CELLULOLYTIC ENZYME SYSTEMS OF MYROTHECIUM VERRUCARIA

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INTRODUCTION

Cellulose is the most widely distributed and abundant organic compound elaborated by living cells (37, 109). As the name suggests, it is the chief constituent of the cell walls of plants. When plants die the carbon in their structures is returned to the atmosphere as carbon dioxide by the processes of decay. In this respect the enzymatic hydrolysis of cellulose plays an important part in nature for it marks the first step in the decay of cellulosic materials.

Aside from its fundamental importance in the maintenance of the carbon cycle, the decomposition of cellulose has other immediate and important implications. Economic pressures have created the impetus for research into the mechanism of the enzymatic hydrolysis of cellulose. The chief economic factor is that annual losses as a result of biological decay run into billions of dollars (114). The destructiveness of these organisms has caused intensive research in the field of cellulose preservation. In addition to dead cellulosic materials, living forests are under constant attack by bacteria and fungi. These disease-producing organisms presumably possess a cellulase which enables them to penetrate the living cell wall of the host plant. A detailed study of the cellulase of these fungi may contribute materially to the eventual control of the diseases caused by these organisms.

Equally important from an economic standpoint are the many beneficial and desirable processes carried out by cellulose-decomposing microorganisms. This type of organism plays an important role in the

formation of humus in the soil (125), and they are playing an expanding role in the decomposition of cellulose in sewage and industrial wastes (47). The possibility of obtaining valuable products from an anaerobic fermentation of cellulose is an obvious extension of current disposal processes (65). In this respect the anaerobic dissimilation of cellulose plays a very important part in ruminant nutrition (99). In the face of a burgeoning world population, organisms which can convert indigestible polysaccharides such as cellulose into digestible sugars or other nutrients are being studied as a potential source of food (120).

In general, the ability of living organisms to degrade cellulose is restricted to the lower forms of life. Many bacteria, fungi, and protozoa are capable of decomposing cellulose. Also, a few invertebrates such as the snail (Helix) (61, 62), the shipworm (Teredo) (40), the clam (Mactra) (67), and the silverfish (Ctenolepisma) (66) possess cellulose-hydrolyzing enzymes. The enzyme is not known to be elaborated by man or other higher animals. However, it appears that most animals do have bacteria or protozoa as part of their intestinal flora which are capable of degrading cellulose. The ability of the termite and the porcupine to digest cellulose is due to the action of bacteria in their intestines. However, in the former connection, Misra (82) has reported that at least one termite (Termes obesus) elaborates the enzyme itself and does not depend on microbial activity for its nutrients. Further studies are necessary to clarify the role played by symbiotic cellulose-decomposing organisms in the nutrition of these

species. In the case of man, it appears that cellulose-decomposing organisms constitute only a very minor part of the intestinal flora and that these organisms do not contribute anything to the nutrition of the host. In this connection, however, it might be pointed out that a few cellulose decomposers have been isolated which are pathogenic to man (123). The role that such organisms play in the etiology of disease has received very little attention.

The ability of ruminant animals to utilize cellulose and related polysaccharides is due to the action of bacteria and protozoa in the digestive tract. These organisms degrade cellulose via an anaerobic mechanism to short chain fatty acids which the host can utilize as nutrients (29, 99).

The occurrence of cellulases in higher plants has been verified but little investigated. The enzyme has been demonstrated in sprouting barley, potatoes, and other germinating plants (100, 113). In view of the fact that plants elaborate cellulose, it is probable that cellulase has a wider distribution in plants than is now generally realized. It would appear mandatory that the plant cell possesses a cellulase in order to allow for growth and expansion of the cell. Cellulase occurs in other species of plants and an extensive list of the occurrence of cellulase in nature has been compiled by Pigman (100).

The enzymatic hydrolysis of cellulose has been studied with varying degrees of intensity for the last 75 years. During the early years the observations were largely qualitative and, because of the crudeness of the methods employed, the results are sometimes difficult

to assess in terms of modern enzymology. Many of the early workers used the plant cell wall as substrate and its dissolution by extracts of germinating seeds and invertebrates was observed. Until about 15 years ago the snail was regarded as the source of the most powerful cellulase and was used almost exclusively. In the last decade, however, the choice source of cellulolytic enzymes has been microorganisms. This choice is largely because of considerations already mentioned as well as the ease and convenience of culturing microbes as compared to snails.

The study of cellulose-hydrolyzing enzymes is complicated by the insolubility and the heterogeneity of cellulose itself. There is virtually no knowledge on the effect of insolubility on substrate-enzyme interactions and it has proved almost impossible to prepare two identical batches of cellulose of known chain length. There is still some question regarding the structure of cellulose, and the purity of native cellulose is debatable. As the result of these complications the fundamental question of whether a single enzyme or several enzymes is involved in the decomposition of cellulose has not been satisfactorily resolved. In spite of intensive and exhaustive studies that have been carried out since the end of the Second World War, there are still many facets of cellulose decay that remain to be elucidated. As of now only one investigator has reported the crystallization of cellulase (87, 92), and this report has not been confirmed. There has not been agreement on the exact mechanism of

the decomposition by even a single organism, nor has there been complete agreement on the end products of the degradation.

In short, it appears that many more years of research will be required before the entire picture of cellulose decomposition in nature is completely elucidated. In view of the many considerations already mentioned, research on the mechanism of cellulose hydrolysis appears justified.

