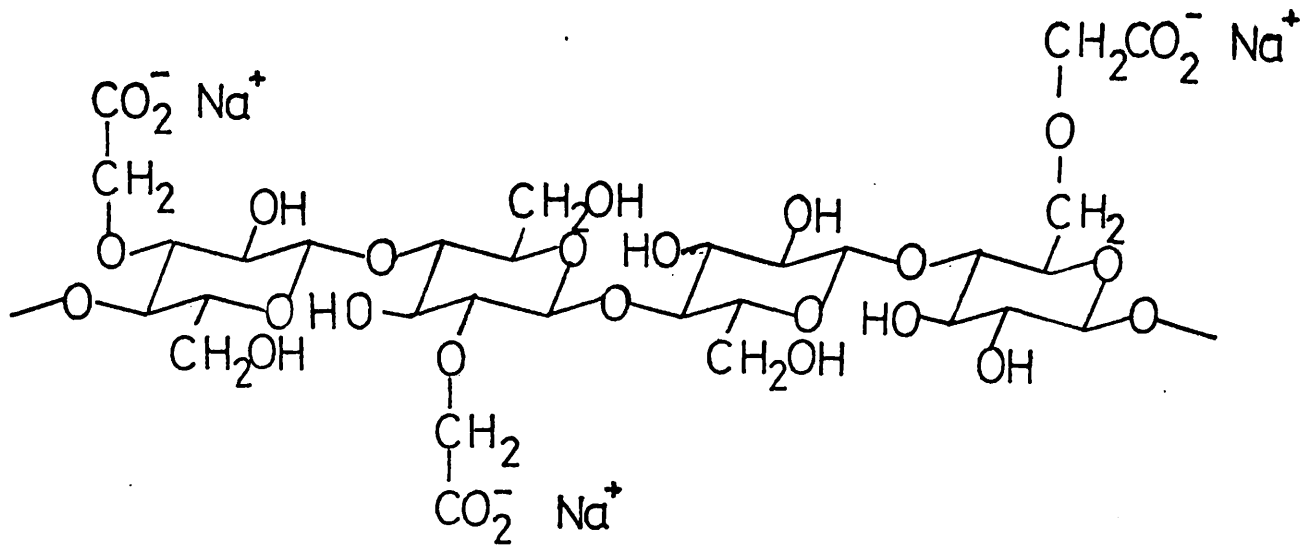
Figure 12 shows the idealised structure of carboxymethyl-cellulose with DS 0.75.

The amount of reducing sugar present was determined by the method of Miller et al. (146).

The reagent was made up as follows:-
3,5-Dinitrosalicylic acid (5g), phenol (1g), sodium sulphate (0.25g) and potassium sodium (+) tartrate (100g) were dissolved in a 2% sodium hydroxide solution (250ml) and the solution made up to 500ml.

The amount of reducing sugar present was determined by taking 2ml of the solution to be analysed and adding 3ml of the dinitro-salicylic acid reagent (DNSA), heating the solution in boiling water for 15 minutes and then measuring the absorbance at 640nm.

A calibration curve was obtained by preparing standard solutions of glucose over the range 0-1mg reducing sugar/ml and measuring the absorbance of the solution at 640nm after treatment with the DNSA reagent. From Figure 13 it can be seen that this gave a direct relationship over this range. The reducing power of the carboxymethyl cellulose solution was determined to be 0.02mg/ml (as glucose). The enzyme assay was carried out at room temperature.



Carboxymethyl cellulose

Idealised portion of chain of DS 0·75.

Figure 12

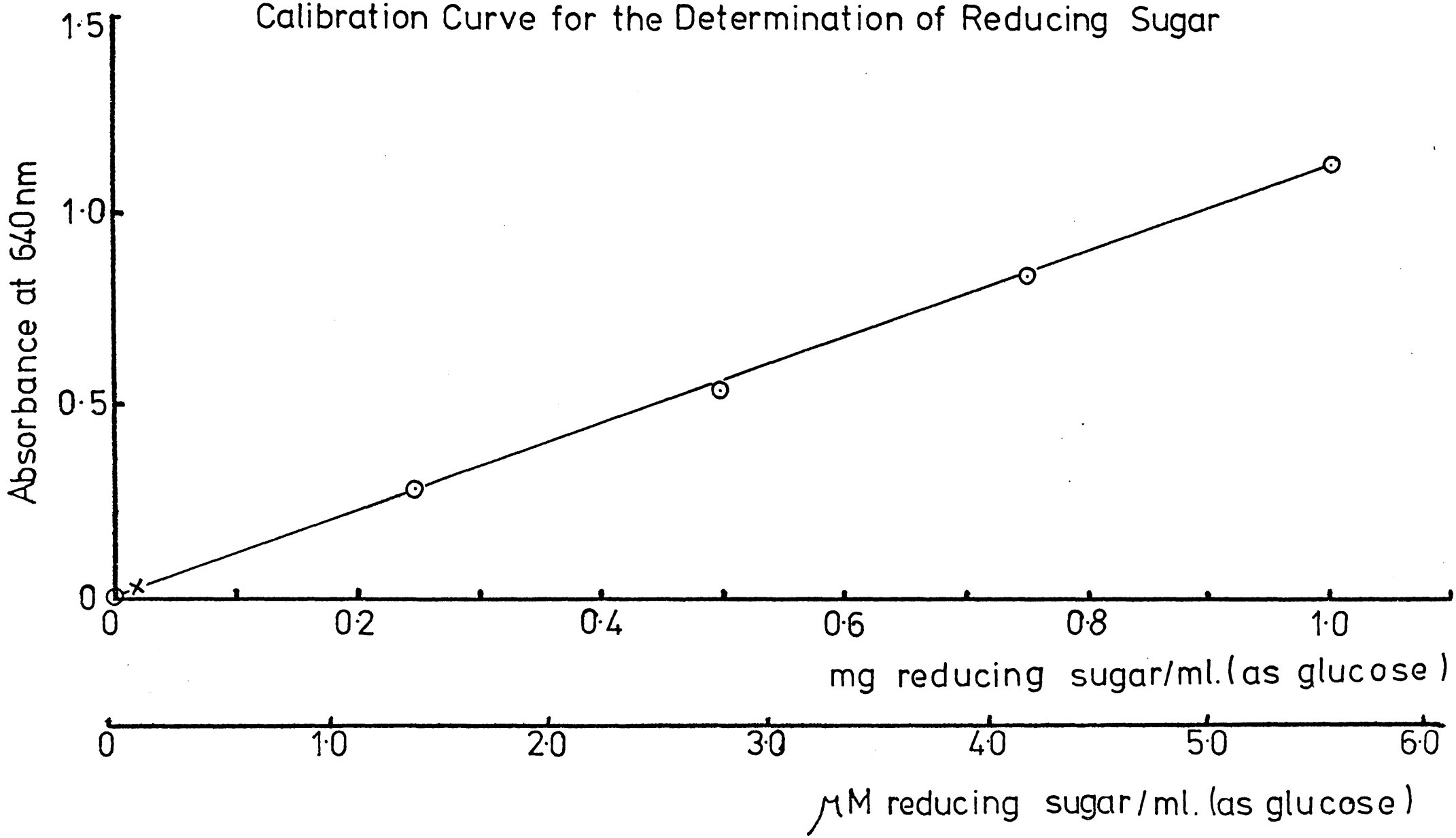

Calibration Curve for the Determination of Reducing Sugar
Absorbance at 640nm
1·5
1·0
0·5
0
0
0·2
0·4
0·6
0·8
1·0
mg reducing sugar/ml. (as glucose)
0
1·0
2·0
3·0
4·0
5·0
6·0
μM reducing sugar/ml. (as glucose)

Figure 13

For enzyme assays, 1ml of the CMC solution was placed in a 10ml conical flask and 1ml of the enzyme solution added. This was incubated for a specific time (usually 15 minutes), treated with the DNSA reagent and the absorbance measured. From the calibration curve the amount of reducing sugar produced could be determined. The units of CMC-ase activity are given in μ moles reducing sugar produced/A_{280} /minute. (Absorbance of the enzyme is before dilution).

Activity towards Avicel (Microcrystalline cellulose)

Avicel-hydrolysing activity was assayed by measuring the amount of reducing sugar produced from Avicel (FMC Corporation, American Viscose Division, Marcus Hook, Pennsylvania). The reaction mixture contained 70mg of Avicel, 3ml of 0.1M acetate buffer, pH 5, and 1ml of the enzyme solution. The mixture was incubated at 30°C for 7 days, then filtered. To 2ml of the filtrate, 3ml of the DNSA reagent was added and the mixture heated in boiling water for 15 minutes, cooled and the absorbance read at 640nm.

One unit of 'Avicelase' activity was defined as μmoles reducing sugar produced/A_{280} /day.

β-Glucosidase activity

This was determined using 3,4-dinitrophenyl β-D-glucopyranoside as substrate. A stock solution of 2×10^{-3}M glucoside was made up in 0.1M acetate buffer, pH 5. 100μl was added to the enzyme solution in a 10mm UV cell. The absorbance at 400nm was measured on the Cecil CE 212 Variable Wavelength Ultraviolet Monitor. The output was connected to the chart recorder, giving a graph of absorbance at 400nm against time. From the graph the β-glucosidase activity can be measured from the initial slope. The β-glucosidase activity is given in μmoles 3,4-dinitrophenol produced/A_{280} /minute. The temperature in the UV cell chamber was between 20 and 25°C.

Activity towards 3,4-dinitrophenyl cello-oligosaccharides

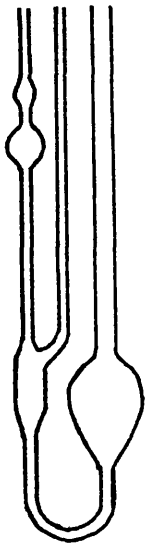
An assay using the series of aryl glycosides was carried out as follows.

Stock solutions (2×10^{-3}M) of 3,4-dinitrophenyl β-D-gluco-

pyStock solutions (2×10^{-3}M) of 3,4-dinitrophenyl β-D-gluco-xyl β-cellotrioside and 3,4-dinitrophenyl β-cellotetraoside were made up in 0.1M acetate buffer, pH 5. 200μl of the enzyme solution to be assayed was added to 2.3ml of 0.1M acetate buffer, pH 5, in a 10mm UV cell. 100μl of the stock solution of the aryl glycoside was added and a graph of the absorbance at 400nm against time, which indicates the rate of release of 3,4-dinitrophenol from the glycoside, obtained. This was done for each substrate in turn and the graphs superimposed. The temperature of the cell chamber was 20 - 25°C.

Viscometric assay

The 1% carboxymethylcellulose solution, pH 5 described earlier was used in the viscosity studies.



Ubbelohde Viscometer

CMC solution (40ml) was poured into an Ubbelohde capillary viscometer and the enzyme solution (0.25-1.0ml) to be assayed, added. The specific viscosity (η_{sp}) was measured as a function of time and the results graphed. The specific viscosity was determined from the equation

$$\eta_{sp} = \frac{t - t_o}{t_o}$$

where t = time for the CMC solution to pass down through the capillary, and

t_o = time for an equivalent solution of the monomer, in this case a 1% glucose solution, to pass through the same capillary. This reaction was performed at room temperature.

Purification of the Enzymes

Commercial cellulase from Trichoderma viride (B.D.H.) was extracted with 0.06M acetate buffer, pH 5 (1g in 30ml). The extract was filtered through a sintered glass filter. This gave the initial crude cellulase solution. Its activity against cellulolytic substrates is shown in Table 5.

From the rate of release of 3,4-dinitrophenol from the aryl glycosides it can be seen that the crude cellulase solution is rich in β-glucosidase activity. The release of the phenol from $34DNPG_2$ and $34DNPG_4$ shows an induction period. This presumably occurs because β-glucosidases remove glucose units from the non-reducing end of the cello-oligosaccharide chain. Only with $34DNPG_1$ is there no discernible induction period. If only β-glucosidases were present the aryl glycoside bond of $34DNPG_2$ should be hydrolysed before $34DNPG_4$ but as can be seen from the graph this is not so. Clearly another enzyme (or enzymes) is present which requires more than two glucose units in the chain for optimum substrate properties. These results are shown in Figure 14.

DEAE-Sephadex A-25 column chromatography

Figure 15 shows the result from chromatography of the enzyme after glass-filtration. The crude cellulase solution (25ml) was applied to a column (2.6cm x 70cm) of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) equilibrated with 0.06M acetate buffer, pH 5. The column was eluted at a rate of 1ml/minute with the same buffer. A salt gradient was applied as shown in the diagram to elute the more tightly bound enzymes. The fractions FI to FVII, as shown in the diagram, were assayed towards the various cellulolytic substrates. The results are shown in Table 5.

From the graph of absorbance at 400nm against time for the

Graph of Absorbance against Time for the Reaction of the 34-Dinitrophenyl Glycosides with the Crude Cellulase

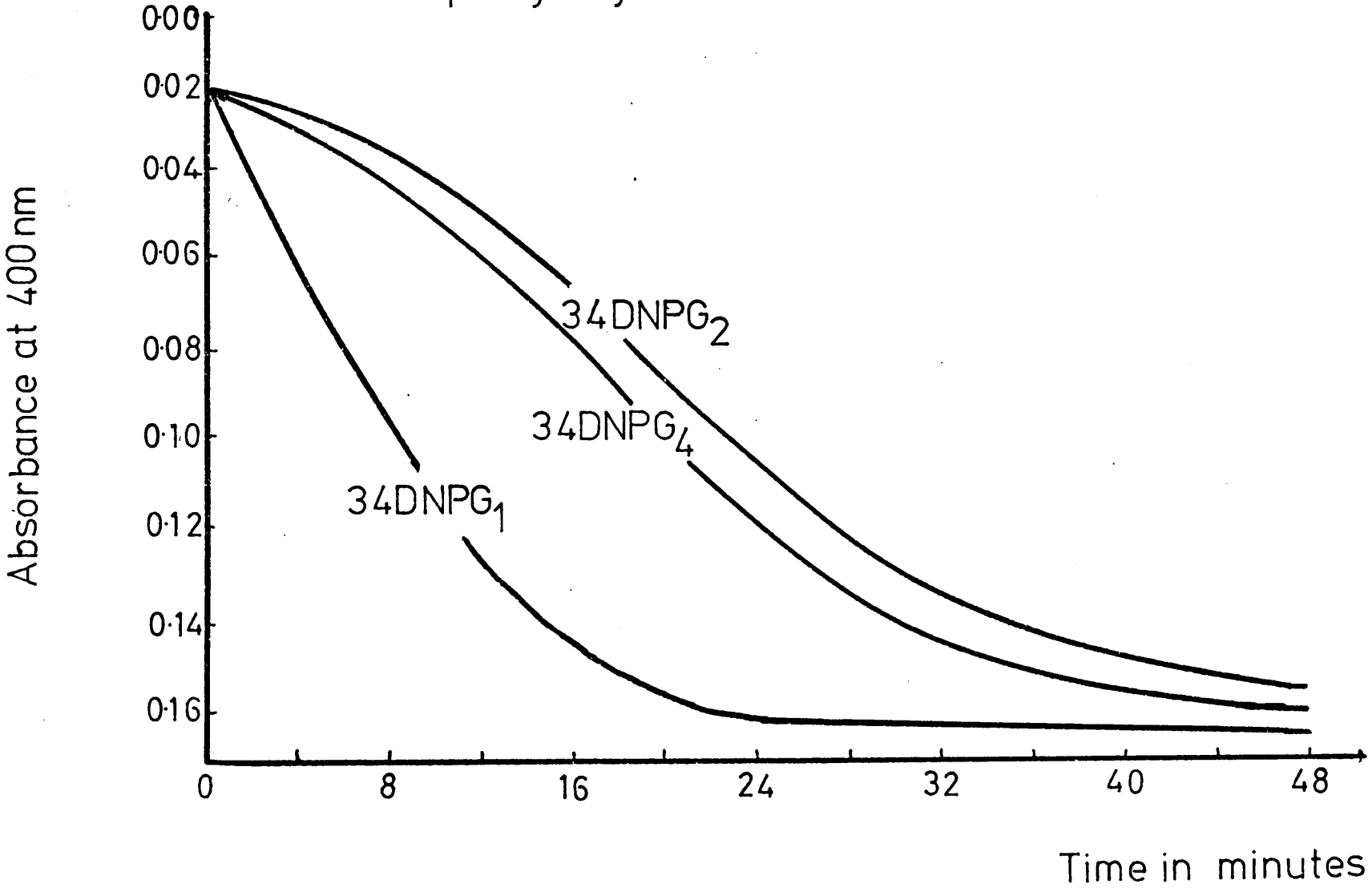


Figure 14

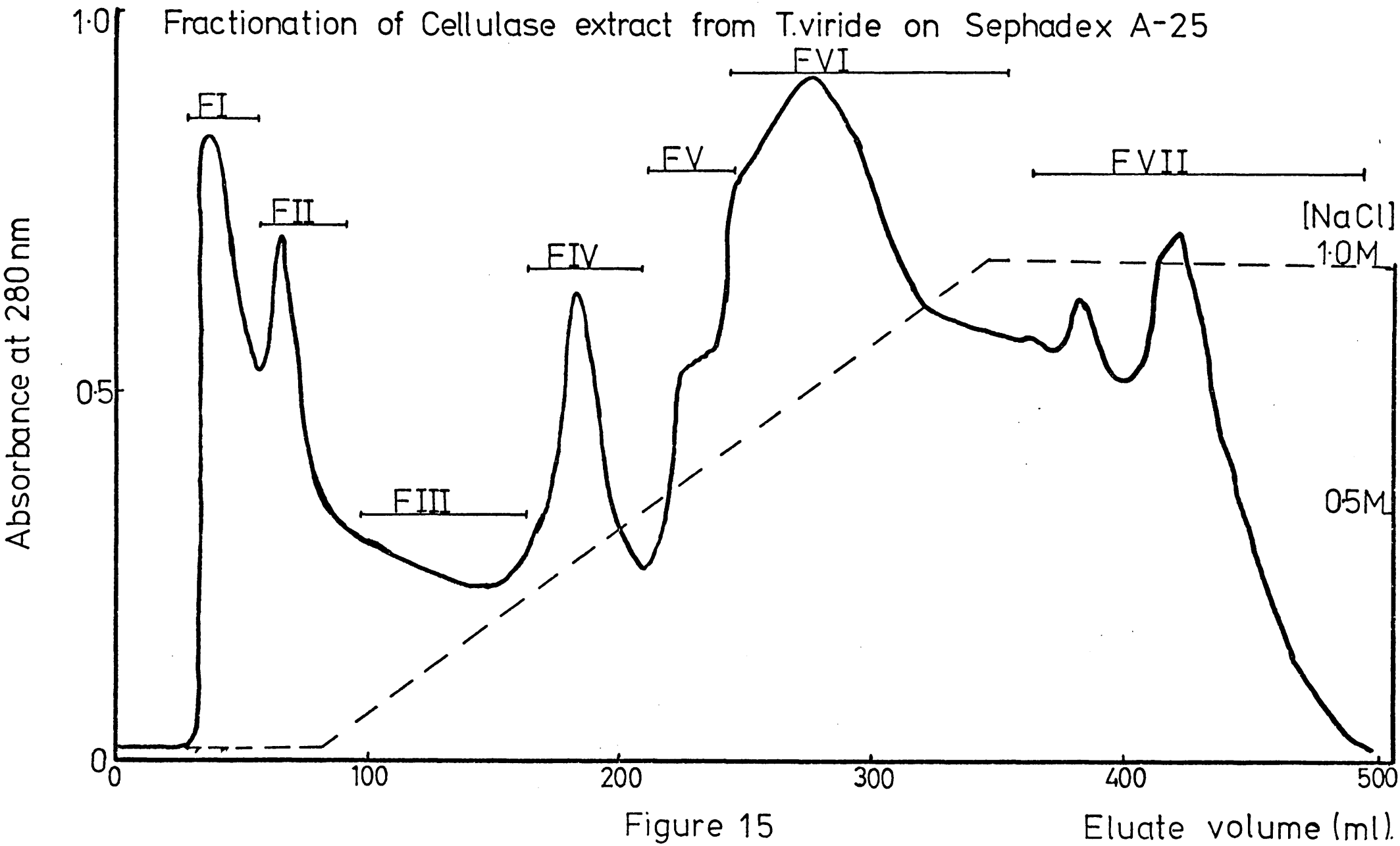

Fractionation of Cellulase extract from T.viride on Sephadex A-25
1·0
0·5
0
Absorbance at 280 nm
FI
FII
FIII
FIV
FV
FVI
FVII
[NaCl]
1·0M
0·5M
0
100
200
300
400
500
Eluate volume (ml)