REVIEW OF THE LITERATURE

Cellulose Structure and Biological Decay

Chemical structure. Because of its industrial importance, the chemical and physical structure of cellulose has been the subject of intensive investigations. As a result, the structure is fairly well known. Some of the latest reviews of current understanding of cellulose structure include those of Aspinall (3), Compton (24), Nickerson (85), and an extensive and comprehensive monograph by Ott (95).

Cellulose occurs in the plant cell wall with a mixture of other polysaccharides and non-carbohydrate substances such as lignin. The principal polysaccharides other than cellulose are the hemicelluloses, an ill-defined mixture of polysaccharides consisting primarily of pentoses and uronic acids. The commercial preparation of cellulose involves drastic treatment to remove the hemicelluloses and lignin, and it is generally thought that some degradation of cellulose accompanies its purification. The purest natural source of cellulose is the seed hairs of the cotton plant (85-97 percent) (37).

Chemically, cellulose is composed of linear chains of D-glucose residues joined mutually by β-1,4 glucosidic bonds. This chemical structure has been confirmed by many types of studies. Native cellulose molecules contain 3000 or more anhydroglucose units while reprecipitated cellulose contains from 200-600 glucose units (115). However, these values must be regarded as minimal since degradation

of the molecule in the course of its isolation and in the molecular weight determination cannot be excluded. It has been found that carefully prepared samples of cellulose contain more than 10,000 glucose units per molecule (38, 45). Pascau (96) has postulated that native cellulose possesses an infinitely high molecular weight and is unintentionally degraded to chain lengths of approximately 3000 during the usual isolation and analysis.

Freudenberg's picture of the cellulose molecule stipulates that the fiber is held together by three types of force (32). The first and most important of these forces is the primary valence bond, the β-1,4 glucosidic linkages which have an energy of approximately 50 kcal per mole. The separate chains are held together by hydrogen bonds whose binding strength is about 15 kcal per mole (76). Much weaker van der Waal's forces also operate in the cellulose fiber and the strength of these is about 8 kcal per mole. All of these figures appear to need revising. Pauling (97) lists 70.0; Pitzer (101), 79; and Dewar (27), 88.2 kcal per mole for a C-O bond. Pauling lists about 5 kcal per mole for the strength of a hydrogen bond and van der Waal's forces are much weaker than the hydrogen bonds.

There are indications of a small number of covalent linkages in cellulose other than the β-1,4 bonds, but to date their occurrence has not been positively demonstrated (115). These other types of bonds would be very important from a biological standpoint since they would provide linkages that must be enzymatically ruptured and their number and distribution would affect the ease with which cellulose is

biologically degraded (123). Reese (109) has suggested that since occasionally a few β-bonds occur in starch, perhaps a few α-bonds occur in cellulose.

Physical structure. It has been established by x-ray studies that the cellulose fiber is essentially a crystalline body with submicroscopic disorganized regions. In the crystalline regions of the fiber the glucose chains are arranged in an orderly and compact fashion. The disorganized or amorphous regions lack this organization.

The physical structure of cellulose has a marked effect on the ease with which it is degraded biologically. It has been established that cellulase preparations preferentially attack the amorphous regions of the cellulose fiber (127). This conclusion appears to be reasonable. The loosely packed areas of the fiber should be more accessible to the large protein enzyme than the tightly oriented chains of the crystalline regions. Many observations are available to substantiate preferential utilization of the amorphous regions of the fiber by the enzyme. Seilliere, quoted by Tracey, (123) observed that native cotton, which was not attacked by the enzyme, was readily attacked after precipitation from copper-ammonia solution. A resistant residue remained, but this residue was totally digested after reprecipitation and retreatment with the enzyme. He also showed that preliminary treatment with zinc chloride or sodium hydroxide made cotton susceptible to the action of enzymes. Karrer and Schubert (60) demonstrated that the rate of enzyme action depends on substrate area

rather than substrate mass. Consequently, any mechanical treatment of cellulose that increases the surface area will increase its susceptibility to enzymic attack. Partial disintegration in a Wiley mill has a marked effect on the ease of hydrolysis which seems to be higher than can be accounted for on the basis of increased surface alone. Possibly depolymerization also occurs with this treatment.

Thus far, two variable attributes of cellulose fibers have been shown to influence its susceptibility to enzyme attack: the substrate area and the degree of orientation of the sub-units making up its structure. The rate of attack has been believed to be independent of the degree of polymerization of the cellulose. However, there is some evidence which suggests that the latter opinion requires revision (109). Evidence bearing on this question will be presented in this thesis.

Methods for Assaying Cellulase Activity

Chemical methods. Because of the diverse chemical and physical properties of cellulose, many methods have evolved for the study of cellulase action on cellulose. As the cleavage of the glycosidic bond releases a potential aldehydic group on carbon atom 1 of the anhydro-glucose unit, reducing sugars accumulate in the enzymatic digests. Therefore an analysis for reducing sugars is a convenient way of measuring enzyme activity and has been used more often than any other method (11, 30, 40, 42, 53, 104, 105, 112, 123, 126, 135).

Physical methods. Most of the other methods used for assaying cellulase activity are based on the physical properties of cellulose. These methods include gravimetric determination of residual cellulose after hydrolysis (126, 127), determination of loss of tensile strength of cotton fabrics (105) and a photometric measurement of the disappearance of cellulose dextrins (93). Another method that has become popular in recent years has been the measurement of changes in viscosity of soluble cellulose derivatives (64, 68, 69, 104, 106, 121, 122). The use of soluble cellulose derivatives has been criticized (93, 123) because they are no longer cellulose and they do not occur naturally. However, this particular method is likely to become the method of choice as many soluble cellulose derivatives are now available commercially. Still other methods include measurement of changes in x-ray diffraction patterns of cellulose (52,86), and measurement of turbidity changes of suspended cellulose (83). The most recent proposal for assaying cellulase activity is to measure the change in birefringence of cellophane films as they undergo enzymatic digestion (121). This method may prove useful where concentrated enzyme solutions are available but appears to be of little value in measuring dilute enzyme solutions.

In this investigation the primary index of cellulase activity has been the accumulation of reducing sugars in enzymatic digests. Occasionally the change in viscosity of a soluble cellulose derivative has also been used.

Multiplicity of the Cellulolytic System

Unienzymatic theory of cellulose hydrolysis. Whitaker (135) has provided evidence that the cellulase of at least one organism consists of a single enzyme. From the culture filtrates of an active cellulose-decomposer, Myrothecium verrucaria, he isolated a protein fraction that was homogeneous when tested by two of the criteria used for protein purity. The protein migrated as a single component in moving boundry electrophoresis at 3 pH's (6.82, 5.03, and 4.33) and was homogeneous when tested in the ultracentrifuge. The protein fraction had approximately the same enrichment in enzyme activity towards the following compounds: untreated cotton linters, swollen cotton linters, a partially degraded cellulose, a soluble cellulose derivative, and cellobiose. In other words a single protein is capable of hydrolyzing native cellulose to glucose. In the purification procedure used by Whitaker (precipitation with ammonium sulfate, fractionation with ethanol, and precipitation with polymethyl acrylic acid) the final protein fraction contained about 45% of the total enzyme activity present in the crude culture filtrate.

Although the unienzymatic theory of cellulose hydrolysis is in conflict with other data that suggest a multienzymatic cellulase system, Whitaker has not abandoned his original conclusion that in Myrothecium verrucaria a single enzyme is responsible for the hydrolysis of cellulose to glucose. In his most recent papers (136, 137, 138, 139, 140, 141) Whitaker has been concerned with the physical chemistry of the

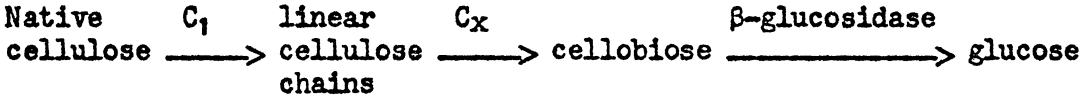
enzyme and the hydrolysis of soluble β-1,4-oligoglucosides. In one of his papers (140) he presented data on the partial denaturation of the enzyme and its subsequent activity on various water soluble members of the cellulose series. For example, heat treatment of the enzyme at 70° C for 30 seconds resulted in a 90% loss in activity toward cellobiose, 73% toward cellotriose, 50% toward cellotetraose, and 18% toward cellopentaose. Such differential effects are usually considered to be an indication of enzymatic heterogeneity, but Whitaker has advanced an explanation which is consistent with the one enzyme theory. He believes that an enzyme can undergo various degrees of denaturation and then the differential effects can be interpreted as indicating that the enzyme underwent structural changes whose effects on enzymic activity varied with the chain length of the substrate.

Other support for the unienzymatic theory. Kooiman et al (64), in an independent study of the cellulase of Myrothecium verrucaria, concluded that a single enzyme hydrolyzed native cellulose to cellobiose and a separate enzyme (cellobiase) hydrolyzed the cellobiose to glucose. Kooiman based his conclusion chiefly on the observation that crude culture filtrates of this organism contained enzyme activities which hydrolyzed native cellulose, partially degraded cellulose, soluble and and insoluble cellulose dextrins, and cellobiose. The activity against cellobiose could be eliminated by boiling the filtrate but the activity against the other compounds was retained. They concluded that such a high degree of thermal resistance of proteins is too rare a property

for two or more enzymes to possess it to exactly the same degree. Obviously, these data are not as powerful as those provided by Whitaker.

Multienzymatic theory of cellulose hydrolysis. Most of the other data which have accumulated on the enzymatic hydrolysis of cellulose have favored a multienzymatic system. Much of this data has been provided by the Pioneering Research Laboratories of the U. S. Army Quartermaster Corps. Although their function is primarily that of an applied research group they have made many contributions to the basic understanding of cellulose decomposition. Part of their program (105) has been the routine screening of organisms for their ability to degrade cellulose, and in their unique position they have been able to collect cellulose-decomposing organisms from all parts of the world. As a result they probably have the largest culture collection of cellulose-decomposing organisms in existence.

The data from the Quartermaster Laboratory have favored a multienzymatic decomposition of cellulose and in their latest paper (109) Reese summarized the views of that laboratory. Reese believes that the decomposition of cellulose proceeds in at least three steps and involves a minimum of three enzymes. The following diagram illustrates the views of the Quartermaster Laboratory.