Figure 15

Graph of Absorbance against Time for the Reaction of the 3 4-Dinitrophenyl Glycosides with Fraction FI

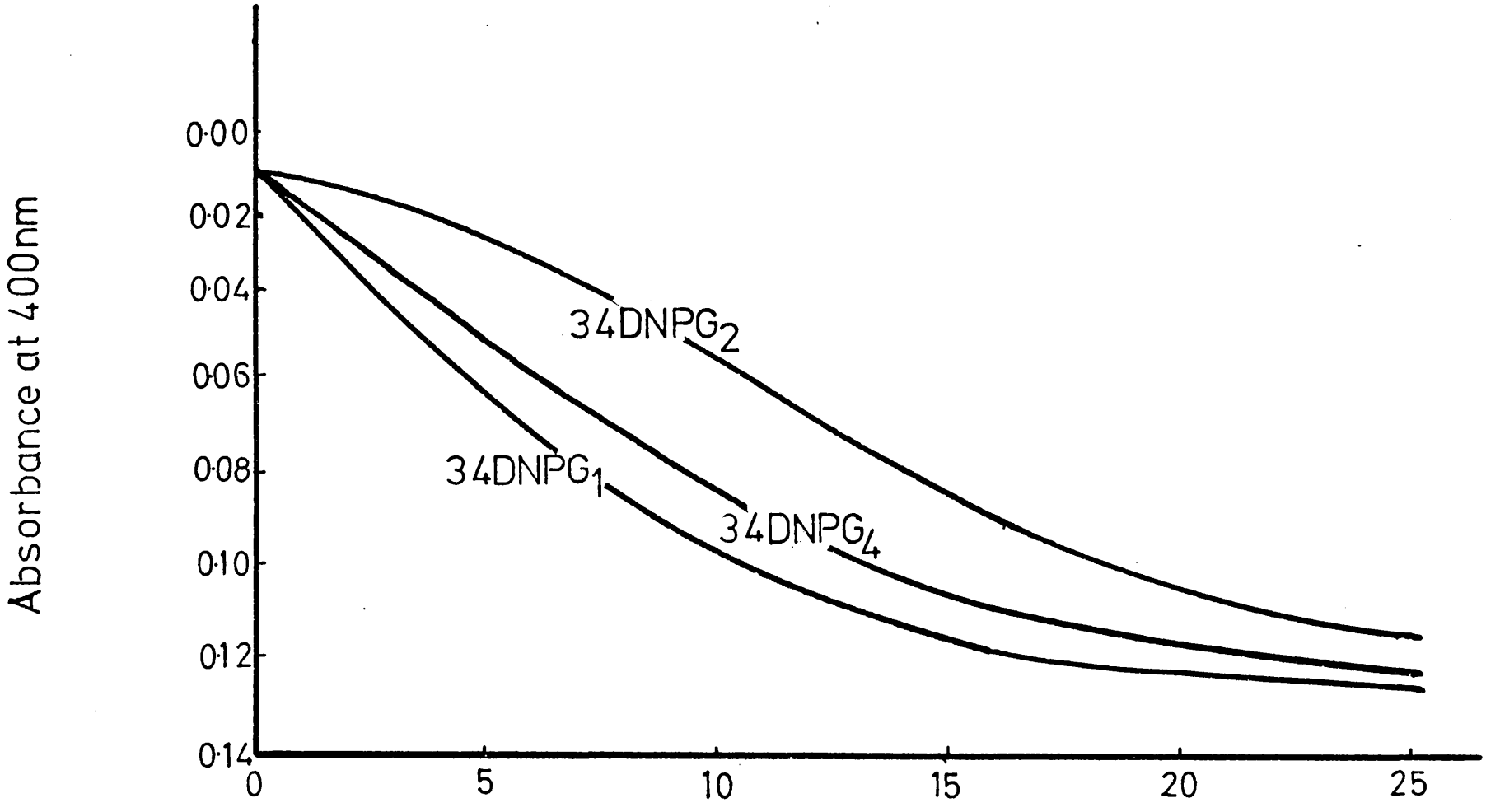


Figure 16

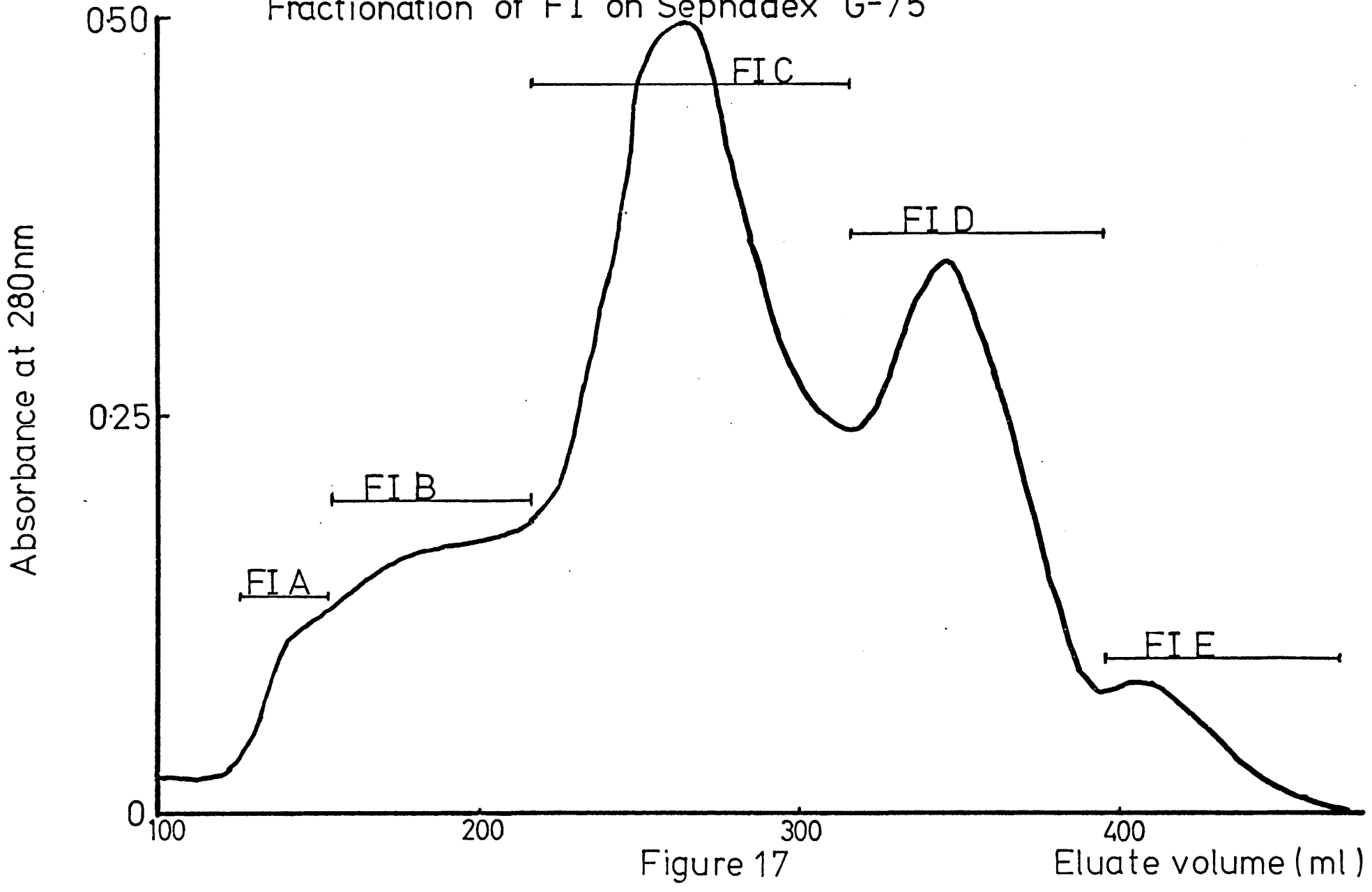

Fractionation of FI on Sephadex G-75
0·50
0·25
0
Absorbance at 280nm
FI A
FI B
FI C
FI D
FI E
100
200
300
400
Eluate volume (ml)

Figure 17

Graph of Absorbance against Time for the Reaction of the 34-Dinitrophenyl Glycosides with Fraction FIC

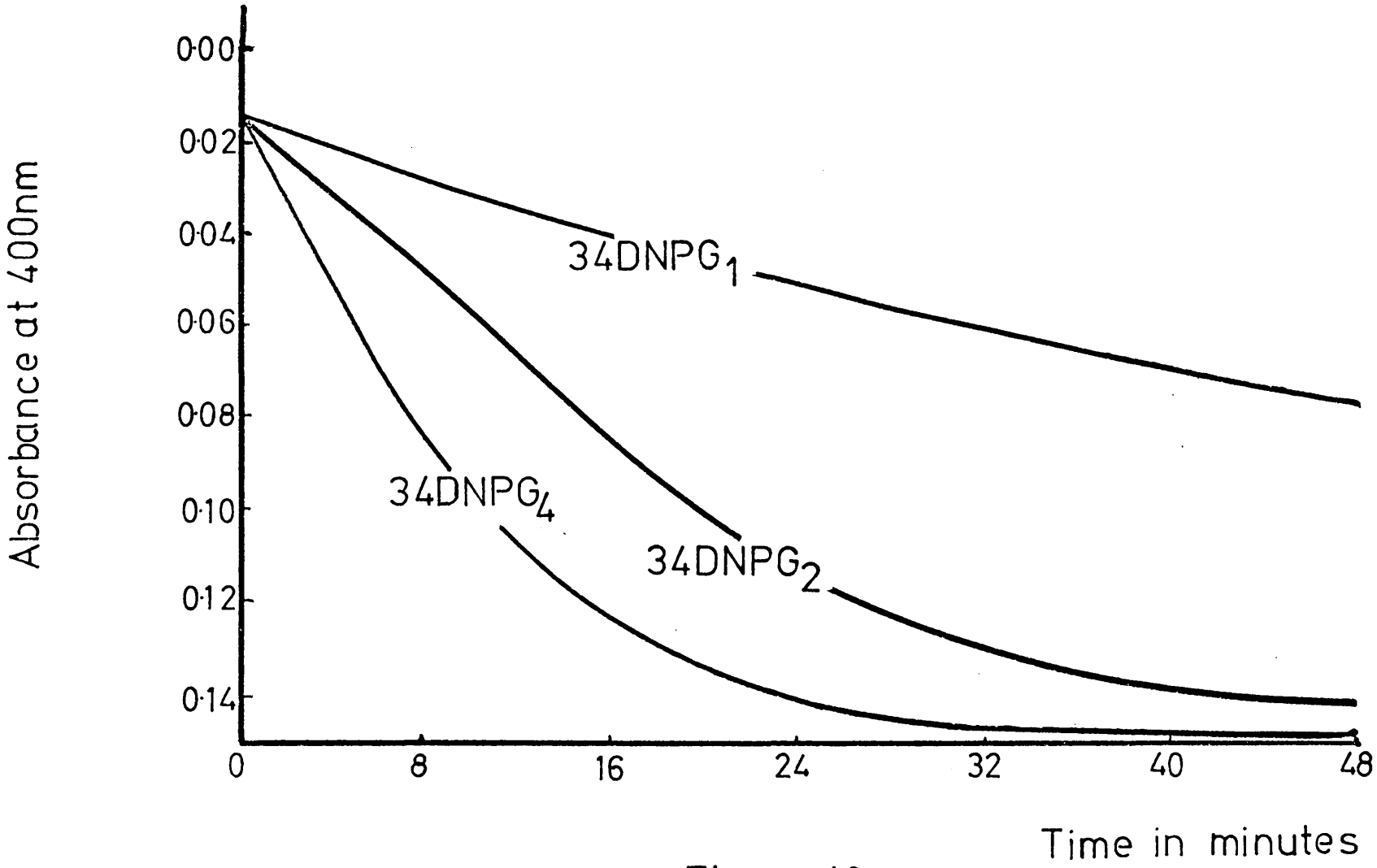


Figure 18

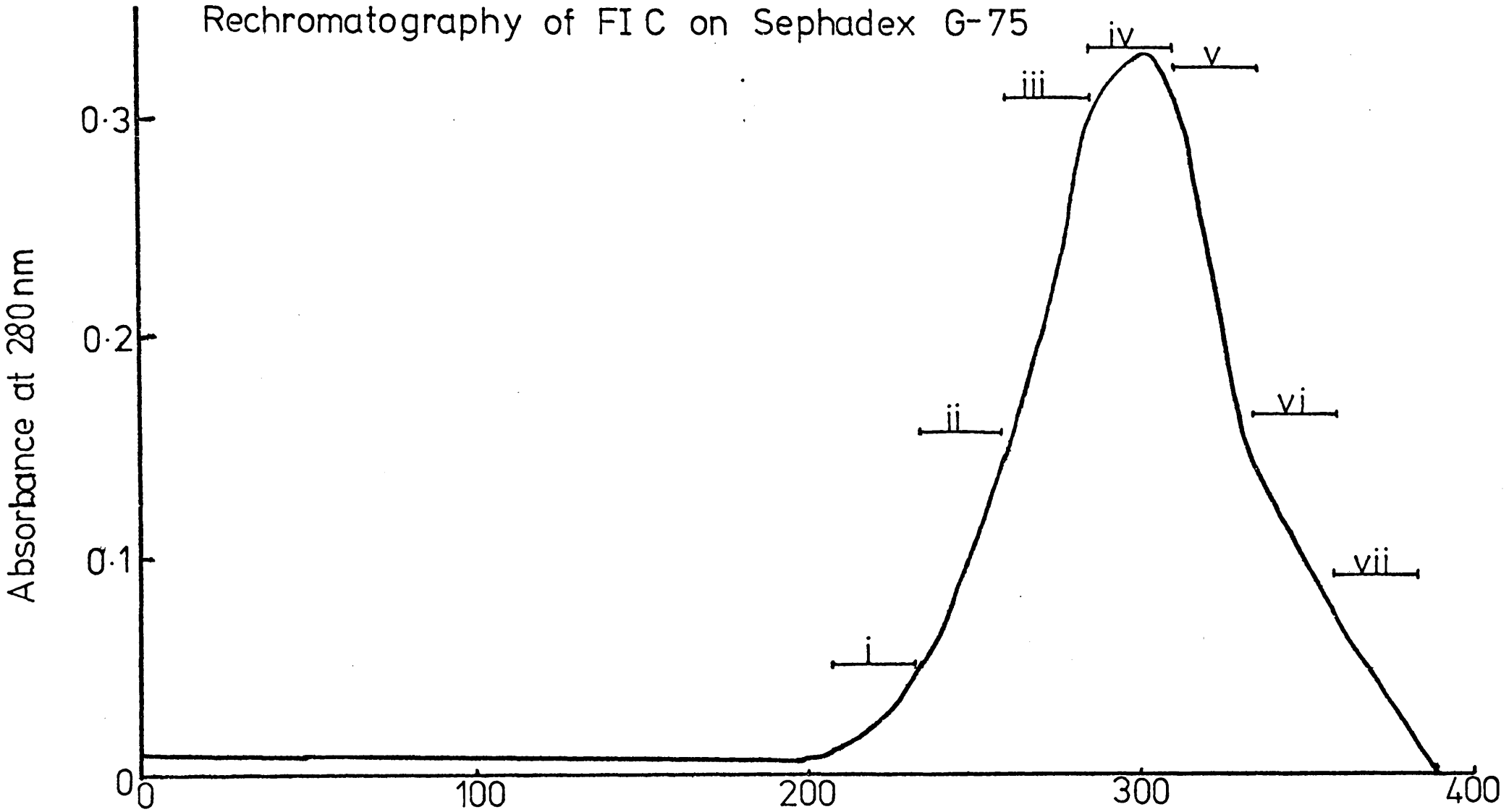

Rechromatography of FIC on Sephadex G-75
Absorbance at 280nm
0·3
0·2
0·1
0
0
100
200
300
400
Eluate volume (ml)
i
ii
iii
iv
v
vi
vii

Figure 19

TABLE 5

CELLULASE ACTIVITIES IN EACH FRACTION AFTER DEAE-SEPHADEX A-25 CHROMATOGRAPHY OF THE CRUDE

	Crude	FI	FII	FIII	FIV	FV	FVI	FVII
Avicelase	1.48	0.91	0.6	0.66	0.08	0.44	0.17	-
CMCase	0.39	0.60	1.74	3.61	0.51	0.20	0.23	-
β-Glucosidase	1.77	5.88	6.00	2.67	-	-	0.40	-
β-Cello-tetraosidase	0.10	3.68	1.25	-	-	-	0.24	-

TABLE 6

CELLULASE ACTIVITIES IN EACH FRACTION AFTER SEPHADEX G-75 CHROMATOGRAPHY OF FRACTION FI

	FI	FIA	FIB	FIC	FID	FIE
Avicelase	0.91	0.09	0.34	0.11	-	-
CMCase	0.60	3.86	0.92	0.49	2.66	0.02
β-Glucosidase	5.88	39.0	9.54	1.01	1.00	-
β-Cellotetrao-sidase	3.68	0.48	1.37	6.91	0.88	-

TABLE 7

CELLOTETRAOSIDASE/GLUCOSIDASE ACTIVITIES FOR EACH FRACTION AFTER RECHROMATOGRAPHY ON SEPHADEX G-75 OF FRACTION FIC

	FICi	FICii	FICiii	FICiv	FICv	FICvi	FICvii
$\frac{\beta\text{-Cellotetraosidase}}{\beta\text{-Glucosidase}}$	0.94	1.79	9.98	18.1	9.50	6.92	3.45

reaction of the aryl glycosides with fraction FI, it can be seen that $34DNPG_2$ still shows an induction period but now $34DNPG_4$ does not. The most important enzyme in this fraction is still a β-glucosidase. This is shown in Figure 16.

Sephadex G-75 column chromatography

Fraction FI was lyophilised and the lyophilised powder dissolved in 10ml of 0.06M acetate buffer, pH 5. There was almost no loss of activity. This was applied to a column (2.6cm x 70cm) of Sephadex G-75 (Pharmacia Fine Chemicals) equilibrated with 0.06M acetate buffer, pH 5. Figure 17 shows the result of this fractionation. Fractions FIA to FIE were assayed towards the cellulolytic substrates. The results are shown in Table 6. From the graph of absorbance at 400nm against time for the reaction of fraction FIC with the aryl glycosides, it can be seen that the aryl glycoside bond of $34DNPG_4$ is now hydrolysed faster than that of $34DNPG_1$. This clearly shows that an enzyme other than a β-glucosidase is present in this fraction. This is shown in Figure 18.

Rechromatography on Sephadex G-75

Fraction FIC was lyophilised and the lyophilised powder dissolved in 10ml 0.06M acetate buffer, pH 5. This was applied to the same Sephadex G-75 column as above. Figure 19 shows the result of rechromatography of FIC. Fractions of 10ml were collected and labelled FICi to FICvii. Table 7 shows the ratio of activities of the various enzyme fractions towards $34DNPG_4$ and $34DNPG_1$. As can be seen fraction FICiv shows an 18 fold increase in activity towards the aryl cellotetraoside compared with the glucoside. To remove the β-glucosidase fraction, fraction FICiv was passed through an affinity column specific for β-glucosidases.

Affinity chromatography

The affinity column used was that described in the Preparative Experimental section.

To be sure that the column was specific for β-glucosidases, a sample of fraction FI was passed through the column. The column was equilibrated with 0.06M acetate buffer, pH 5. By means of the 4-way valve, fraction FI (2ml) was applied to the column and eluted with the same buffer. The column was successively eluted with 0.2M glucose, pH 5 and 0.2M cellobiose, pH 5. The result is shown in Figure 20. The glucose solution was given time to equilibrate before being used but, as can be seen, was ineffective in removal of the enzyme. Elution of the column with cellobiose solution caused gradual release of the enzyme as measured by its absorbance at 280nm. When the column was eluted with 1M sodium chloride, pH 5 instead of cellobiose solution the enzyme was released from the column in a small volume. The protein which passed through the column showed little activity towards 3,4-dinitrophenyl β-D-glucopyranoside compared with the original fraction. The enzyme eluted with cellobiose caused hydrolysis of the aryl glucopyranoside but the activity was somewhat reduced. This reduced activity is presumably caused by cellobiose acting as a competing substrate. The column therefore acts as an affinity column for β-glucosidases.

The column was regenerated by eluting with 0.06M acetate buffer, pH 5.

Fraction FICiv (10ml) was applied to the column and eluted with the acetate buffer. The majority of the protein passed through the column and, on elution with 0.2M cellobiose, a small amount of β-glucosidase was released. This can be seen in Figure 21. The enzyme fractions were labelled FICivα and FICivβ respectively.

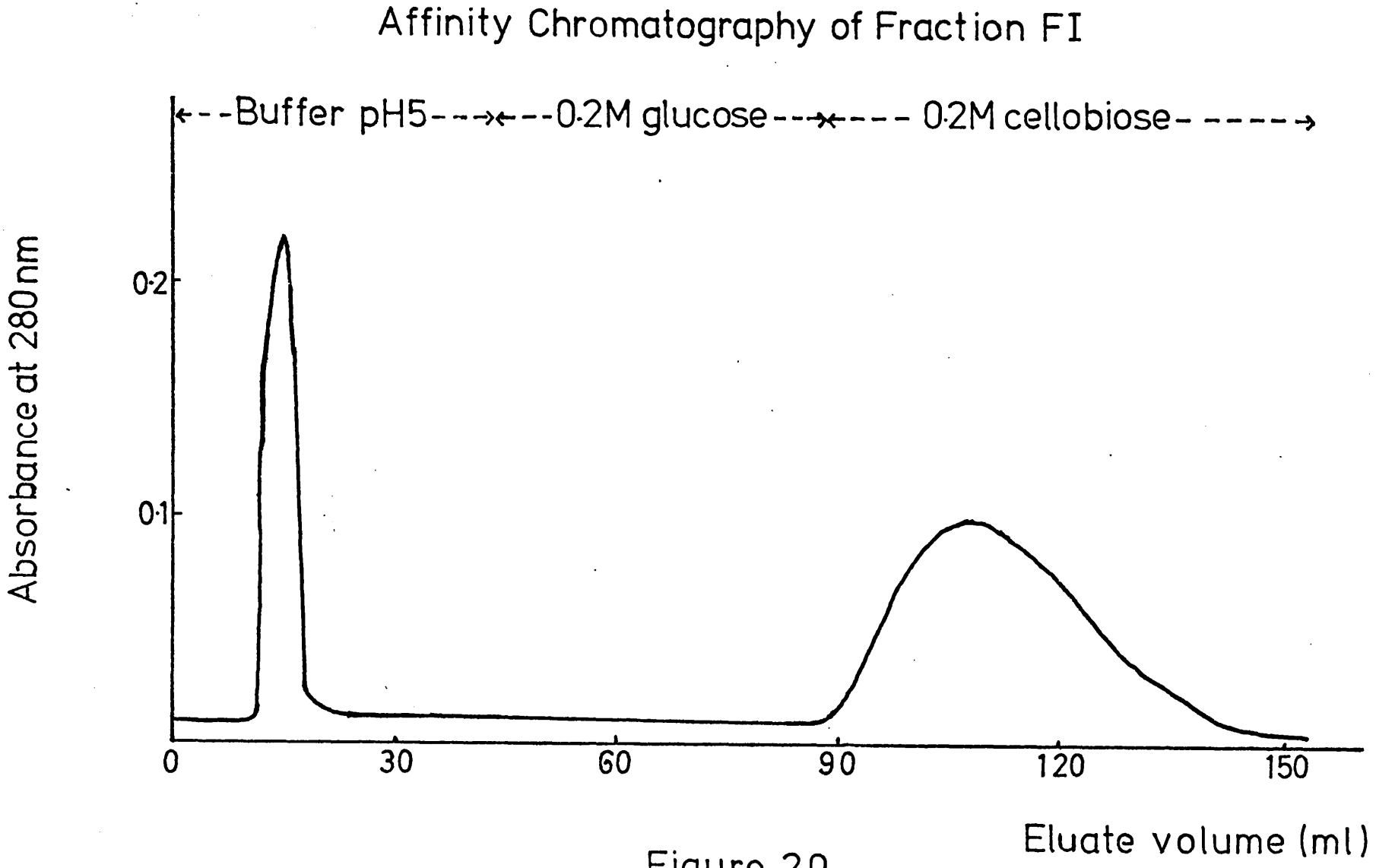

Affinity Chromatography of Fraction FI
←--Buffer pH5--→←--0·2M glucose--→←---- 0·2M cellobiose- ----→
Absorbance at 280 nm
0·2
0·1
0
30
60
90
120
150
Eluate volume (ml)

Figure 20

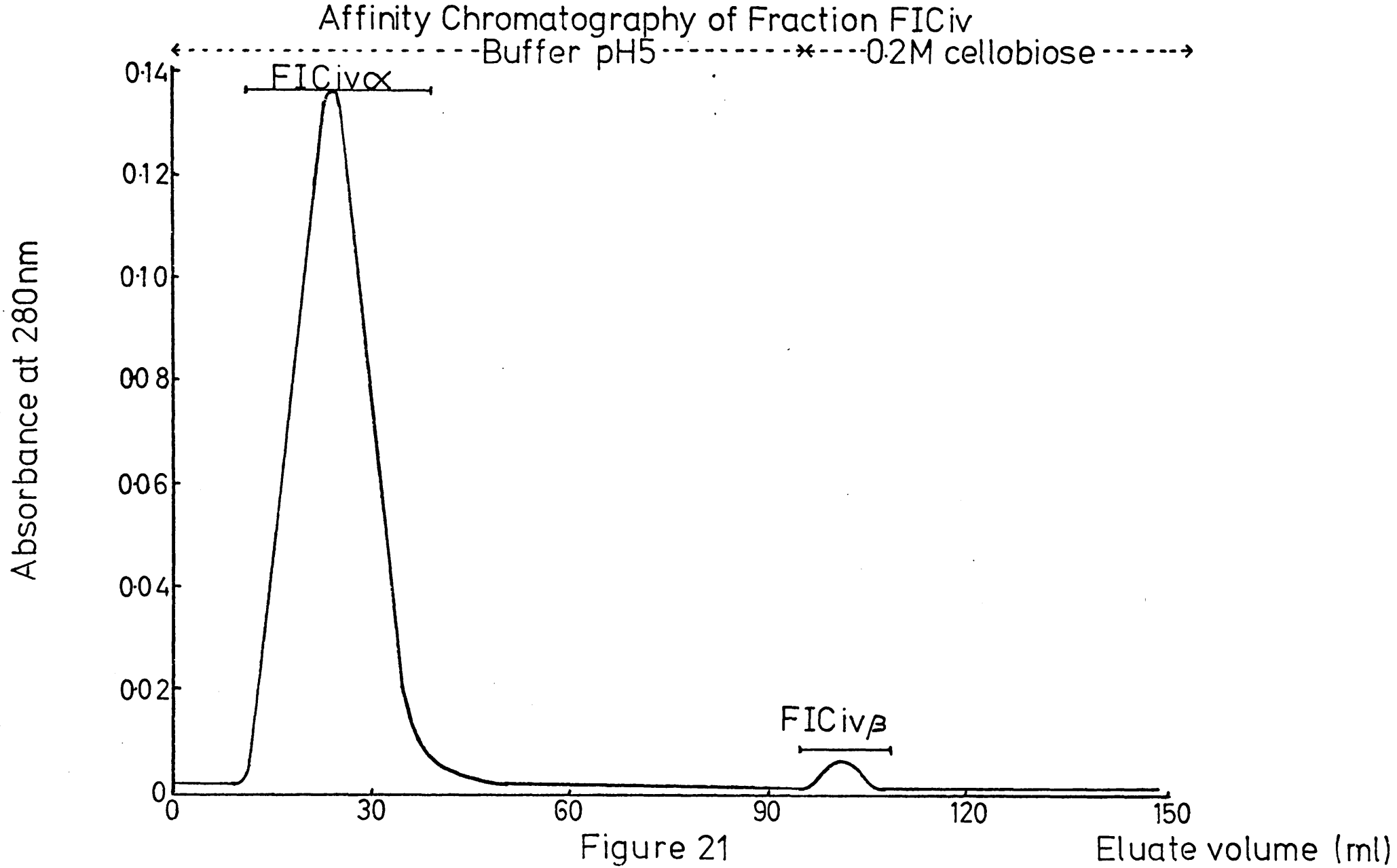

Affinity Chromatography of Fraction FICiv
Buffer pH5
0·2M cellobiose
FICivα
FICivβ
Absorbance at 280nm
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
0
30
60
90
120
150
Eluate volume (ml)

Figure 21

TABLE 8

CELLULASE ACTIVITIES OF FRACTION FICivα

Avicelase	-
CMCase	0.68
β-Glucosidase	0.078
β-Cellotetraosidase	10.4
$\frac{\beta\text{-Cellotetraosidase}}{\beta\text{-Glucosidase}}$	133

TABLE 9

PURIFICATION OF CELLULASE EI FROM TRICHODERMA VIRIDE

Purification Step	Activity towards $34DNPG_4$	
1. Crude	0.10	(1)
2. DEAE-Sephadex A-25	3.68	(36.8)
3. Sephadex G-75	6.91	(69.1)
4. Rechromatography on Sephadex G-75		
5. Affinity column	10.40	(104)

Graph of Absorbance against Time for the Reaction of the 34-Dinitrophenyl Glycosides with Fraction FIC iv α

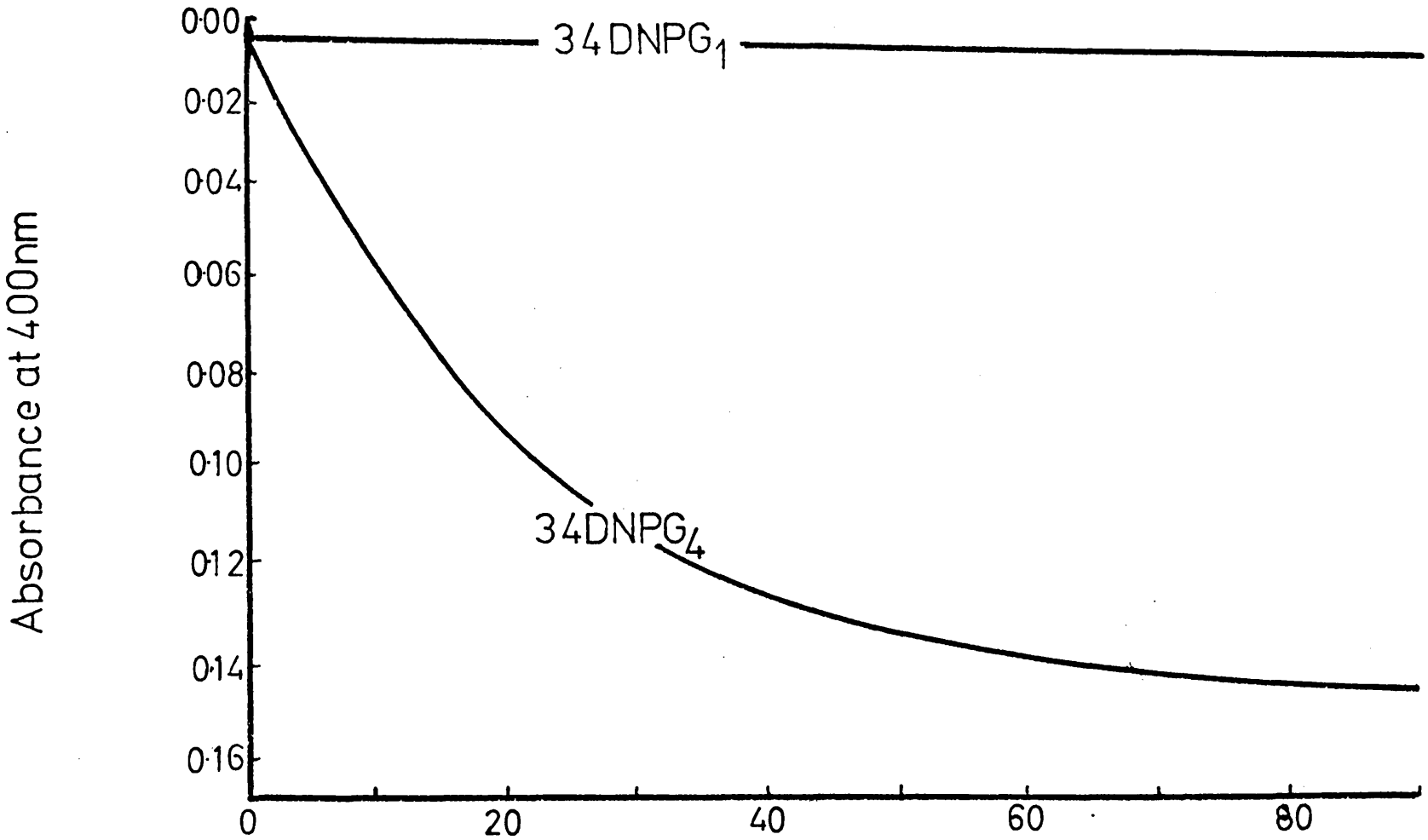


Figure 22

Protein peak FICivα was designated as Cellulase EI. Cellulase EI was assayed towards the various cellulolytic substrates. The results are shown in Table 8 and Figure 22.

Enzyme Purity 1) SDS-Gel Electrophoresis (147).

The purified cellulase was subjected to electrophoresis in polyacrylamide gel (7.5%, w/v) in the presence of sodium dodecyl sulphate (SDS) buffer, pH 6.6. The pre-cast gel was obtained from Bio-Rad Laboratories and measured 5.5mm x 100mm.

The protein was mixed with glycerol, SDS buffer and bromophenol blue (Bio-Rad Laboratories). Bromophenol blue acted as tracker dye. The sample (100 µl) was applied to the top of the gel. Electrophoresis was conducted at a constant current of 12mA per gel for about 3 hours. The gel was stained with Coomassie Brilliant Blue (Bio-Rad Laboratories) and destained by standing in 7.5% acetic acid, 5.0% methanol. The results showed only one band to be present at the lower end of the gel.

2) Molecular Sieve Chromatography

Cellulase EI behaved as a single protein on passage through both Sephadex G-75 and Sephadex G-100 columns (2.6cm x 70cm).

Determination of Molecular Weight of Cellulase EI

A column (2.6cm x 70cm) of Sephadex G-100 was equilibrated with 0.06M acetate buffer, pH 5. The void volume (Vo) of the column was estimated with Blue Dextran 2,000 (mol. wt. 2,000,000; Pharmacia Fine Chemicals). The column was calibrated with three marker proteins (Sigma); cytochrome c (mol. wt. 12,400), trypsin (mol. wt. 23,000) and ovalbumin (mol. wt. 45,000).

A few milligrams of the marker proteins were dissolved in 2.0ml of the purified enzyme solution, applied to the column and then eluted with the same buffer at a rate of 1ml/5minutes. The proteins were located in the effluent by their absorbance at 280nm.