According to this scheme, native cellulose is first attacked by some factor which has been designated C_1 by these investigators. Presumably, the function of C_1 is to prepare the cellulose for action by the second enzyme, C_X. The second enzyme hydrolyzes the linear chains to the disaccharide, cellobiose. The cellobiose is cleaved to glucose by cellobiase, an enzyme which is loosely termed β-glucosidase by these authors. Cellobiase is the preferred name for this enzyme, inasmuch as the term β-glucosidase would include all enzymes which hydrolyze β-glucosides. As has been observed by Jermyn (56) and as will be shown in this paper, there are enzymes in cellulase preparations which hydrolyze various β-glucosides but which do not hydrolyze cellobiose.

Role of C_1. The role of C_1 is very obscure and to date there has not been a positive demonstration of its actual existence. The support for including this factor in the above scheme is largely from indirect evidence. Its presence was deduced from the fact that many species of bacteria and fungi can grow on native cellulose, partially degraded cellulose, and cellobiose, while other species can grow only on the degraded celluloses and cellobiose (105). The former group presumably possesses C_1, C_X, and cellobiase, whereas the latter group possesses only the factors C_X and cellobiase. This factor is thought to act by disrupting the crystalline areas of the cellulose fibers, thereby making the linear chains more accessible to the second enzyme, C_X.

"Hydrogen bondases". Siu (114) has boldly postulated that C_1 is a "hydrogen bondase," the function of which is to break hydrogen bonds in the oriented cellulose fiber. Since there are no known analogous enzymes, most authors have avoided postulating such an enzyme. However, as Rodebush (110) points out, cellulose is one of the most heavily hydrogen bonded substances in existence. Even though the strength of the individual hydrogen bond is small, the enormous numbers of such bonds in a cellulose fiber greatly increase the strength of the fiber. In the course of converting the cellulose molecule to glucose, these bonds must be broken. Whether or not there is an enzyme which specifically catalyzes the rupture of these weak bonds is purely speculative at this time.

"Swelling factor". Another enzymatic factor which attacks cellulose in the early stages of decomposition has recently been discovered (77, 78, 79, 80, 108). This factor has been termed the "swelling factor" and its mode of action is very obscure. It was found that cellulose fibers which had been treated with this factor swelled greatly in alkali. Untreated cellulose fibers do not possess the same degree of swelling. It has been established that the "swelling factor" has its activity on the primary wall of the cellulose fiber. It has been further established that the site of action is on the cellulose and not the non-cellulosic components of the fiber wall such as the pectin, wax, and protein which are components of the primary wall of the fiber (108). It is not known what type of

linkage the "swelling factor" attacks. As a matter of fact, it has not been established that it attacks any linkage. No reducing sugars are formed by its action.

The enzymatic nature of this factor has been definitely established. The factor is destroyed by heat, is non-dialyzable, is precipitated by protein precipitants, and exhibits pH optima in regard to its stability and activity. Furthermore, the factor is adaptive; that is, it is produced only when cellulose is included in the growth medium of the organism.

It has not been possible to establish whether the "swelling factor," "C_1," and "hydrogen bondase" are the same enzyme. In this connection it is of interest to examine Reese's data (108) regarding the "swelling factor" more closely. Trichoderma viride, a highly cellulolytic organism (both C_1 and C_x) produces a great deal of the "swelling factor." Penicillium pusillum, a weakly cellulolytic organism (some C_1 and C_x) also produces appreciable amounts of "swelling factor," whereas Aspergillus niger, a non-cellulolytic organism (only C_x) produces small but definite amounts of the "swelling factor." On the basis of this limited evidence it would appear that the "swelling factor" and C_1 are different, but that the "swelling factor" and C_x might be identical. However, Reese has established that the latter two factors are different. Further studies with many species of cellulolytic and non-cellulolytic organisms in regard to their production of the "swelling factor" may serve to distinguish completely between this factor and C_1. Probably, only

the Quartermaster Laboratories have a sufficiently large culture collection of these organisms to make such a survey.

Other factors in the initial stages of degradation. One other fact concerned with the initial stages of degradation should be pointed out and emphasized. To date there have been no cell-free preparations of cellulase that will attack crystalline cellulose rapidly. This failure has been attributed to the inability of the large protein molecule to penetrate the tightly packed areas of the crystalline fiber. Yet the organism itself, growing in contact with the cellulose particle, can dissolve cellulose rapidly. An active cellulose decomposer can consume more than 50% of the native cellulose (0.5% suspension) in shake flasks in 3-4 days. Yet the same solution, free of the organism, can achieve only a tiny fraction of this hydrolysis, even though the solution is very active against degraded celluloses (109). From this type of evidence it appears that not all cellulolytic enzymes are secreted into the medium. It has been established that for rapid dissolution to take place the organism must be in intimate contact with the cellulose particle (115). It is quite possible that cell-surface enzymes are responsible for this phenomenon and that one member of the cellulase complex is such a cell-surface enzyme. However, cell-surface chemistry is an extremely difficult field of study and it will probably be some time before any information of this nature becomes available.

An attempt has been made in the previous sections to point out the difficulties involved in studying the initial stages of cellulose decomposition and to emphasize the tenuous nature of the present theories concerning this part of the attack. Time and further studies are needed to clarify the role of C_1, "hydrogen bondase," "swelling factor," and any other factors.

Role of C_x. The factor C_x is the component of the cellulolytic system which has received the greatest attention. It is the activity of this enzyme or enzymes that is being measured by most of the various methods previously mentioned. It is the enzyme that cleaves the β-1,4 glucosidic bonds and is consequently easier to study than the hypothetical C_1 or "hydrogen bondase." A major question regarding C_x is whether there is only one enzyme involved or whether there are many C_x's, whose specificity depends on the chain length of the cellulose molecule. There is some evidence to support the idea of many C_x's but to date a good separation of these factors has not been achieved. In this respect, the inavailability of cellulose of known chain length has been a limiting factor as has been the lack of techniques for fractionating closely related protein species.