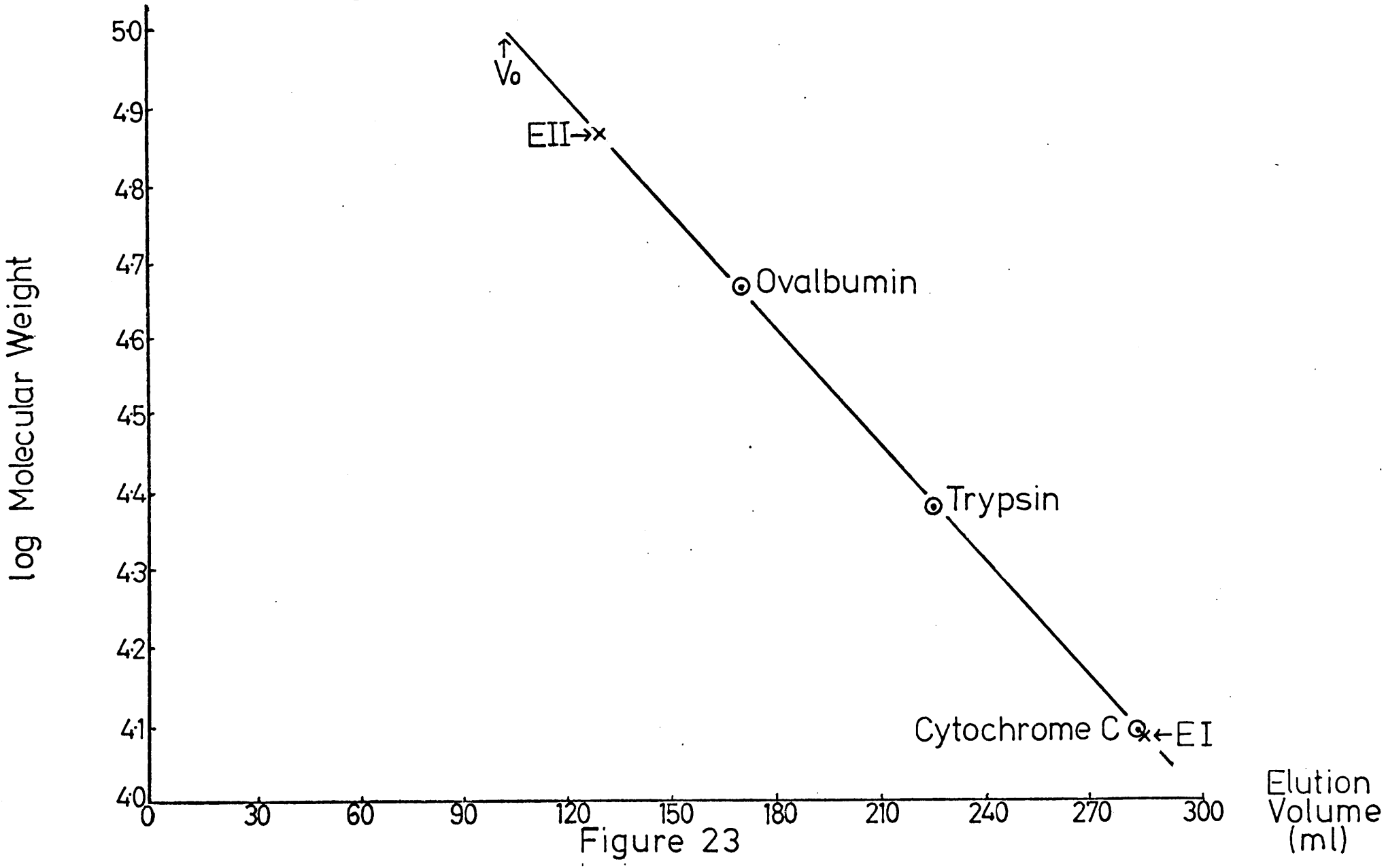

Molecular Weight Estimation of EI and EII by Sephadex G-100 Chromatography
log Molecular Weight
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
Vo
EII
Ovalbumin
Trypsin
Cytochrome C
EI
0
30
60
90
120
150
180
210
240
270
300
Elution Volume (ml)

Figure 23

The elution volume (Ve) was calculated from the mid-point of the peak. The result is shown in Figure 23. The molecular weight of cellulase EI was estimated to be about 12,000.

See introduction to 'KINETIC EXPERIMENTAL' for the computerised methods of determining the rates of reaction using the spectrophotometric substrates.

pH Optimum

Figure 24 shows the effect of pH on the hydrolytic activity of cellulase EI towards 3,4-dinitrophenyl β-cellotetraoside at 40°C. The release of the phenol was measured by its absorbance at 400nm. The extinction coefficient of 3,4-dinitrophenol at the various pHs was determined by adding 20μl of a stock solution of the phenol to 2.5ml of the buffer and measuring its absorbance directly on the Cary 16. The pH, extinction coefficient of 3,4-dinitrophenol and the rate of hydrolysis of $34DNPG_4$ are listed in Table 10.

pH optimum is between 4.5 and 5.5. The activity falls off more rapidly on the acidic side than the basic side of the plateau.

Temperature Optimum

Acetate buffer (2.3ml) pH 5.02, I = 0.1 was thermostatted in the Cary 16 cell chamber at the temperature under study. 5μl of the stock enzyme solution was added to the UV cell. The system was left for 10 minutes and then 200μl of the stock solution of $34DNPG_4$ added.

Figure 25 shows the effect of this treatment on the activity of cellulase EI from 20-70°C. The temperature/rate data is listed in Table 11.

Optimum temperature for the activity of cellulase EI was found to be 60°C.

No loss of activity was observed after 24 hours at 40°C.

Effect of pH and Temperature on the Activity of Cellulase EI

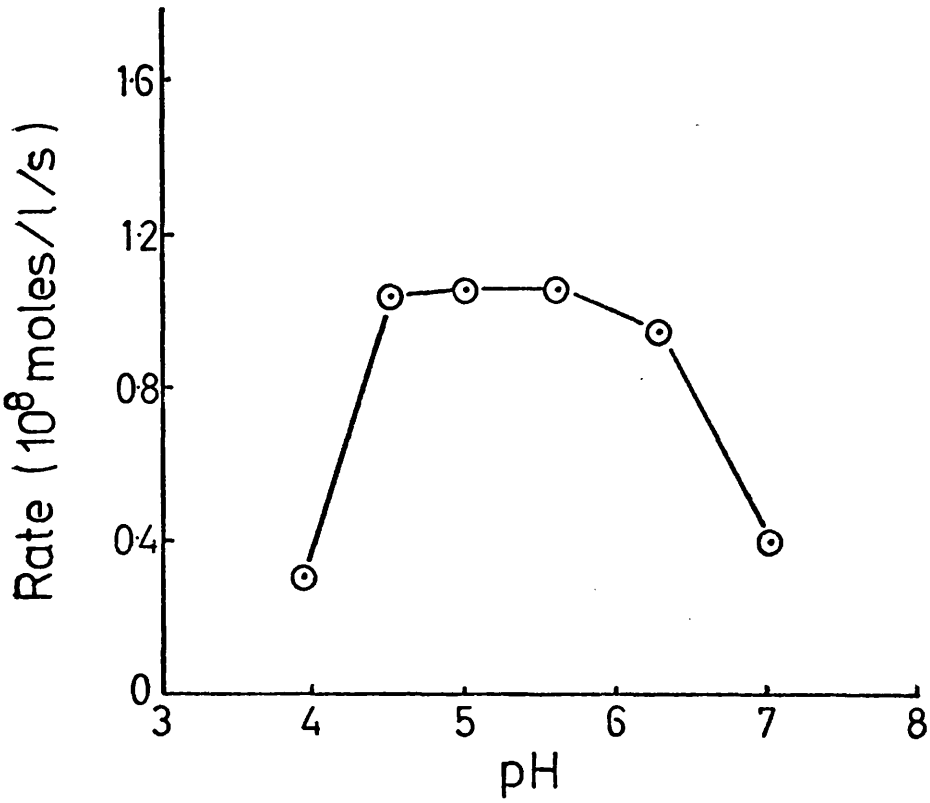


Figure 24

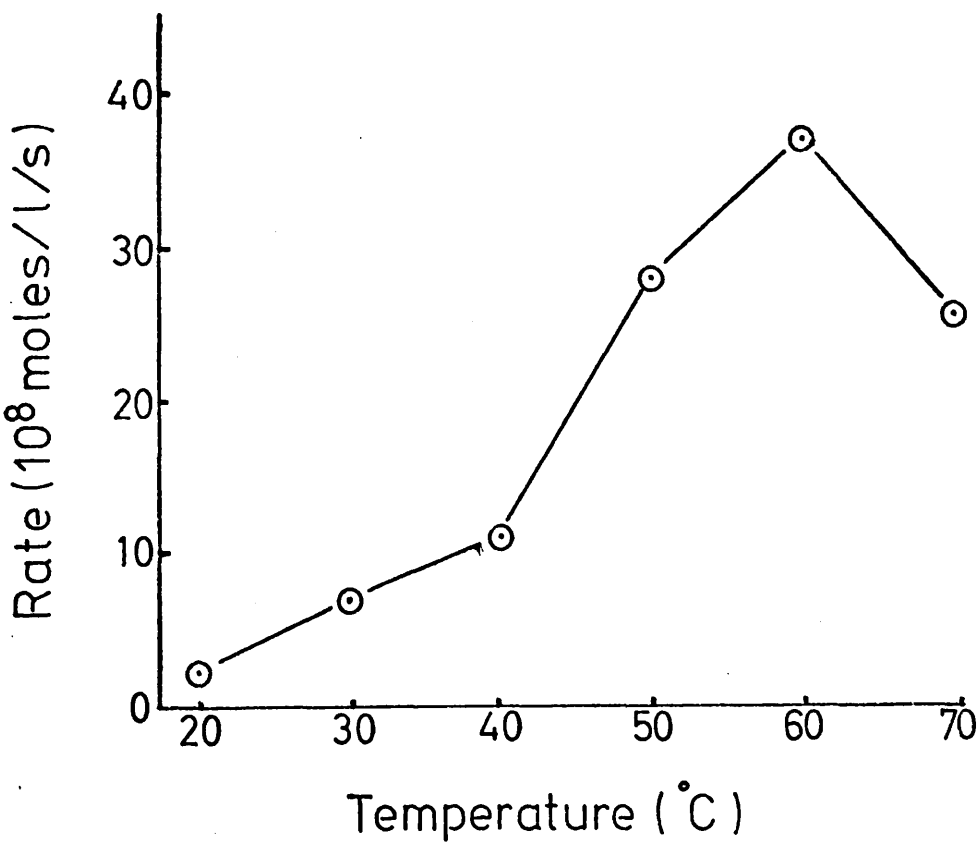


Figure 25

TABLE 10

Effect of pH on the Activity of Cellulase EI

pH	400 (3,4-dinitrophenol)	Rate (10^8M/l/s)
3.96[a]	1000	0.301
4.56[a]	2780	1.046
5.01[a]	5450	1.061
5.63[a]	10500	1.064
6.30[b]	11750	0.952
7.02[b]	13000	0.403

[a] Acetate buffer I = 0.1 T = 40°C.

[b] Phosphate buffer I = 0.1

TABLE 11

Effect of Temperature on the Activity of Cellulase EI

Temperature (°C)	Rate (10^8M/l/s)
20	0.242
30	0.695
40	1.124
50	2.803
60	3.738
70	2.587

Acetate buffer, pH = 5.02

Enzyme concentration = 1.04×10^{-7}M/l.

Substrate concentration = 2.38×10^{-4}M/l.

Substrate Specificity

Cellulase EI was incubated with a 1% xylan (β-1,4-linked xylose units) solution and a 1% amylose (α-1,4-linked glucose units) solution. An increase in the amount of reducing sugar indicated enzyme activity.

With the amylose solution no hydrolysis took place even after incubations of up to 2 hours. With the xylan solution activity was as large as that with carboxymethyl cellulose. No real comparison can be made since all the xylose residues of the xylan are unsubstituted whereas with CMC there are substituents on the glucose rings which will probably reduce the activity. With 3,4-dinitrophenyl β-chitotrioside or 3,4-dinitrophenyl β-chitotetraoside as substrates under the conditions employed for the assay with $34DNPG_4$ no enzymic release of 3,4-dinitrophenol was observed.

Randomness of CMC-saccharifying Activity of Cellulase EI

Cellulase EI was incubated with CMC under the assay conditions already described. The decrease in viscosity of the CMC solution by cellulase EI is compared with that of cellulase EII. Cellulase EII will be shown to be an exo-cellulase which removes glucose residues successively from the non-reducing end of cello-oligosaccharide chains.

The conditions for the viscosity studies are such that cellulases EI and EII have the same activity towards carboxymethyl cellulose. The graph of viscosity against time during the hydrolysis of CMC by cellulases EI and EII is shown in Figure 26.

The results show that cellulase EI produces a rapid decrease in viscosity indicating random cleavage of CMC whereas cellulase EII shows little change in viscosity on hydrolysis of CMC.

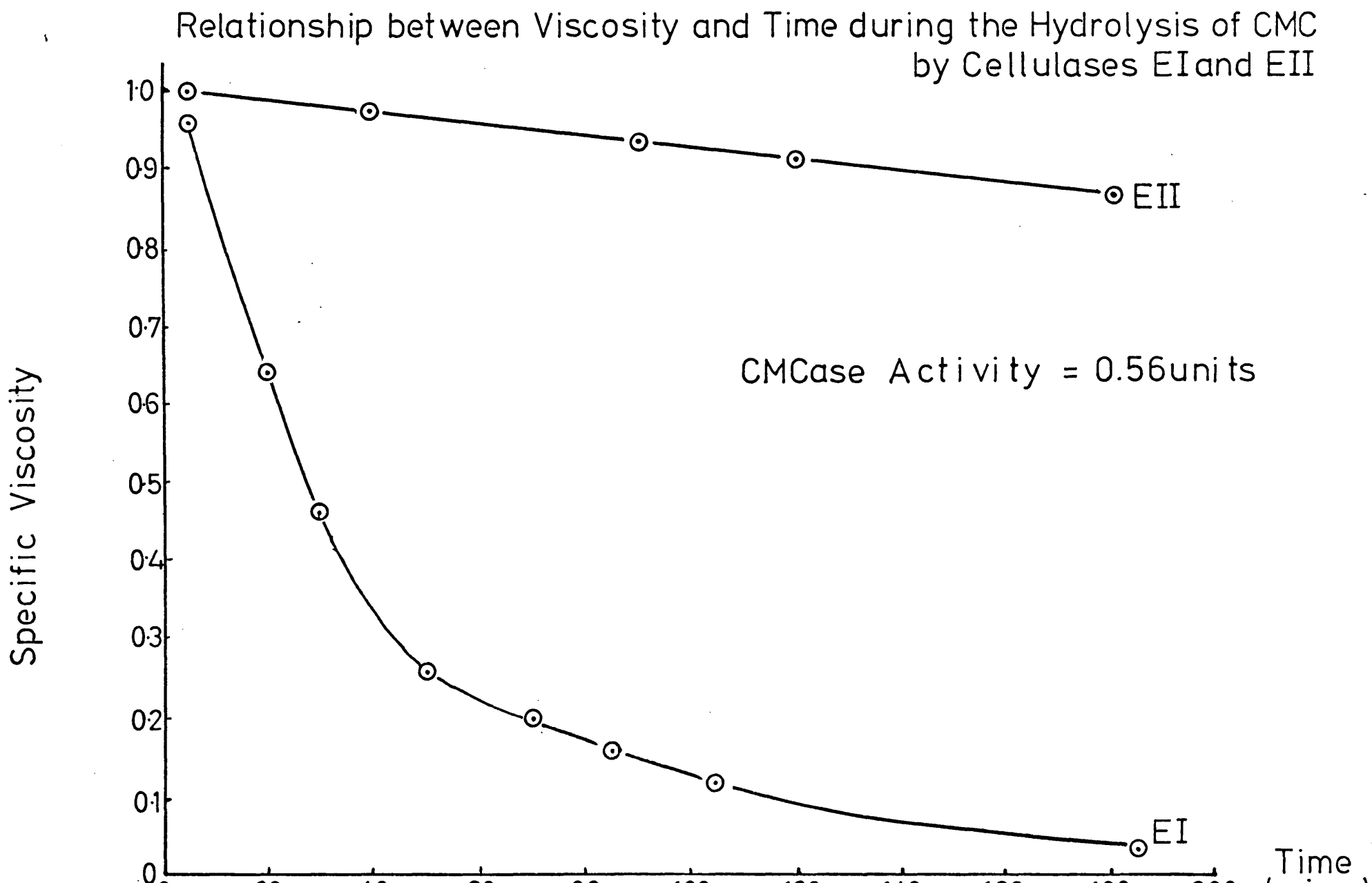

Relationship between Viscosity and Time during the Hydrolysis of CMC by Cellulases EI and EII
Specific Viscosity
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
20
40
60
80
100
120
140
160
180
200
Time (mins)
EII
EI
CMCase Activity = 0.56units

Figure 26

The above results strongly suggest that the enzyme isolated from the commercial sample of a cellulase extract of Trichoderma viride is a 1,4 β -D-glucan 4-glucanhydrolase [EC 3.2.1.4]. The enzyme may also be referred to as an endo-cellulase.

The fraction labelled FIA shows a vast increase in β-glucosidase activity over the crude. This enzyme fraction was looked at in closer detail.

Sephadex G-100 chromatography

Fraction FIA was lyophilised and the lyophilised powder dissolved in 5ml of acetate buffer, pH 5. This was applied to a column (2.6cm x 70cm) of Sephadex G-100 (Pharmacia Fine Chemicals) equilibrated with the same buffer. Figure 27 shows the result of this fractionation. This cellulase component was designated cellulase EII. The enzyme was assayed towards the various cellulolytic substrates. The results are shown in Table 12. Figure 28 shows the reaction of cellulase EII with the aryl glycosides.

The purification of the enzyme is summarised in Table 13.

Enzyme Purity 1) SDS-Gel Electophoresis (147).

Cellulase EII was subjected to the same gel electrophoretic conditions as for cellulase EI. A single band was observed.

2) Molecular Sieve Chromatography

Cellulase EII behaved as a single protein on passage through both Sephadex G-75 and Sephadex G-100 columns (2.6cm x 70cm).