Role of cellobiase. This enzyme (the β-glucosidase of Reese and Siu) is widely distributed among bacteria and fungi. Grassman et al (39), Nishizawa (90), Ploetz (102), and Reese et al (105) partially separated the cellobiase from the cellulase of various microorganisms. The

latter investigators concluded that although the amount of cellobiase present in culture filtrates is low, it is the enzyme responsible for the hydrolysis of cellobiose to glucose.

Other evidence for the multienzymatic theory. Whitaker (135) does not consider the data of the Quartermaster Laboratory to be at variance with the unienzymatic theory. He points out that the properties of a cellulase must include not only β-glucosidase activity but also physical properties which permit its adsorption onto an insoluble cellulose surface. Then, the data of the Quartermaster Group can be interpreted that one group of organisms possesses an enzyme with both properties (according to Reese they have C_1 and C_x), and the other group of organisms possesses an enzyme with only the former property (according to Reese they have only C_x). In regard to the adsorbability of cellulase on cellulose, the surface potential of the cellulose particle has been emphasized by Whitaker (134) and Basu et al (11) who demonstrated that various proteins and dyes adsorbed on the fiber had a pronounced effect on enzyme action. Whitaker found that traces of albumin increased the rate of hydrolysis of cotton linters as much as 500 percent. The dyes were either stimulatory or inhibitory, depending on the pH of the reaction mixture (11).

In spite of this apparent reconciliation of the two theories of cellulose hydrolysis, most of the latest work favors the multienzymatic theory. By subjecting the filtrates of many organisms to separation by paper chromatographic procedures (107), and cellulose and calcium

phosphate gel column chromatography (34), Reese and Gilligan were able to demonstrate several components with cellulase activity in the filtrates. However, it has not been established that these components actually represent different enzymes. By use of starch gel electrophoresis, Miller and Blum (81) were able to separate the cellulase activity of Myrothecium verrucaria (the organism used by Whitaker) into several components. Using the same organism, Grimes (41) also found multiple components of cellulase on electrophoresis. These observations serve to demonstrate that various ionic species of cellulase are present in culture filtrates but again it has not been possible to call each component a separate enzyme. The lack of well-defined substrates of known chain length has seriously handicapped the characterization of these components.

In a study of the cellulase of Aspergillus oryzae, Jermyn (53, 54) and Gillespie and Woods (33) have shown the presence of several electrophoretically distinct proteins with cellulase activity in culture filtrates of the organism. Jermyn also established the presence of several β-glucosidases in these filtrates, some of which he believed were active on rather long cellulose chains. Reese (109) is inclined to doubt the latter possibility since Grassman et al (39) established in 1933 that β-glucosidase (cellobiase) was active on the cellulose series from cellobiose to cellohexaose and had no activity on cellulose dextrins with a chain length of more than six. Reese's objection to Jermyn's conclusion is in turn subject to other

objections. First of all, there is no assurance that the preparation used by Grassman was a single enzyme and, even assuming that it was, the assumption that all other cellobiases have exactly the same specificities appears unwarranted. Secondly (as has already been pointed out) the notion that all β-glucosidases are cellobiases is not valid. There are many β-glucosidases (cellulase is a β-glucosidase) whose specificities vary and there is no theoretical reason why there could not be several β-glucosidases whose specificities depend only upon the nature of the substituents and the chain length. Future studies may well establish that the entire cellulase complex consists of a series of β-glucosidases whose specificities are for a particular range of chain lengths. Reese (109) has provided data which suggest that the enzymes do have a preferred chain length and further information on this subject is presented in this thesis. This hypothesis may account for the inability of current techniques to achieve a clear cut separation of the proteins in culture filtrates of cellulolytic organisms. Large physical or chemical differences between the protein molecules need not be expected. Again the lack of cellulose substrates of known chain length has seriously handicapped the characterization of such proposed enzymes.

β-aryl glucosidase. In addition to "C_1," "hydrogen bondase," "swelling factor," "C_X," and cellobiase activities which are present in culture filtrates, there are other enzymes present in the same filtrates whose action is also on β-glucosidic linkages. The relationship of these enzymes to the cellulase complex is not clear, but they

will be included in this section because their activity is on β-glucosidic bonds.

One of these enzymes is called β-aryl glucosidase, since it is active on β-aryl glucosides. This enzyme has been demonstrated in culture filtrates of many cellulose-decomposing microorganisms and has been erroneously identified as cellobiase (42, 69, 105). Jermyn (56) was the first to show that this type of enzyme (which he calls β-glucosidase) was not a cellobiase. The enzyme hydrolyzes a large number of aryl glucosides such as the phenyl- and naphthyl-β-glucosides. For example, p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-cellobioside have been used extensively as substrates by Nishizawa (87, 88, 89, 90, 91, 92). Reese and Levinson (105) used salicin (o-hydroxymethyl phenyl-β-D-glucoside) and cellobiose as substrates for β-glucosidase activity and observed that filtrates of highly cellulolytic organisms were more active on salicin than on cellobiose. It is probable that they were observing the activities of two separate enzymes: a cellobiase and a β-aryl glucosidase. It is also probable that other data published on cellobiase activity are erroneous because salicin was used as substrate in most of these experiments (105). It follows that in order to measure the concentration of cellobiase accurately, cellobiose must be used as substrate. The function of a β-aryl-glucosidase in culture filtrates of cellulolytic organisms is obscure, but it will be shown in this thesis that these enzymes act as gluco-transferases and transfer the glucose part of the aryl glucoside to a variety of alcohols.

Transglucosidases. Another enzyme in culture filtrates of cellulolytic organisms whose activity is on β-glucosidic linkages is a glucose-transferring enzyme, or simply a transglucosidase. This enzyme transfers a glucose molecule from one cellobiose molecule to another cellobiose molecule forming a trisaccharide and free glucose. There is no net synthesis of glucosidic bonds, and presumably this transfer is possible because of a low energy barrier encountered in the reaction. This reaction can be thought of as an enzyme-catalyzed displacement reaction, a type common in organic chemistry. The reaction sequence is diagramed as follows:

glucose-glucose + enzyme ——> enzyme-glucose + glucose
(disaccharide-donor)

glucose-glucose + enzyme-glucose ——> glucose-glucose-glucose + enzyme
(disaccharide-acceptor) (trisaccharide)

In some still unknown fashion, one of the glucose residues is transferred to the enzyme so that the energy of the bond is preserved. The glucose is then transferred to a suitable acceptor (cellobiose or another sugar) and the enzyme is freed to catalyze another transfer. In the same fashion the newly formed trisaccharide could act as acceptor and a tetrasaccharide would be formed. This process could be repeated until a high polymer is formed. As there are several hydroxyl groups on the acceptor molecule to which the glucose residue could be transferred as well as two types of configuration for the new linkage (α or β), the theoretical number of such sugars is enormous. Since the number of sugars found in nature is limited, the specificity of the enzyme for the position and the configuration must be restricted. Virtually

nothing is known about the synthesis of cellulose in nature but it would seem that transglucosidases would offer a good clue to its synthesis.

These transglucosidases have been observed only recently. They were first reported in cellulolytic culture filtrates by Crook and Stone (26) in 1953. Since that time there have been numerous reports concerning transglucosidase activity in cellulolytic and non-cellulolytic culture filtrates. The cellulolytic organisms include Myrothecium verrucaria (26, 109), Trichoderma viride, Pestalotia palmarum, Stachybotrys atra (109), and Chaetomium globosum (19, 20, 21). The reaction has been observed in non-cellulolytic culture filtrates of Aspergillus niger (5, 6, 8, 9, 18, 98), Aspergillus luchuensis (109), Aspergillus flavus (35), and Aspergillus oryzae (55).

It has been suggested that transglucosidase and β-glucosidase may be the same enzyme (109), with the acceptor molecule determining the nature of the reaction. If the acceptor molecule is water the reaction will be a hydrolysis, and if the acceptor is a compound other than water the reaction will be a transglucosidation.

The exact role that transglucosidases play in cellulolytic culture filtrates has not been clarified. It seems probable that these enzymes do not participate in the actual degradation of cellulose, but they could have a profound effect on the final products that are found in cellulose hydrolyzates. Information on the nature of the products of these enzymes will be presented in this thesis.

Cellobiose phosphorylase. This enzyme cleaves cellobiose in the presence of inorganic phosphate to give glucose and glucose-1-phosphate. It is included in this discussion because it has been observed in cellulose-decomposing organisms (50, 116). It appears to be an intracellular enzyme and as such does not participate in the extracellular decomposition of cellulose.

Cellulose phosphorylase. This enzyme would cleave cellulose in the presence of inorganic phosphate to give glucose-1-phosphate and a cellulose chain with one less glucose unit. As far as is known this type of enzyme has never been reported. It is included here because an analogous enzyme, starch phosphorylase, is well known (129). It seems plausible that such an enzyme exists for cellulose. It is also probable that it would be an intracellular enzyme. In all the cases studied, this enzyme was absent and most assays for cellulase activity were made in the absence of inorganic phosphate.

Other polysaccharases. The culture filtrates of cellulolytic organisms that are commonly used for studies on cellulose decomposition are exceedingly complex mixtures of enzymes. In addition to the enzymes of the cellulase complex, there are other enzymes, which degrade polysaccharides, present in crude filtrates of these organisms. Independent investigations of the filtrates of Myrothecium verrucaria have revealed the presence of several other polysaccharases. Miller and Blum (81) reported amylase; Grimes (41), hemicellulase; and Aitken et al (2), an enzyme which cleaves the bonds of laminarin,

a β-1,3 linked glucosan. The latter investigators also found that culture filtrates of this organism hydrolyzed a barley β-glucosan which consisted of β-1,4 and β-1,3 linkages. The action on the latter is presumably the result of cellulase and the β-1,3-glucosidase. Thomas (121) found that a cellulase preparation from Stachybotrys atra hydrolyzed a β-1,4 xylosan. Whistler and Masak (130) reported that fractionated enzymes from Aspergillus foetidus hydrolyzed both xlyan and cellulose. In view of the structural similarities of xylan and cellulose (xylan consists of β-1,4 xylopyranose units; cellulose, β-1,4 glycopyranose units), it seems probable that the same enzyme could hydrolyze both substrates. Tracey (124) reported the presence of chitinase in cellulolytic soil protozoa and it appears probable that even more polysaccharases will be found in future studies.