Determination of Molecular Weight of Cellulase EII

The column of Sephadex G-100 which was used in the molecular weight estimation of cellulase EI was used. The same marker proteins were also used. The result is shown in Figure 23. The molecular weight was estimated to be about 74,400.

pH Optimum

Figure 29 shows the effect of pH on the enzyme activity assayed towards 3,4-dinitrophenyl β-D-glucopyranoside at 40°C. The measured pH, extinction coefficient of 3,4-dinitrophenol at 400nm at this pH and the measured rate of release of the phenol are

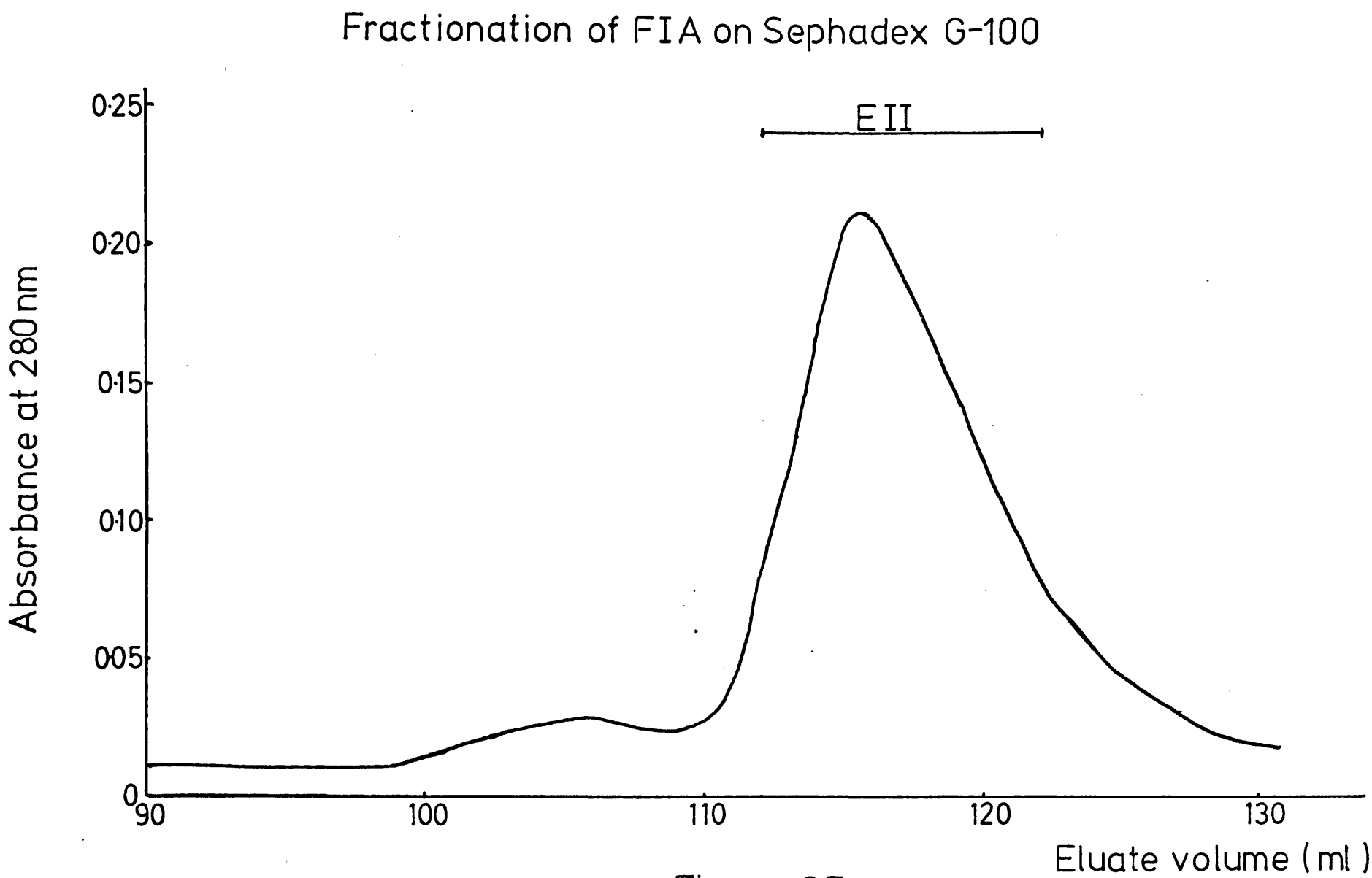

Fractionation of FIA on Sephadex G-100
EII
0·25
0·20
0·15
0·10
0·05
0
Absorbance at 280 nm
90
100
110
120
130
Eluate volume (ml)

Figure 27

TABLE 12

CELLULASE ACTIVITIES OF CELLULASE EII

Avicelase	-
CMCase	4.06
β-Glucosidase	44.0

TABLE 13

PURIFICATION OF CELLULASE EII FROM TRICHODERMA VIRIDE

Purification Step	Activity towards 34 $DNPG_1$	
1. Crude	1.77	(1)
2. DEAE-Sephadex A-25	5.88	(3.32)
3. Sephadex G-75	39.0	(22.0)
4. Sephadex G-100	44.0	(24.9)

Graph of Absorbance against Time for the Reaction of the 34-Dinitrophenyl Glycosides with Cellulase EII

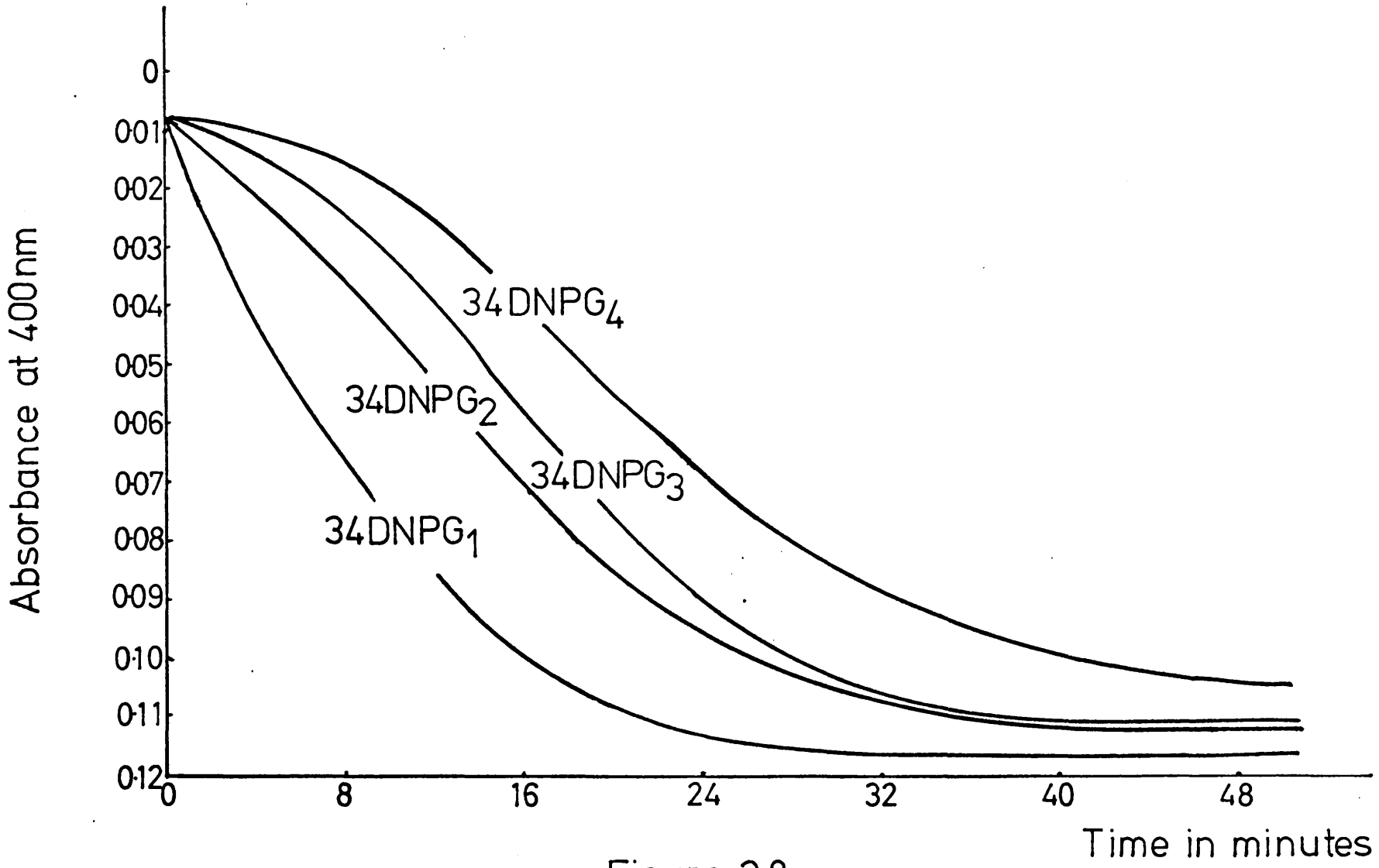


Figure 28

Effect of pH and Temperature on the Activity of Cellulase EII

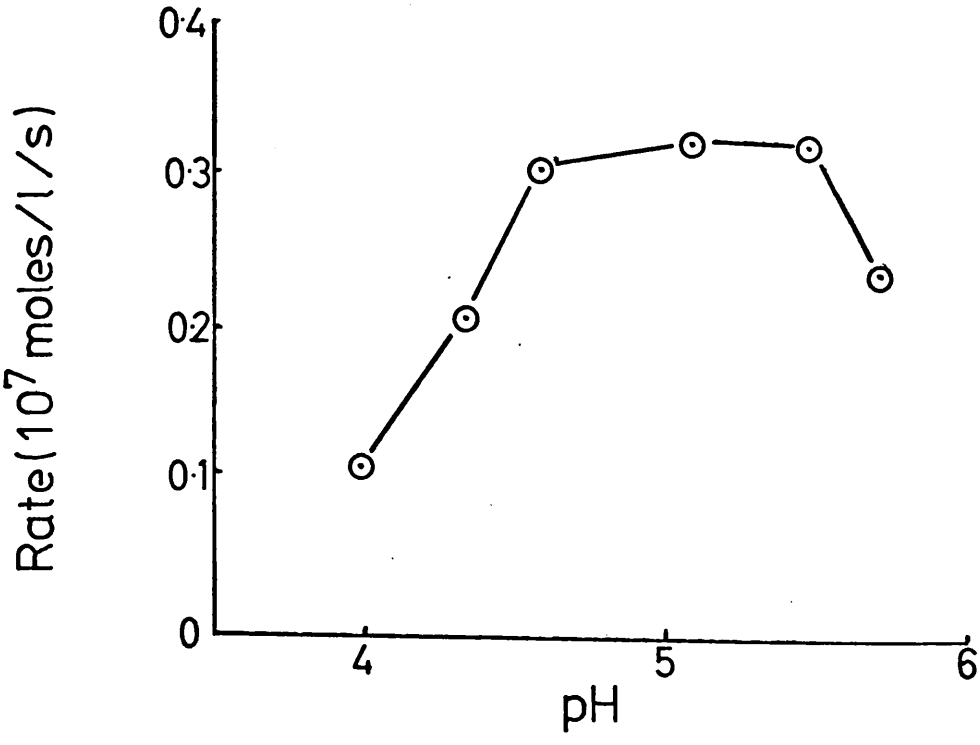


Figure 29

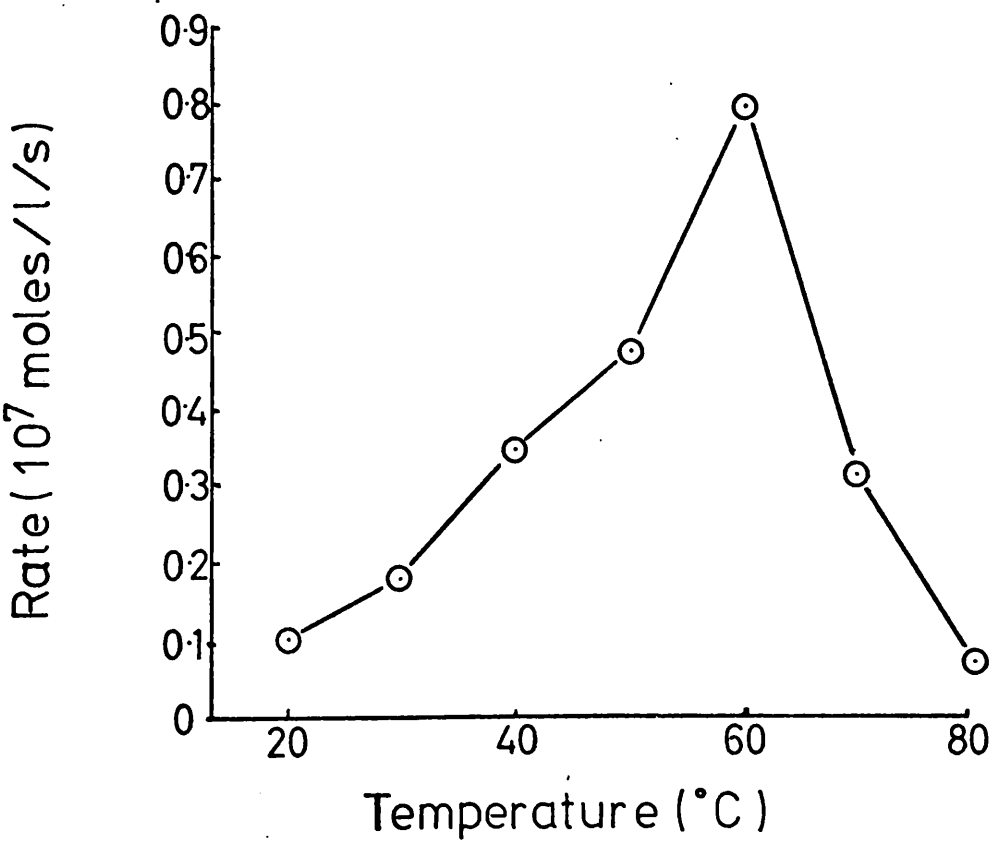


Figure 30

TABLE 14

Effect of pH on the Activity of Cellulase EII

pH	400 (3,4-dinitrophenol)	Rate (10^7M/l/s)
4.02[a]	1025	0.110
4.29[a]	1750	0.208
4.61[a]	2970	0.307
5.19[a]	6760	0.331
5.47[a]	8530	0.328
5.67[b]	10650	0.240

[a] Acetate I = 0.1 T = 40°C.

[b] Phosphate I = 0.1

TABLE 15

Effect of Temperature on the Activity of Cellulase EII

Temperature (°C)	Rate (10^7M/l/s)
20	0.105
30	0.186
40	0.327
50	0.464
60	0.804
70	0.311
80	0.075

Acetate buffer, pH = 5.02

Enzyme concentration = 2.3×10^{-8}M/l.

Substrate concentration = 1.208×10^{-4}M/l.

given in Table 14.

The pH optimum was found to form a plateau between 4.5 and 5.5.

Temperature Optimum

The reaction of the enzyme with $34DNPG_1$ was carried out at pH 5.02 at different temperatures (20-80°C) in the Cary 16 spectrophotometer. The results are shown in Figure 30. The measured values are given in Table 15. Optimum temperature for the activity of cellulase EII was found to be 60°C.

Substrate Specificity

Cellulase EII was incubated with the 1% xylan and the 1% amylose solution but no increase in the amount of reducing sugar was observed with either substrate.

The enzyme was also allowed to react with a series of aryl glycosides. The experiments were carried out at 40°C in the Cary 16 spectrophotometer. The activity of cellulase EII on the aryl glycosides was determined by measuring the rate of release of the phenol. The results are given in Table 16 . Only with 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was there any spontaneous hydrolysis observed. This was negligible compared to the enzymic rate of hydrolysis.

The enzyme was assayed towards the aryl cello-oligosaccharides. The result is shown in Figure 28. As can be seen there is no induction period with $34DNPG_1$ but with $34DNPG_2$, $34DNPG_3$ and $34DNPG_4$ an increasing induction period is found. This is consistent with the enzyme successively removing glucose residues from the non-reducing end of the cello-oligosaccharide before the phenol can be released.

TABLE 16

Cellulase EII catalysed hydrolysis of aryl glycosides.

T = 40°C; pH = 5.02 (acetate buffer, I = 0.1)

Extinction coefficient of 3,4-dinitrophenol at 400nm = 5500

Extinction coefficient of 4-nitrophenol at 350nm = 2550

Substrate	Rate ($\times 10^{8}$ M/l/s)	% of Glucopyranoside
3,4-Dinitrophenyl β-D-glucopyranoside	1485	100
3,4-Dinitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside	59.09	3.97
3,4-Dinitrophenyl 6-deoxy-β-D-glucopyranoside	8.66	0.58
3,4-Dinitrophenyl β-D-xylopyranoside	1.52	0.10
3,4-Dinitrophenyl 6-chloro-6-deoxy-β-D-glucopyranoside	1.04	0.07
3,4-Dinitrophenyl 6-O-methyl-β-D-glucopyranoside	0.23	0.01
4-Nitrophenyl β-D-glucopyranoside	1153	100
4-Nitrophenyl β-D-galactopyranoside	4.33	0.37
4-Nitrophenyl 2-deoxy-β-D-glucopyranoside	0	0

Enzyme concentration = 2.3×10^{-4} M/l.

Substrate concentrations = $1.2 \pm 0.1 \times 10^{-4}$ M/l.

Randomness of CMC-saccharifying Activity of Cellulase EII

Cellulase EII was incubated with CMC under the viscosity assay conditions already described for cellulase EI. The viscosity results for cellulase EI and EII are shown in Figure 26 and taken together show that cellulase EII does not cause a rapid decrease in viscosity although hydrolysis is at a rate similiar to that of cellulase EI. This indicates that cellulase EII behaves like an exo-cellulase.

Induced Hydrolysis

Experiments were carried out to see if the endo-cellulase possessed any transglycosylation properties.

Cellotriose, cellotetraose or cellopentaose and 3,4-dinitrophenyl β-D-glucopyranoside were dissolved in acetate buffer, pH 5.02, I = 0.1 (2.3ml) in a UV cell and a solution of the enzyme (200µl) added. The final concentrations are given in Table 17. The absorbance of the reaction mixture was monitored at 400nm. Any release of phenol was taken as an indication that transglycosylation was indeed taking place since incubation of the substrate with the enzyme, but without any oligosaccharide, under the conditions described did not result in any observable release of the phenol. The rate of release of 3,4-dinitrophenol after various time intervals and the percentage reaction are given in Table 17 for each of the cello-oligosaccharide in turn. The amount of 3,4-dinitrophenol released compared to that of the starting concentration of $34DNPG_1$ gives the percentage reaction.