It is difficult to assess the role that these enzymes play in cellulose decomposition. If cellulose does contain small numbers of covalent linkages other than β-1,4 bonds, some of these enzymes could conceivably act on those linkages. The possibility that these enzymes could exert an influence on the observed course of cellulose hydrolysis seems to have been ignored.

Cofactors and activators. The enzymes that degrade cellulose do not appear to require any accessory factors, either organic or inorganic. Most of the enzyme preparations used by various workers have been dialyzed exhaustively and such dialysis does not decrease

the activity of the enzyme. Thomas (121) even subjected his cellulase preparation from Stachybotrys atra to exhaustive electrodialysis without loss of activity.

The Mechanism of Cleavage of the Cellulose Molecule

Endwise or random cleavage? There is an abundance of information in the literature which indicates that most of cellulases degrade cellulose in a completely random manner. These data include: (A) Cotton fabric and cellophane strips lose most of their tensile strength before reducing sugars are formed, (B) depolymerization of regenerated cellulose occurs without a concomitant increase in reducing sugar, and (C) there is a rapid decrease in the viscosity of soluble cellulose derivatives before reducing sugars are formed. To date there has been only one cellulase reported which appears to cleave the cellulose molecule in an endwise fashion from the end of the molecule (87, 90).

There are two types of amylase that degrade starch in the manners mentioned above and, as a great deal of information on these enzymes has accumulated, it may be valuable to compare the cellulose and starch hydrolyzing enzymes further. α-amylase hydrolyzes the starch chains (α-1,4 linkages) in a random fashion producing α-maltose and traces of glucose as the final product (13). Since the attack is random, intermediate compounds such as the tri-, tetra-, penta-, and hexasaccharides of the maltose series accumulate in the hydrolyzate. β-amylase acts on starch in a stepwise fashion to produce β-maltose, the action proceeding from the non-reducing end of the molecule. Why there is an inversion of the α-configuration to produce β-maltose is as yet unknown. As individual maltose molecules are split from the chain, there are no intermediate compounds formed.

Until recently it has been impossible to determine the configuration of the cellobiose that was liberated enzymatically from cellulose chains because mutarotation was faster than the hydrolytic reaction. The difficulty lay in finding a substrate that was attacked by the enzyme fast enough to produce measurable amounts of sugars before mutarotation established equilibrium mixtures of the α and β forms. Whitaker found that cellopentaose was hydrolyzed rapidly enough by his enzyme to provide this information (137). Although the differences were very small, he found an upward mutarotation of the sugars in the hydrolyzate of cellopentaose. The upward mutarotation indicates that the β-configuration is retained when the molecule is hydrolyzed. In this respect this cellulase resembles the action of α-amylase and the observation is compatible with other evidence indicating a random cleavage of the cellulose molecule. Nishizawa (87) has not yet reported whether Irpex lacteus cellulase (which appears to be analogous to β-amylase) liberates cellobiose in the α or β configuration. In the majority of cases studied, the evidence indicates that cellulases are analogous to the α-amylases; that is, they hydrolyze cellulose in a completely random manner.

End products of cellulase action. The end products of cellulase have long been a subject of interest. Certainly glucose is the ultimate end product and in all cases studied glucose appears in the enzymatic digests. However, according to the hypothesis set forth by Reese (109), the glucose arises as the result of contaminating

cellobiases and actually cellobiose is the end product of cellulase action. There is some evidence to support the idea. Cellobiose inhibits the hydrolysis of cellulose by cellulase but glucose does not (94, 106). It is a common experience for end products of enzyme action to inhibit the reaction. For example, glucose inhibits invertase, maltose inhibits amylase, and inorganic phosphate inhibits phosphatase. Hungate (51) found that Clostridium cellobioparus, an anaerobic cellulose decomposer, produced only cellobiose and no glucose as the final product.

Whitaker (135) questions the data of Reese by showing that his single protein enzyme from Myrothecium verrucaria hydrolyzed cellulose to an approximately equimolar mixture of glucose and cellobiose. He contends that glucose formation does not depend on the prior formation of cellobiose. In other words, the enzyme is capable of removing single glucose units from the chain. If Whitaker's observation is correct, then it is difficult to explain why cellobiose but not glucose inhibits the enzymatic reaction. The inhibition may be related to the presence of glucosidic bonds, and cellobiose is the smallest member of the cellulose series which contains this linkage. Thomas (121) found that the cellulase from Stachybotrys atra was inhibited by neither glucose nor cellobiose, and Nishizawa (87) found that the crystalline enzyme from Irpex lacteus (analogous to β-amylase?) was inhibited by glucose. He did not mention inhibition by cellobiose. However, all of Nishizawa's papers have been read only in abstract as the originals have not been seen in English translation.

Intermediate sugars. Thus far, the analogy between the α-amylases and the majority of the cellulases studied appears valid. However, there is one facet that needs further investigation. It has been pointed out that intermediate sugars accumulate in hydrolyzates of starch by α-amylases. These intermediate sugars do not normally accumulate in hydrolyzates of cellulose. Levinson (69) postulated that these sugars were more available to the enzyme and consequently were hydrolyzed more rapidly than the insoluble chains. In other words, the rate limiting reactions are those concerned with the insoluble molecule. This hypothesis has been strongly substantiated by Whitaker (138), who found that the water soluble members of the cellulose series were rapidly attacked by his enzyme preparation.