The results show that as the chain length of the oligosaccharide increases the induction period before hydrolysis begins is reduced. The results also show that the rate of release of the phenol from the transglycosylation product with G_5 as the oligosaccharide is the greatest. Cellopentaose was therefore used in future experiments.

Similar experiments were carried out with the modified aryl glucosides to investigate the features which make the aryl oligosaccharides substrates for the enzyme. The substrate concentration was increased by a factor of 5. The experimental results and the conditions employed are given in Table 18.

TABLE 17

Rate of Formation of 3,4-Dinitrophenol During Incubation of Cello-oligosaccharides and 3,4-Dinitrophenyl β-D-glucopyranoside with Cellulase EI

	Time (mins)	Rate ($\times 10^{10}$ M/l/s)	Percentage Reaction
G_3	5	0	0
	10	0	0
	20	0	0
	30	2.69	0.02
	60	2.72	0.61
	120	2.75	1.50
G_4	5	0	0
	10	0	0
	20	2.75	0.30
	30	3.14	0.46
	60	3.69	0.78
	120	4.04	1.66
G_5	5	0	0
	10	3.03	0.02
	20	3.87	0.46
	30	4.54	0.61
	60	4.95	0.91
	120	5.02	2.58

Enzyme concentration = 4.16×10^{-6} M/l.

Oligosaccharide concentration = $6.0 \pm 1.0 \times 10^{-3}$ M/l.

Substrate concentration = 1.208×10^{-4} M/l.

Temperature = $20 \pm 2^{\circ}$C.

TABLE 18

Rate of Formation of 3,4-Dinitrophenol During Incubation of Cellopentaose and Modified 3,4-Dinitrophenyl β-D-glucopyranosides with Cellulase EI

Time(mins)	Rate($\times 10^{10}$ M/l/s)	Percentage Reaction
3,4-Dinitrophenyl β-D-glucopyranoside		
0	0	0
5	0	0
30	6.06	0.17
60	8.48	0.40
90	10.90	0.73
120	12.25	1.07
150	12.23	1.33
3,4-Dinitrophenyl β-D-xylopyranoside		
0	0	0
5	0	0
30	10.38	0.40
60	12.12	0.80
90	12.12	1.17
3,4-Dinitrophenyl 6-deoxy-β-D-glucopyranoside		
0	0	0
15	0	0
30	14.54	0.37
60	20.60	0.97
90	22.51	1.67

Time(mins)	Rate($\times 10^{10}$ M/l/s)	Percentage Reaction
3,4-Dinitrophenyl 6-chloro-6-deoxy-β-D-glucopyranoside		
0	0	0
45	0	0
60	2.20	0.23
120	3.89	0.40
240	4.12	0.63
600	4.66	1.33
3,4-Dinitrophenyl 6-O-methyl-β-D-glucopyranoside		
0	0	0
50	0	0
60	3.03	0.13
90	3.03	0.20
120	3.03	0.27
4-Nitrophenyl β-D-glucopyranoside		
0	0	0
5	0	0
30	4.58	0.13
60	6.67	0.33
120	10.33	0.90

4-Nitrophenyl 2-deoxy-β-D-glucopyranoside

No phenol was liberated even after incubation of the reaction for 3 hours.

Enzyme concentration = 4.16×10^{-6} M/l.

Concentration of cellopentaose = 5.7×10^{-3} M/l.

Substrate concentration = $6.0 \pm 0.5 \times 10^{-3}$ M/l.

Temperature = $20 \pm 2^{\circ}$C.

KINETIC EXPERIMENTAL

General

Kinetic experiments with the aryl glycosides were carried out in a Cary 16 spectrophotometer operating on line to a Digico Micro 16 P computer. Quartz cells with a path length of 10mm were used in a thermostatted cell block. The majority of the reactions were carried out at 40°C. The kinetic procedure was generally: equilibration of the buffer in the UV cell for 15 minutes, addition of the enzyme (usually 5µl) then addition of the substrate solution to give a total volume of 2.50 ±0.05ml. After initiation of the reaction the absorbance values at λ max of the phenol (400nm for 3,4-dinitrophenol and 350nm for 4-nitrophenol) were gathered on line at a predetermined time interval. The initial rate of release of phenol was determined by fitting the data to a quadratic equation of the form:

$$A_t = \underline{a} + \underline{b}t + \underline{c}t^2$$

by the generalised least squares method(148). A listing of the program is given in Appendix I.

Two methods for the determination of the Michaelis-Menten constants Km and Vmax from the values of initial rate and substrate concentration were available. One was the generalised least squares method of Wentworth (148), the other was a weighted linear least squares method of Cornish ~~—~~ Bowden (149). These two programs are listed in Appendix II and Appendix III respectively.

The enzyme concentrations were calculated from their respective extinction coefficients. For cellulase EI the measured extinction coefficient was 17,800 $1.M^{-1}.cm^{-1}$ and 21,100 $1.M^{-1}.cm^{-1}$ for cellulase EII. These values were obtained by de-salting the enzyme on a Sephadex G-15 column (1.6cm x 40cm), lyophilising the free enzyme and then measuring the absorbance at 280nm of a measured concentration of the enzyme using the estimated molecular weight.

TABLE 19

Cellulase EI catalysed hydrolysis of $34DNPG_2$

T = 40°C; pH 5.02 acetate I = 0.1

Extinction coefficient of 3,4-dinitrophenol at 400nm = 5500

Substrate conc. ($x10^4$ M/l)	Initial rate. ($x10^8$ M/l/s)
0.985	0.154
1.477	0.206
1.970	0.267
2.463	0.312
2.955	0.339
3.448	0.401
5.420	0.525
7.450	0.606

Enzyme concentration = 1.04×10^{-7} M/l.

Vmax = 1.01×10^{-8} M/l/s[a] Standard deviation = 5.2%.

0.89×10^{-8} M/l/s[b]

Km = 6.02×10^{-4} M/l[a] Standard deviation = 7.8%.

4.68×10^{-4} M/l[b]

[a] Calculated by method of Wentworth (148) - Appendix 2.

[b] Calculated by method of Cornish — Bowden (149) - Appendix 3.

TABLE 20

Cellulase EI catalysed hydrolysis of $34DNPG_3$

T = 40°C; pH 5.02 acetate I = 0.1

Extinction coefficient of 3,4-dinitrophenol at 400nm = 5500

Substrate conc. ($\times 10^{4}$ M/l)	Initial rate. ($\times 10^{8}$ M/l/s)
0.475	0.172
0.980	0.237
1.459	0.364
1.929	0.451
2.392	0.538
3.518	0.896
4.600	1.107
5.863	1.055
6.900	1.151

Enzyme concentration = 1.04×10^{-7} M/l.

Vmax = 2.11×10^{-8} M/l/s[b]

2.9×10^{-8} M/l/s[a] Standard deviation = 28%.

Km = 6.08×10^{-4} M/l[b]

9.8×10^{-4} M/l[a] Standard deviation = 36%.

[a] Calculated by method of Wentworth (148) - Appendix 2.

[b] Calculated by method of Cornish — Bowden (149) - Appendix 3.

TABLE 21

Cellulase EI catalysed hydrolysis of $34DNPG_4$

T = 40°C; pH 5.02 acetate I = 0.1

Extinction coefficient of 3,4-dinitrophenol at 400nm = 5500

Substrate conc. ($\times 10^{4}$ M/l)	Initial rate ($\times 10^{8}$ M/l/s)
0.595	0.148
0.952	0.283
1.190	0.351
1.488	0.447
1.785	0.536
2.083	0.543
2.380	0.682
2.975	0.875
3.272	1.161
4.362	1.200
6.543	1.259

Enzyme concentration = 1.04×10^{-7} M/l.

Vmax = 1.97×10^{-8} M/l/s[a] Standard deviation = 31%.

2.26×10^{-8} M/l/s[b]

Km = 6.37×10^{-8} M/l[a] Standard deviation = 37%.

5.13×10^{-8} M/l[b]

[a] Calculated by method of Wentworth (148) - Appendix 2.

[b] Calculated by method of Cornish — Bowden (149) - Appendix 3.

TABLE 22

Cellulase EII catalysed hydrolysis of $34DNPG_1$

T = 40°C; pH 5.02 acetate I = 0.1

Extinction coefficient of 3,4-dinitrophenol at 400nm = 5500

Substrate conc. ($\times 10^{4}$ M/l)	Initial rate. ($\times 10^{8}$ M/l/s)
0.1253	0.196
0.2496	0.334
0.4951	0.426
0.7366	0.564
0.9742	0.897
1.2080	0.979
1.4381	1.045
1.6646	1.033
1.8875	0.973
2.1070	1.028
2.3231	1.107

Enzyme concentration = 2.3×10^{-8} M/l.

Vmax = 1.51×10^{-8} M/l/s[a] Standard deviation = 6.1%.

1.44×10^{-8} M/l/s[b]

Km = 8.3×10^{-5} M/l[a] Standard deviation = 12.4%.

7.09×10^{-5} M/l[b]

[a] Calculated by method of Wentworth (148) - Appendix 2.

[b] Calculated by method of Cornish — Bowden (149) - Appendix 3.

DISCUSSION

The enzymes which are responsible for the breakdown of native cellulose consist of 3 main types:-

1) a β-1,4-glucan cellobiohydrolase which removes cellobiose residues from the non-reducing end of the cellulose polymer. This enzyme type has been isolated from culture filtrates of Trichoderma viride (73, 93,94,95,101), Trichoderma koningii (81,82), Irpex lacteus (105) and Fusarium solani (110). This enzyme is strongly inhibited by its product, cellobiose, but when this was removed continuously it extensively degraded native cellulose (94).

2) a β-1,4-glucan glucanohydrolase which randomly cleaves the cellulose chain. This enzyme is extensive in its occurrence being present in probably every species utilising cellulose. From kinetic experiments these enzymes require the cellulose substrate to contain at least 5 or 6 glucose residues for efficient hydrolysis. Most reports say that the enzyme has no activity towards native cellulose, but it could be that the enzyme is strongly inhibited by medium length cello-oligosaccharides. No one has performed a similar experiment to that of Pettersson (94) with the β-1,4-glucan cellobiohydrolase, that is, continuous removal of the products (if any) to ensure there is no inhibition.

3) a cellobiase and/or a β-glucosidase which degrades small cello-oligosaccharide chains to glucose by successive removal of a glucose residue from the non-reducing end.

To study the complete cellulase complex the obvious choice of substrates to use is cotton and Avicel. But to study the enzymes independently and determine their enzymic properties more fully, the substrates themselves must be fully characterised. In this respect substrates like phosphoric acid-swollen cellulose and

partially degraded cellulose are poor. Carboxymethyl cellulose which has been the most important substrate in determining cellulolytic activity is again a poor substrate in that substitution of the hydroxyl groups on carbons 2,3 and 6 cause large deviations from the natural substrate. Parfondry and Perlin (150) have recently shown by ^{13}C NMR studies on CMC with DS 0.7 that the hydroxyl groups of cellulose react in the order OH-2 > OH-6 ≫ OH-3. They also showed that residues released by enzymic action did not contain substituents on the 2 position. More useful are the cello-oligisaccharides or the methyl β-cello-oligosaccharrides first used by Whitaker (31) in that they can be fully characterised and are fully soluble. The problem here is that in order to determine the position of cleavage lengthy separations of the reaction products have to be undertaken.

Spectrophotometric substrates have found little favour in determining the activity of cellulolytic enzymes. Nisizawa has used p-nitrophenyl β-cellobioside in his studies of cellulases (53,100) but their use has only been for determining the position of cleavage of the two possible sites. The longer chained spectrophotometric substrate, p-nitrophenyl β-cellotetraoside has also been used by Nisizawa (34) but again this was only used to determine the position of cleavage by the enzyme. This type of substrate has not been pursued by Nisizawa or any other workers.

From the present knowledge of the enzymes in the cellulase system it was thought that a series of aryl β-cello-oligosaccharides could be used to determine the presence of various enzymes present in a crude cellulase mixture and that these substrates could be used to determine the enzymic properties of each of these enzymes. Aryl β-D-glucopyranoside would be a substrate for a cellobiase or a β-glucosidase. It was hoped that an aryl β-cellobioside would

serve as a substrate for a β-1,4-glucan cellobiohydrolase and an aryl β-cellotetraoside would be a substrate for a β-1,4-glucan glucanohydrolase.

Ballardie and Capon (127), working on catalysis by lysozyme, found that the best leaving group from synthetic aryl chito-oligosaccharides in terms of activity and stability was 3,4-dinitrophenol. For this reason 3,4-dinitrophenyl β-cello-oligosaccharides with DP from '1' to 4 were synthesised. These were prepared by coupling the acetobromo-sugar and 3,4-dinitrophenol in dry acetone in the presence of potassium carbonate. Figure 31 shows the ^{1}H NMR spectrum of 3,4-dinitrophenyl tri-O-acetyl-β-D-xylopyranoside. The splitting pattern of the proton resonances for the aryl group is typical of all the compounds incorporating this aromatic system. The acetylated aryl glycosides were de-O-acetylated by the method of Zemplén (135) without much methanolysis of the aryl glycoside bond. The structure of 3,4-dinitrophenyl β-cellotetraoside is shown in Figure 32.

The use of aryl glycosides possesses the obvious advantage of ease of kinetic measurements and the fact that only one process is measured spectrophotometrically, namely fission of the glycosyl-aryloxy bond. The disadvantages are that the mechanism of hydrolysis may be different from that of the natural substrate and that the aryl residue will bind differently (if at all) from a glucose residue in the enzyme/substrate complex. Nevertheless, any substrates for which the mechanism of action of cellulolytic enzymes can be more fully understood would be valuable. In this respect an enzyme was sought for which 3,4-dinitrophenyl β-cellotetraoside would be a substrate.

A commercial cellulase from Aspergillus niger (Koch-Light Laboratories) and two from Trichoderma viride (B.D.H. and Worthington

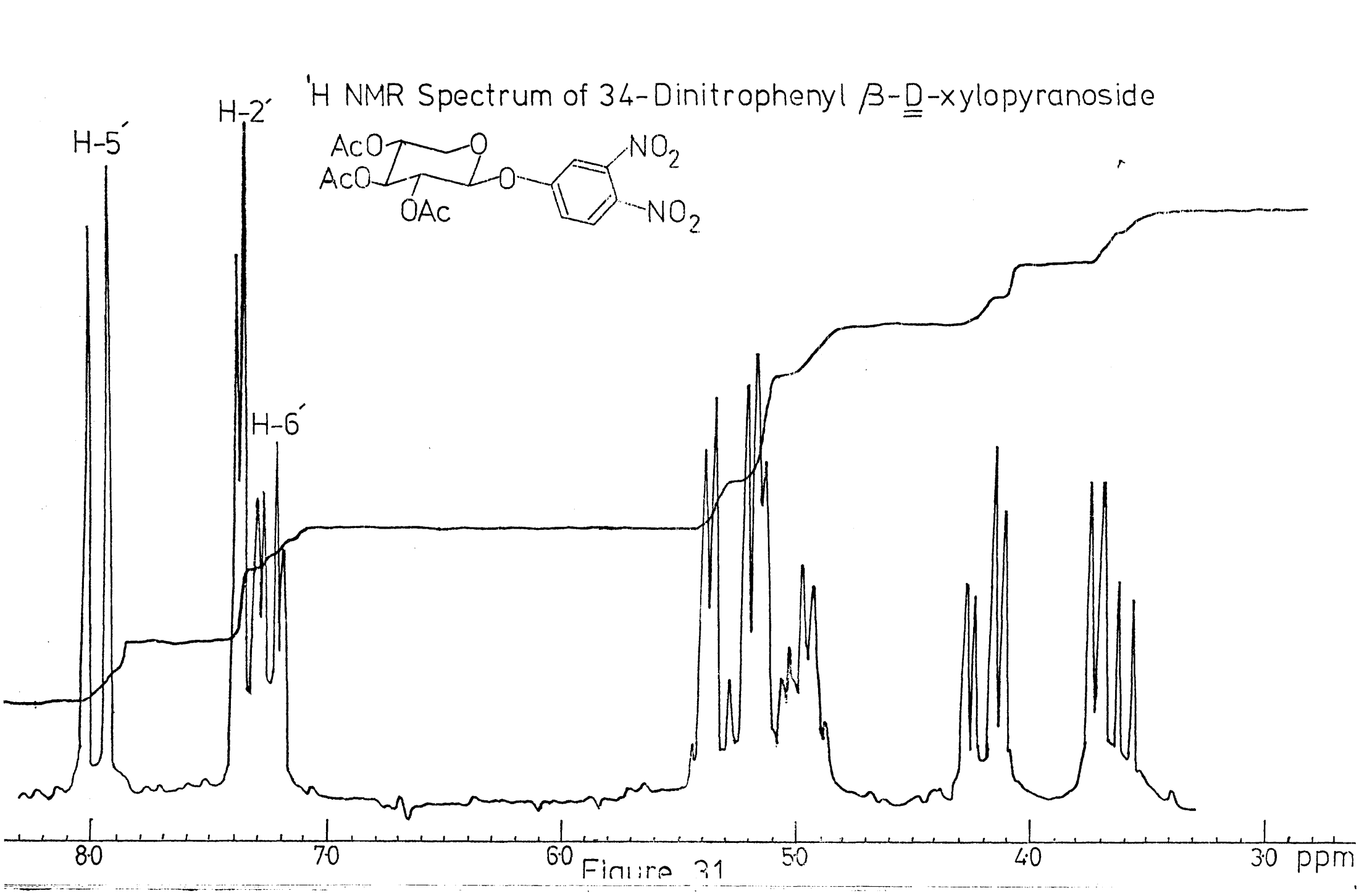
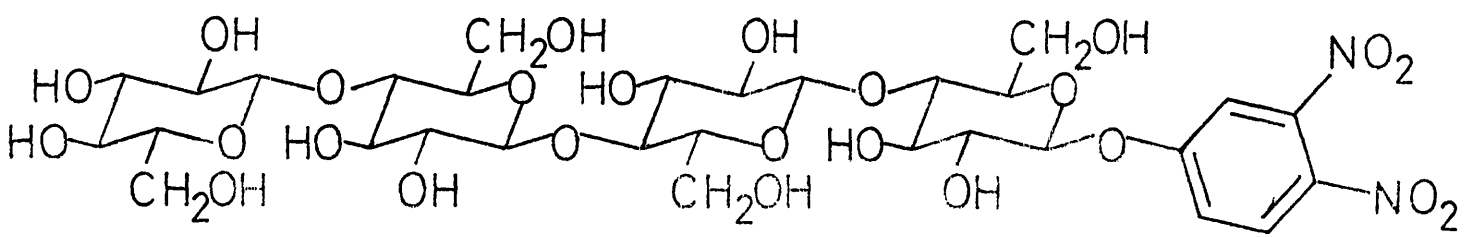

'H NMR Spectrum of 34-Dinitrophenyl β-D-xylopyranoside
H-5´
H-2´
H-6´
AcO
AcO
OAc
O
O
NO2
NO2
8·0
7·0
6·0
5·0
4·0
3·0
ppm

Figure 31



34DNPG$_4$

Figure 32

Enzymes) were assayed towards 34DNPG$_1$, 34DNPG$_2$ and 34DNPG$_4$. Only with the crude cellulase from B.D.H. was there any evidence of a component which might use 34DNPG$_4$ as substrate. This assay is shown in Figure 14. The crude cellulase was fractionated on a DEAE-Sephadex column to give 7 fractions as shown by the absorbance at 280nm of the eluate. This fractionation is shown in Figure 15. All 7 fractions were assayed towards 34DNPG$_1$, 34DNPG$_2$ and 34DNPG$_4$ as well as the conventional substrates. Fraction FI showed a greater increase in activity towards 34DNPG$_4$ than towards 34DNPG$_1$ although the rate of release of 3,4-dinitrophenol from the aryl glucoside was still the greater (Figure 16). From Table 5 it can be seen that Fraction II was very rich in β-glucosidase activity and possibly this is due to a different enzyme than the enzyme with β-glucosidase activity in fraction FI. This possibility was not investigated. Fraction FIII showed an increased activity towards 34DNPG$_4$. Is this activity due solely to the β-glucosidase component? No activity towards 34DNPG$_1$ was found in fractions FIV and FV but reappeared in fraction FVI. Another β-glucosidase must be present in the crude cellulase.

Since fraction FI showed the most promise it was further fractionated on a Sephadex G-75 column into 5 other components. This is shown in Figure 17. All fractions were again assayed towards 34DNPG$_1$, 34DNPG$_2$ and 34DNPG$_4$ and it was found that the activity of fraction FIC was now greater towards 34DNPG$_4$ than towards 34DNPG$_1$. This is shown in Figure 18 and in Table 6. Also interesting was the 7-fold increase in β-glucosidase activity of fraction FIA over that that of fraction FI. This fraction was investigated further.

Fraction FIC was subjected to another passage through the same Sephadex G-75 column and 7 fractions were collected as shown in Figure 19. Table 7 shows the ratio of activities towards 34DNPG$_4$

and 34DNPG$_1$ for each of these 7 fractions. Fraction FICiv showed the largest ratio of activity between the two substrates.

Passage of this fraction through an affinity column specific for β-glucosidases successfully removed most of the β-glucosidase activity giving a ratio of 133 : 1 for activities towards 34DNPG$_4$ and 34DNPG$_1$ respectively. The affinity column was regenerated and the enzyme fraction passed through again but still the enzyme showed a ratio of 133 : 1 for rates of hydrolysis of the spectrophotometric substrates. Since the column has been shown to be effective in removal of β-glucosidases it must be concluded that the enzyme which is hydrolysing 34DNPG$_4$ readily is also responsible for the slight β-glucosidase activity found in this fraction.

This fractionation procedure has resulted in the isolation of an enzyme with activity towards 34DNPG$_4$ and 34DNPG$_1$ in the ratio 133 : 1 from a crude cellulase mixture with activity towards the substrates in the ratio 1 : 17. The purity of the enzyme, designated cellulase EI, was ascertained by SDS-gel electrophoresis and by its behaviour on both Sephadex G-75 and G-100. In all three cases the enzyme behaved as a single protein. The molecular weight of the enzyme was estimated at 12,000 by comparison with other proteins of known molecular weight as shown in Figure 23. The pH optimum was found to be in the range 4.5-5.5 which is comparable to the pH optimum for the majority of cellulolytic enzymes. This suggests that the mechanism of hydrolysis of the aryl glycoside bond is the same as hydrolysis of the natural substrate. The temperature optimum is also similar to that of other cellulolytic enzymes, being 60°C.

The enzyme caused a rapid decrease in the viscosity of a CMC solution indicating a random hydrolysis action on the polymeric substrate. This is typical of a β-1,4-glucan glucanohydrolase.

In addition to CMC as substrate the enzyme also hydrolysed xylan which has β-1,4-linked xylose residues. It did not hydrolyse amylose which has α-1,4-linked glucose residues, or substrates for measuring chitinase activity. It was not expected that the enzyme would hydrolyse the aryl β-chito-oligosaccharides since the cell walls of the fungus are composed mainly of chitin. It appears that the enzyme is specific for β-1,4-linked glucose and xylose residues. The enzyme's activity is susceptible to substitution on carbon 2 of the glucose rings but does not require any substituent on carbon 5.

These results will be discussed further when considering the induced hydrolysis results.

By virtue of its enzymic properties, molecular weight and its source, cellulase EI is probably the same enzyme as that isolated by Pettersson et al. (99), page 30, and Selby and Maitland (78,79), page 31.

Kinetic studies of the enzyme with $34DNPG_2$, $34DNPG_3$ and $34DNPG_4$ as substrate were carried out under the conditions described on page 115. Values of Km and Vmax were obtained for each substrate. These results are summarised in the following table.

	$34DNPG_2$	$34DNPG_3$	$34DNPG_4$
Vmax ($\times10^{8}$M/l/s)	1.01	2.11	2.26
Km ($\times10^{4}$M/l)	6.02	6.08	5.13
$k_{cat}(s^{-1})$	0.097	0.203	0.217
k_{cat}/Km ($l.M^{-1}.s^{-1}$)	161	334	423

The values of k_{cat}/Km for the lysozyme catalysed hydrolysis of 3,4-dinitrophenyl β-chito-oligosaccharides are given for comparison.

	$34DNP(NAG)_2$	$34DNP(NAG)_3$	$34DNP(NAG)_4$
k_{cat}/Km ($l.M^{-1}.s^{-1}$)	0.064	7.9	232

The k_{cat}/Km value increases as the chain length of the cello-oligosaccharide increases. The value doubles on going from 2 glucose residues to 3 but shows a less marked increase on going from 3 to 4. This is in contrast to the k_{cat}/Km values for the lysozyme catalysed reaction of 3,4-dinitrophenyl β-chito-oligosaccharides at pH 5.08 and 40°C shown in the table above, where there is a large increase on going from 3 N-acetyl glucosamine residues to 4.

The natural substrate for lysozyme is the cell wall of Gram-positive bacteria. This has the general structure of alternating β-1,4-linked N-acetyl-D-glucosamine (NAG) and N-acetyl-muramic acid (NAM) residues which are cross linked by short polypeptide chains. Lysozyme degrades this cell wall by hydrolysing the polysaccharide between a NAM and a NAG residue. Oligosaccharides with just β-1,4-NAG residues are also hydrolysed. The maximum reaction rate is attained with the hexasaccharide, $(NAG)_6$, which is hydrolysed into $(NAG)_4$ and $(NAG)_2$. Philips and his co-workers (151) formulated their mechanism of action in terms of $(NAG)_6$. The X-ray studies of the enzyme with the non-productive binding complex with $(NAG)_3$ led them to the model for productive binding shown in Figure 33. It was

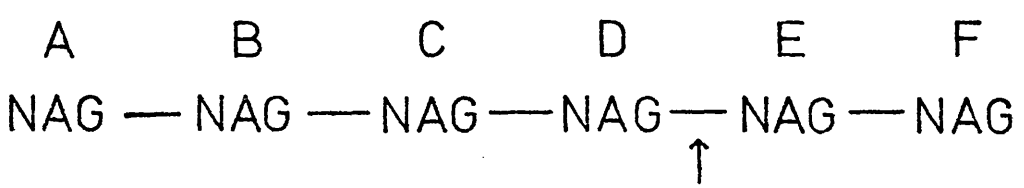


Figure 33

postulated that one residue of the $(NAG)_6$ occupied each of the binding sites labelled A to F and that hydrolysis was catalysed by the close proximity of two residues, namely, glutamic acid 35 and aspartic acid 52.

From the kinetic studies of cellulase EI it appears that the

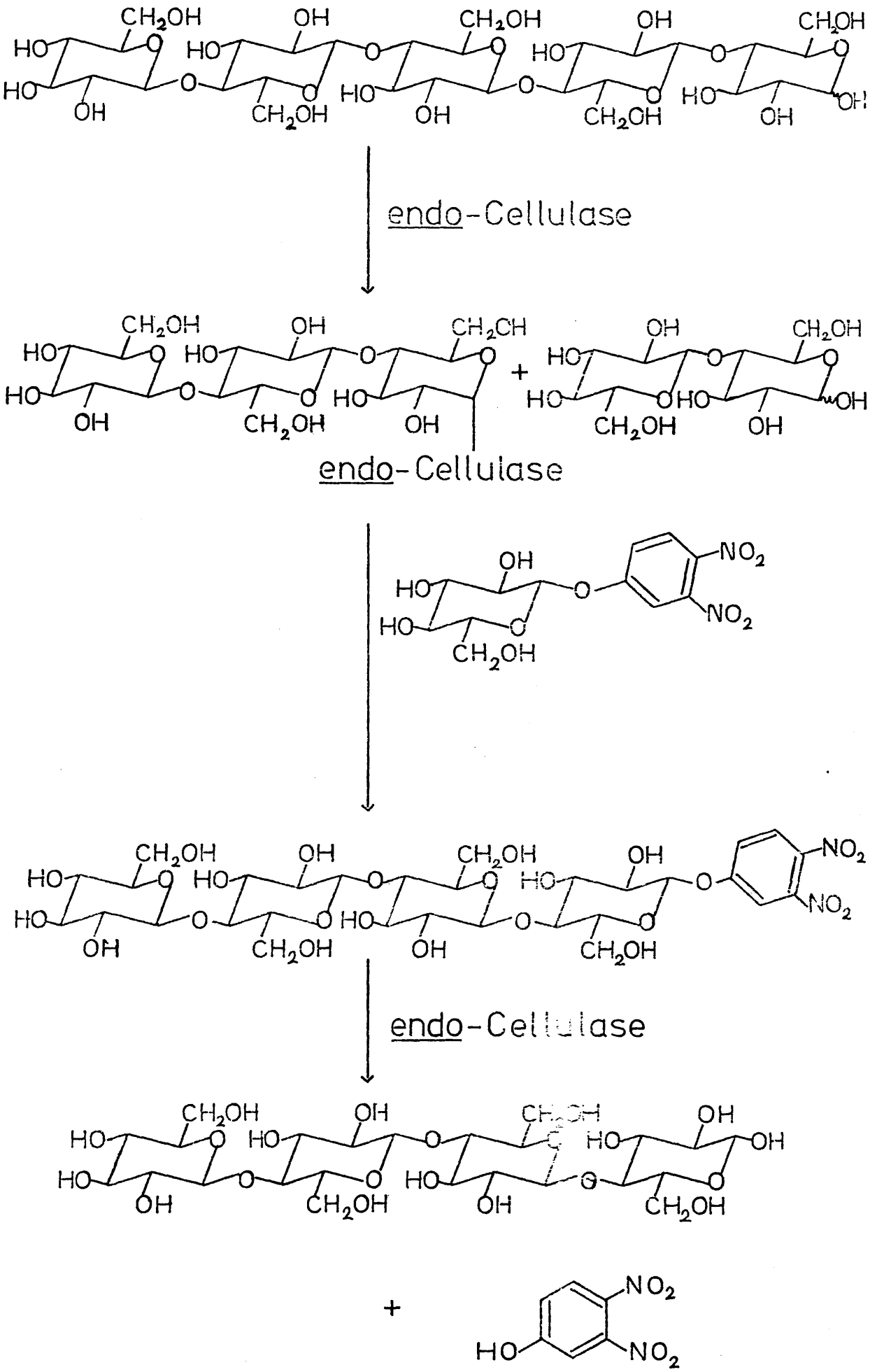
number of binding sites on the enzyme on the the glycone side with the aryl glycosides as substrate is probably 3.

The composite values for cellulase EI catalysed hydrolysis of $34DNPG_4$ at pH 5.02 and 40°C and those for lysozyme catalysed hydrolysis of $34DNP(NAG)_4$ at pH 5.08 and 40°C is shown below.

	Cellulase EI + $34DNPG_4$	Lysozyme + $34DNP(NAG)_4$
k_{cat} (s^{-1})	0.217	2.01×10^{-3}
Km (Ml^{-1})	5.13×10^{-4}	9.83×10^{-6}
k_{cat}/Km ($l.M^{-1}s^{-1}$)	423	205

Km for $34DNP(NAG)_4$ with lysozyme is 50 times smaller than Km for $34DNPG_4$ with cellulase EI, that is, the substrate appears to be more strongly bound with lysozyme. The value of k_{cat} is 100 times greater with cellulase EI and $34DNPG_4$ than with lysozyme and $34DNP(NAG)_4$. This probably is a result of the greater importance of non-productive binding with lysozyme since the values of k_{cat}/Km which are equal to k_2/k_s differ only by a factor of 2.

Experiments were carried out to investigate the transglycosylation properties of the enzyme. The experimental conditions are described on page 111 and in Table 17. The induction period before release of 3,4-dinitrophenol decreased from 20 minutes to 10 minutes to 5 minutes on increasing the length of the cello-oligosaccharide chain from 3 to 4 to 5. Also the rate of release of phenol increased as chain length increased. If the number of subsites on the enzyme on the glycone side is 3, then, since G_5 shows the highest tendancy to cause induced hydrolysis (and also a high rate of release of 3,4-dinitrophenol) it could be that the number of subsites on the aglycone side is 2. These ideas lead to the mechanism shown in Scheme 1 for the appearance of 3,4-dinitrophenol from 3,4-dinitrophenyl β-D-



Scheme 1

glucopyranoside and cellopentaose in the presence of cellulase EI.

In order to test whether small modifications of the substrate would cause any effect on the rate of the enzymatically catalysed reaction, the induced hydrolysis of modified 3,4-dinitrophenyl β-D-glucopyranosides was studied in the presence of cellopentaose and cellulase EI. The preparation of the modified glycosides is described in the 'Preparative Experimental' section. The conditions and results are given in Table 18.

The results show that for enzymic hydrolysis to occur the requirements around carbon 6 are very few, in fact, removal of the hydroxyl or even the hydroxymethyl substituent on carbon 5 to give the xylopyranoside, slightly increased the hydrolysis rate. Replacement of the 6-hydroxyl by a chlorine atom lengthened the induction period and lowered the hydrolysis rate. Replacement of the hydroxyl proton by methyl caused an even greater lengthening of the induction period and lowering of the rate. These results suggest that the last two compounds form poor aryl oligosaccharide substrates due to a steric effect rather than an electronic interaction effect. These results as well as the fact that the enzyme hydrolyses xylan stimulate the question : is the enzyme a cellulase or a xylanase? One method may be to compare the enzyme activity towards substrates such as cellopentaose and xylopentaose or 3,4-dinitrophenyl β-cellotetraoside and 3,4-dinitrophenyl β-xylotetraoside. One other way may be to ask what the purpose of the enzyme is? Organisms appear to produce enzymes in response to the substrates which are present. It is not known what the growth conditions for this commercial cellulase preparation from Trichoderma viride were. If it were grown on wheat bran-sawdust then both cellulases and xylanases would be produced. If, however, the sole carbon source were cellulose then the enzyme

would most likely be a cellulase. It could be that cellulases and xylanases are the same and that some organisms may produce an enzyme capable of hydrolysing both β-1,4-linked glucose and xylose polymers. This possibility has not been fully examined since most 'cellulase' workers, when isolating the various enzymes assay them towards cellulose, and 'xylanase' workers assay their enzymes towards xylan.

Similar experiments have been carried out with lysozyme (152) using the 3,4-dinitrophenyl glycosides of 2-acetamido-2,6-dideoxy-β-D-glucose, 2-acetamido-2,6-dideoxy-6-chloro-β-D-glucose, 2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucose as well as 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-xylopyranoside, 3,4-dinitrophenyl β-D-glucopyranoside and the 'natural' substrate 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in the presence of both $(NAG)_4$ and $(NAG)_5$. Only with the last two aryl glucosides was any induced release of 3,4-dinitrophenol observed. Clearly the mechanism of action of the two enzymes differs greatly, at least in their requirements for substituents on carbon 6 and carbon 6 itself.

As stated earlier, fraction FIA showed a large increase in β-glucosidase activity. This fraction was further purified by Sephadex G-100 gel filtration. This is shown in Figure 27. The enzyme fraction designated cellulase EII was judged to be pure by SDS gel electrophoresis and gel filtration. The molecular weight of this enzyme was estimated to be about 74,400. The pH and temperature optima were similar to that of cellulase EI. The increase in activity of the enzyme over the crude cellulase was 25 fold. Cellulase EII showed little ability to cause a reduction in the viscosity of a CMC solution although hydrolysis was taking place. This suggests that the enzyme is of the exo-type removing residues from the end of the polymer chain. With the series of 3,4-dinitrophenyl β-cello-oligosaccharides the enzyme rapidly hydrolysed 34DNPG$_1$ and on going from 34DNPG$_2$ to 34DNPG$_4$ the release of the phenol was characterised by an increasing induction period. This is shown in Figure 28. The results are consistent with an enzyme which removes glucose residues from the non-reducing end of the oligosaccharide chain. From these results it seems feasible that the enzyme isolated is similar to that isolated by Li, Flora and King (73) or one of three chromatographically distinct, yet kinetically similar, cellobiase fractions of molecular weight about 76,000 isolated by Gong et al. (155).

The enzyme was very specific in its substrate requirements. No activity was found with either xylan or amylose as substrate as determined by the increase in reducing power. The enzyme is very sensitive to modification at the carbon 6 position of the aryl glucoside. Replacement of the hydroxyl group by hydrogen caused a 172-fold decrease in activity and complete removal of the CH_2OH group reduced the activity 1000 times. Replacement of the hydroxyl by a chlorine atom was even more severe resulting in a 1430-fold

decrease in activity. When a methyl group was substituted for the proton of the hydroxyl group on carbon 6 the rate of release of 3,4-dinitrophenol dropped by a factor of 10^{-4} compared with the parent aryl glucopyranoside. Substitution on carbon 2 also affected the activity. Replacement of the hydroxyl group by a proton resulted in complete loss of activity and replacement by an acetamido group caused a 25-fold decrease in activity compared with the parent glucopyranoside. Altering the configuration of the hydroxyl on carbon 4 from equatorial to axial caused a 270-fold decrease in activity. These results show that the β-glucosidase is very specific in its substrate requirements. The constraints imposed on the atoms around carbon 6 are very rigid.

Substitution of a methyl group for a hydrogen atom on the hydroxyl group of carbon 6 reduces the rate of hydrolysis by cellulase EII almost to zero suggesting that either the steric requirements are well defined or that the hydroxyl group is required for hydrogen bonding to the enzyme. With chlorine in place of the hydroxyl group the rate is again drastically reduced. This may be due to the loss of the hydrogen for association with the enzyme or that the size of the chlorine atom is not allowing efficient substrate binding. Both oxygen and chlorine are hydrogen bond acceptors and the important fact could be the size of the chlorine atom. This may be resolved by using 3,4-dinitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside since oxygen and fluorine have similar atomic radii. On removal of the hydroxyl group on carbon 6 or the hydroxymethyl group itself the activity is greatly reduced. This emphasises the need for the CH_2OH group on the pyranose ring. The hydroxyl on carbon 2 is important since removal causes complete loss of enzymic action. However, replacement by an acetamido group still leads to

enzymic activity. The reduced activity may be due to the steric effects of the acetamido group and it may be that the 2-amino-2-deoxy-glucopyranoside would be a good substrate. The inversion of configuration of the hydroxyl group on carbon 4 leads to a much reduced activity. Again this may be a steric effect and by using 3,4-dinitrophenyl 4-deoxy-β-D-glucopyranoside as substrate this question may be answered. Another compound which would be worth preparing as a probe to the enzyme/substrate requirements would be 3,4-dinitrophenyl 3-deoxy-β-D-glucopyranoside.

These relative rates of hydrolysis may be compared with the results obtained by Gough (156) for a series of deoxy-methyl β-D-glucopyranosides hydrolysed by Almond emulsin and by Reese et al. (157) for p-nitrophenyl β-D-glucopyranosides and methyl β-D-glucopyranosides substituted at carbon 6 hydrolysed by β-glucosidases from various sources. These results as well as the results obtained for the β-glucosidase from Trichoderma viride are given in Table 23.

The most obvious difference is for that of the 6-deoxy compounds where the relative rate with Trichoderma viride is much less than the relative rates found with the other enzymes. Jermyn (158) using a β-glucosidase from Strachybotrys atra found the ratio of k_{cat}/Km for p-nitrophenyl β-D-glucopyranoside and the 6-deoxy compound was 240:1.

The Michaelis-Menten constants for the enzyme were determined using $34DNPG_1$ as substrate. The results are shown in the following table.

Vmax ($M.l^{-1}.s^{-1}$)	1.44×10^{-8}
Km ($M.l^{-1}$)	7.09×10^{-5}
k_{cat} (s^{-1})	0.63
k_{cat}/Km ($l.M^{-1}.s^{-1}$)	8830

Percentage Relative Rates of Enzymic Hydrolysis of Modified Glucosides Compared to the Parent Glucoside

Methyl β-D-glucopyranoside	2-deoxy	3-deoxy	4-deoxy	6-deoxy	6-O-Me	6-chloro	[xyloside]
Almond emulsin[a]	0.04	0.03	2.3	8.4	-	-	-
Almond emulsin[b]	-	-	-	14.0	-	3.5	-
Aspergillus niger[b]	-	-	-	3.0	-	0	-
Cellulase 9X fraction, Miles Lab.[b]	-	-	-	3.0	-	0	-
Penicillium melinii[b]	-	-	-	3.0	-	0.5	-
p-Nitrophenyl β-D-glucopyranoside							
Almond emulsin[b]	-	-	-	-	0.1	-	-
Aspergillus niger, fraction[b]	-	-	-	-	<0.6	-	-
Cellulase 9X, fraction[b]	-	-	-	-	<1.0	-	-
Penicillium melinii, fraction[b]	-	-	-	-	4.0	-	-
Trichoderma viride, fraction[c]	0	-	-	-	-	-	-
3,4-Dinitrophenyl β-D-glucopyranoside							
Trichoderma viride, fraction[c]	-	-	-	0.58	0.01	0.07	0.1

a = Reference 156 b = Reference 157 c = This thesis

TABLE 23

The aryl glucoside is therefore a reasonable substrate for the enzyme having a 10 fold smaller Km value than $34DNPG_4$ with cellulase EI and a value of k_{cat}/Km 20 times greater.

From the experiments performed on cellulase EII it is not possible to say if the enzyme is truly a β-glucosidase or a cellobiase. The key difference between these enzymes is the number of binding sites on the enzyme. A β-glucosidase will only have one binding site whereas a cellobiase will have a binding site for each residue of cellobiose. The k_{cat}/Km value for a β-glucosidase should be larger for p-nitrophenyl β-D-glucopyranoside (or 3,4-dinitrophenyl β-D-glucopyranoside) compared with that of cellobiose due to the aryloxy group being a better leaving group. A cellobiase, on the other hand, would have a more comparable k_{cat}/Km value for cellobiose and p-nitrophenyl β-D-glucopyranoside due to the extra enzyme activity obtained from the binding of the second glucose moiety.

An example of this argument is the enzyme isolated by Berghem and Pettersson (102). The catalytic constants for the enzymic action on p-nitrophenyl β-D-glucopyranoside and cellobiose is given below.

	Cellobiose	p-nitrophenyl glucoside
Vmax ($x10^{-6}$ M.s^{-1})	0.55	536.7
Km ($x10^{3}$ M)	1.5	0.28
k_{cat} (s^{-1})	25.8	25,200
k_{cat}/Km ($M^{-1}.s^{-1}$)	17,200	9×10^{7}

There is no doubt from this analysis that the enzyme isolated by Berghem and Pettersson is a β-glucosidase.

Another example of this type of analysis can be worked out for the enzyme isolated by Maguire (153). The ratio of the k_{cat}/Km values for p-nitrophenyl β-D-glucopyranoside and cellobiose is

5.5 : 1. This small variation strongly suggests that the enzyme isolated by Maguire is indeed a cellobiase.

An indication to the nature of cellulase EII may be obtained from the relative rates of hydrolysis of 34DNPG$_1$ and 34DNPG$_2$ as shown in Figure 25. After 4 minutes the amount of 3,4-dinitrophenol released from 34DNPG$_1$ is only about double that of 34DNPG$_2$. This implies that the cleavage of the first glucose residue from the cellobioside proceeds at a similar rate to the cleavage of the aryl glucoside bond. p-Nitrophenyl β-D-glucopyranoside, as a leaving group, must be similar to glucose itself and therefore some assistance probably arises from binding of the second glucose residue in the cellobioside which suggests that the enzyme is a cellobiase.

This is only tentative and obviously a more detailed kinetic study of the hydrolysis of cellobiose is required.

From the work undertaken in this project it appears that 3,4-dinitrophenyl β-cello-oligosaccharides will be useful substrates for the rapid determination of the presence and activity of cellulase enzymes. The substrates may find a use in determining the ratio of β-1,4-glucan glucanohydrolase activity to β-glucosidase activity since they have the same leaving group. This is in contrast to substrates used at present, namely, carboxymethyl cellulose and p-nitrophenyl β-D-glucopyranoside whose 'structures' and 'leaving groups' are very different.

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APPENDIX 1 - INITIAL SLOPE PROGRAM

```
WRITEA
  7T !"TYPE RUN NUMBER"
  9A RN
 10VECTOR AB(1,1,255)
 12T !"TYPE NUMBER OF POINTS TO BE COLLECTED"
 13A N
 14F I=1,N;S Y(I)=0
 15T !"TYPE CARY 16 SCALE"
 16A OB
 17S I=1;S V=0
 18T !
 20S Y(I)=0
 30ZIP1,1,10,0,AB
 40FORJ=1,10;S Y(I)=Y(I)+.0001*OB*AB(J)
 45IF(V-6),50,46,46
 46S V=0;T !
 50T %5.04,Y(I),"//"
 60IF(N-I),140,140,65
 65S V=V+1
 70S I=I+1
 75S X=4000
 80F P=1,X;S KL=0
 90GO20
140S TC=29.25
145S EC=5500
150S PL=1
170T !!
180S P=0
210S A(1,1)=Y(1)
220S A(2,1)=(Y(6)-Y(1))/5*TC
230S A(3,1)=(Y(N)-A(1,1)*TC*(N-1))/(TC*(N-1)↑2)
320S EY=.001
330S ER=1
340S P=P+1
345F R=1,4;F Q=1,4;S B(R,Q)=0
346S X=0
350F I=2,N;DO369/485
360GO 490
370S X=X+TC
380S OF(4)=Y(I)-A(1,P)-A(2,P)*X-A(3,P)*X↑2
390S OF(1)=-1
400S OF(2)=-X
410S OF(3)=-X↑2
420S OY=1
430S OP=-A(2,P)-2*A(3,P)*X
440S L=OX/ER+OY/EY
450S R=0
460S R=R+1
470F Q=1,4;S B(R,Q)=B(R,Q)+OF(R)*OF(Q)/L
481IF(R-4),460,485,485
485
490S BO=B(1,1)*(B(2,2)*B(3,3)-B(2,3)↑2)
501S BO=BO-B(1,2)*(B(1,2)*B(3,3)-B(2,3)*B(1,3))
502S BO=BO+B(1,3)*(B(1,2)*B(2,3)-B(2,2)*B(1,3))
```

```
503S D(1,1)=(B(2,2)*B(3,3)-B(2,3)↑2)/BO
510S D(1,2)=(-B(1,2)*B(3,3)+B(2,3)*B(1,3))/BO
520S D(1,3)=(B(1,2)*B(2,3)-B(2,2)*B(1,3))/BO
525S D(2,2)=(B(1,1)*B(3,3)-B(1,3)↑2)/BO
530S D(2,3)=(-B(1,1)*B(2,3)+B(1,2)*B(1,3))/BO
540S D(3,3)=(B(1,1)*B(2,2)-B(1,2)↑2)/BO
550S D(2,1)=D(1,2)
560S D(3,1)=D(1,3)
570S D(3,2)=D(2,3)
580S Q=P+1
590FOR R=1,3;DO 610/620
600GO 630
610S DL(R)=D(1,R)*B(1,4)+D(2,R)*B(2,4)+D(3,R)*B(3,4)
620S A(R,Q)=A(R,P)-DL(R)
630S S=B(4,4)-B(1,4)*DL(1)-B(2,4)*DL(2)-B(3,4)*DL(3)
635T !,%,A(1,Q),A(2,Q),A(3,Q),
640IF(FABS(P-1)-10↑(-1000)),340,650,650
650IF(FABS(P-4)-10↑(-1000)),670,660,660
660IF(FABS(A(2,Q)-A(2,P))-.01*A(2,P)),670,340,340
670
810T !"ENZYMIC HYDROLYSIS OF 34 DINITROPHENYL CELLOTETRAOSIDE"
815T !"JOHN W. THOMSON"
820GO 840
840
845T " RUN NUMBER ",%4.00,RN
850T !"ABSORBANCE DATA"
860
870S P=P+1
880T !"INITIAL SLOPE PROGRAM"
890T !,%1.00,P-1," ITERATIONS NEEDED"
899F I=1,3;S SD(I)=FSQT(S*D(I,I)/(N-5))
900T !"A=",%,A(1,P),"    EST A",A(1,1),!,"ST.DEV.",SD(1),
910T !"         ",100*SD(1)/A(1,P),"PER CENT"
915
920T !!"B=",A(2,P)," A.U. PER SEC","    EST B=",A(2,1),
921T !"  ",60*A(2,P)," A.U. PER MIN",!,"  ",A(2,P)/EC*PL,
922T "MOLE/LITRE/SEC",!,"ST. DEV.","=",SD(2),
923
930T !"       ",100*SD(2)/A(2,P)," PER CENT",
940T !!,"C=",A(3,P),"    EST C",A(3,P),!,"ST DEV",SD(3)
950T !"      ",100*SD(3)/A(3,P)," PERCENT
955G07
960T !!"FULL DATA LIST YES TYPE1/,NO TYPE-1/"
970A CO
980IF(CO),1009,990,990
990T !!!"TIME/S                ABS       CALABS    RESIDUALS"
991S X=0
1000F I=2,N;DO1002/1004
1001G01009
1002S X=X+TC
1003S SR=Y(I)-A(1,P)-A(2,P)*X-A(3,P)*X↑2
1004T !,%,X,"  ",%5.04,Y(I),"  ",Y(I)-SR,"  ",SR
1009QUIT
```

APPENDIX 2 - MICHAELIS-MENTEN PROGRAM

```
WRITEA
   5COMMENT MICHAELIS MENTEN PROGRAM
   6T !"TYPE RUN NUMBER"
   7A RN
  10T !"TYPE NUMBER OF SUBSTRATE CONCENTRATIONS"
  20A N
  30
  35T !
  40T !"TYPE POWER FACTOR FOR RATES FORMAT 1E-X"
  45A PR
  47T !"TYPE POWER FACTOR FOR SUBSTRATE CONCS. FORMAT 1E-Y"
  48A PS
  49T !"TYPE INITIAL RATES(M/L/S),%ST.DEVS.,SUBSTRATE CONCS.(M/L)"
  50T !!
  52F I=1,N;D055/64
  53GO 65
  55A V(I),EV(I),S(I);T "  //",!
  60S V(I)=V(I)*PR
  62S EV(I)=EV(I)*V(I)/100
  64S S(I)=S(I)*PS
  65T !
  70T !"TYPE ESTIMATED K-M"
  80A A(1,1)
  81S A(1,1)=A(1,1)*PS
  85T !"TYPE ESTIMATED V-MAX"
  90A A(2,1)
  91S A(2,1)=A(2,1)*PR
  95S P=0
 100S P=P+1
 110F R=1,3;F Q=1,3;S B(Q,R)=0
 120F I=1,N;D0140/270
 130GO 280
 140
 150S OF(3)=V(I)-(A(2,P)*S(I)/(A(1,P)+S(I)))
 160S OF(2)=-S(I)/(A(1,P)+S(I))
 170S OF(1)=A(2,P)*S(I)/((A(1,P)+S(I))↑2)
 180S OX=A(2,P)*S(I)/((A(1,P)+S(I))↑2)-A(2,P)/(A(1,P)+S(I))
 190S OY=1
 200S WY=1/(EV(I)↑2)
 210S WX=1/(S(I)*.01)↑2
 220S L=OX↑2/WX +OY↑2/WY
 230S Q=1
 240F R=1,3;S B(Q,R)=B(Q,R)+OF(Q)*OF(R)/L
 250S Q=Q+1
 260IF(Q-4),240,270,240
 270
 280S BO=-B(1,1)*B(2,2)+B(1,2)↑2
 290S DA(1)=(B(1,2)*B(2,3)-B(2,2)*B(1,3))/BO
 291
 300S DA(2)=(B(1,2)*B(1,3)-B(1,1)*B(2,3))/BO
 301
 310S Q=P+1
 315F R=1,2;S A(R,Q)=A(R,P)-DA(R)
 320IF(P-5),100,330,100
 330
```

```
340
350S SO=B(3,3)-B(3,1)*DA(1)-B(3,2)*DA(2)
351S SO=-SO
355S SD(1)=FSQT(B(2,2)*SO/BO*(N-2))
357S SD(2)=FSQT(B(1,1)*SO/BO*(N-2))
360
365T !"JOHN W. THOMSON          RUN NUMBER",%4.00,RN
370T !!!"SUBSTRATE CONC.       EXPERIMENTAL RATE          CALC.RATE"
371T !"  MOLES/LITRE          MOLES/LITRE/SEC            M/L/S"
380F I=1,N;D0400/410
385S P=P+1
390G0420
400S BA(I)=A(2,P)*S(I)/(A(1,P)+S(I))
410T !,%,S(I),"    ",V(I),"     ",BA(I)
415
416
420T !"  VMAX                          K-M          ITERATED"
430F I=1,P;T !A(2,I),"    ",A(1,I)
440T !"                VMAX                    K-M"
450T !"CALCULATED",A(2,P),"    ",A(1,P)
460T !"ESTIMATED ",A(2,1),"    ",A(1,1)
470T !"STAND.DEV.",SD(2),"    ",SD(1)
500T !!!"FULL DATA LIST YES TYPE 1 /NO TYPE -1/"
502A Z
503IF(Z),550,550,505
505T !"RESIDUALS      VEXPT-VCALC."
510F I=1,N;D0520/530
515G0540
520S BB(I)=V(I)-BA(I)
530T !,BB(I)
540
550QUIT
```

APPENDIX 3 - MICHAELIS - MENTEN PROGRAM

```
!
WRITEA
  10COMMENT ]] EQUATION CORNISH-BOWDEN P179
  20T !"NO OF POINTS";A N
  25T !"POWER FACTOR FOR VELOCITIES";A Y
  30T !"VELOCITIES"!;F I=1,N;A V(I);S V(I)=V(I)*10↑Y
  35T !"POWER FACTOR FOR CONCENTATIONS";A X
  40T !"CONCENTATIONS"!;F I=1,N;A C(I);S C(I)=C(I)*10↑X
  50F I=1,5;S S(I)=0
  60F I=1,N;D080/120
  70G0130
  80S S(1)=S(1)+V(I)↑4/C(I)↑2
  90S S(2)=S(2)+V(I)↑4
 100S S(3)=S(3)+V(I)↑3
 110S S(4)=S(4)+V(I)↑4/C(I)
 120S S(5)=S(5)+V(I)↑3/C(I)
 130S D=S(1)*S(3)-S(4)*S(5)
 140
 150T !,%,(S(1)*S(2)-S(4)↑2)/D
 160S K(1)=(S(2)*S(5)-S(4)*S(3))/D
 170T K(1)
 180S P=1
 190F I=1,5;S S(I)=0
 200F I=1,N;D0220/270
 210G0280
 220S L(I)=(K(P)+S(I))↑2
 230S S(1)=S(1)+V(I)↑2/L(I)
 240S S(2)=S(2)+(C(I)↑2)*(V(I)↑2)/L(I)
 250S S(3)=S(3)+C(I)*V(I)↑2/L(I)
 260S S(4)=S(4)+(C(I)↑2)*V(I)/L(I)
 270S S(5)=S(5)+C(I)*V(I)/L(I)
 280S D=S(1)*S(4)-S(3)*S(5)
 285S P=P+1
 290S U(P)=(S(1)*S(2)-S(3)↑2)/D
 300T !,U(P)
 310S K(P)=(S(2)*S(5)-S(3)*S(4))/D
 320T K(P)
 330IF(P-7);190,334,334
 332T !"JOHN W. THOMSON"
 334T !"CONCENTRATION          VOBS                    VCALC"
 335F I=1,N;D0337/338
 336G0339
 337T !,C(I)," ",V(I)," "
 338T U(P)/(1+K(P)/C(I))
 339IF(P-7);340,370,370
 340S K(P)=0
 350S P=P+1
 360G0190
 370QUIT
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