In an attempt to provide a positive demonstration of the intermediate sugars, Hash and King (43) used a dialysis technique to allow the intermediate sugars to accumulate. Small quantities of a higher saccharide, which they concluded was a tetrasaccharide, were isolated from the dialysate. Since the enzyme showed no transferase activity on cellobiose at concentrations equivalent to the total hydrolysis of the cellulose, they concluded that the product was a true intermediate dextrin. This demonstration was accepted as further support of the random cleavage hypothesis by Whitaker (141), but has been rejected by Reese (109) who believes it to be the product of a transglucosidase rather than a cellulase. Kooiman et al (64) also reported sugars which they considered to be intermediate dextrins, but this observation, too, has been rejected by Reese who believes

them to be substituted sulfate esters. For substrate, Kooiman used cellulose which had been partially hydrolyzed by 72% sulfuric acid. It is known that sulfate esters do form with cellulose under these drastic conditions. Both Kooiman, and Hash and King relied heavily on paper chromatographic methods for identification and neither was able to isolate sufficient amounts of the sugars to characterize them rigidly.

Objectives of the Research

An attempt has been made in the previous discussion to present the major points of view of various investigators. The evidence for the theories concerning the unienzymatic versus the multienzymatic nature of cellulase has been examined. It is clear that both groups have support for their views and that further studies are necessary to reconcile the differences between the two.

The question of whether cellobiose is an obligatory precursor of glucose or whether glucose can arise independently of cellobiose has not been settled definitely. Likewise, the question of whether the higher saccharides which have been reported (43, 64) are true intermediate dextrins has not been satisfactorily confirmed. It appears that in order to answer the first question unequivocally, the cellobiase must be separated from the cellulase, if possible, and the end products of cellulase determined in the absence of cellobiase. To answer the second question, substituted celluloses as substrates must be avoided and the transglucosidases must be separated from the

cellulases. Then if higher saccharides are observed, their source could be only from the random cleavage of the cellulose molecule.

The present investigation was proposed in an attempt to provide information on the following phases of cellulose decomposition: (A) the multiplicity of the cellulase system; (B) the formation of glucose in the course of hydrolysis; (C) the significance of the intermediate dextrins; and (D) the properties of β-aryl glucosidase in culture filtrates of Myrothecium verrucaria.

There are two techniques, not yet fully investigated, which may yield information on these aspects of the problem. These methods are paper electrophoresis and column chromatography. Paper electrophoretic methods have been applied to the cellulase of Stachybotrys atra by Jermyn (53, 54) and Thomas (121), and to the cellulase of Aspergillus oryzae by Gillespie and Woods (33), but apparently have not been applied to other cellulolytic systems except in this laboratory (44). Column chromatography has been used by Gilligan and Reese (34) for the study of cellulase activity in filtrates of various organisms. This method, however, is capable of many innovations and refinements and a more detailed study of the cellulase of one organism by such techniques appears warranted. In view of the recent successes in separating proteins by chromatographic methods (48, 103), this approach appeared especially promising.

The paper electrophoretic studies have been used primarily to establish the existence of differently charged species of cellulolytic

enzymes and the column methods have been used in an attempt to separate cellobiase, transglucosidase, and β-aryl-glucosidase from cellulase in order to establish the nature of the end products of cellulase action.

MATERIALS

Selection of Enzyme Source

Myrothecium verrucaria. The mold, Myrothecium verrucaria (Alb. and Schw.) Ditm. ex. Fr. strain 1334.2 of the U. S. Department of Agriculture, was selected as the biological agent for the production of cellulose-decomposing enzymes. This mold is an active cellulose decomposer and has been used extensively as a test organism in the production of fungicides for use in the protection of textiles (114). Many investigators have selected this organism for their studies on cellulose decay and as a result there is more extensive literature on this particular organism than any of the other cellulose decomposers. Saunders (112), Whitaker (133, 134, 135, 136, 137, 138, 139, 140, 141), Crook and Stone (26), Hash and King (43), Kooiman et al (64), Aitken et al (2), Basu and Whitaker (11), Grimes (41), Miller and Blum (81), and Reese et al (105, 108) have used this organism as an enzyme source. It is this organism that Whitaker has used to advance the single enzyme theory, and it is the same organism that Miller and Blum used in an attempt to expand the multienzymatic theory.

Also, investigations by Mandels (71, 72, 73, 74, 75) on the metabolism of Myrothecium verrucaria contribute to a greater biochemical knowledge of this organism than any other active cellulose decomposer.

A culture of this organism was obtained from the American Type Culture Collection, Washington, D. C. It was maintained on a mineral salts-agar medium whose composition is listed in the Appendix.

Enzyme production. The organism was grown in mass culture as follows: Two liters of a cellulose-mineral salts medium were sterilized in a 4-liter serum bottle for 1 hour at 121° C. The composition of the medium and a diagram of the culture apparatus are in the Appendix. The aeration train was sterilized separately and inserted into the bottle as it was removed from the autoclave. After cooling, the medium was inoculated with a spore suspension of Myrothecium verrucaria. The inoculum was a four-day culture of the organism grown on filter paper slants. Sterile water was added to the slant, the spores were scraped off, and the suspension was transferred aseptically to the bottle of sterile medium by way of the inoculating orifice. Air was forced through the sterile cotton-filled tube and the bottle of sterile water before it entered the diffusion stone in the culture medium. The cotton filter sterilized the air, the water saturated the air, and the diffusion stone produced a stream of fine bubbles for maximum aeration of the medium. The rate of aeration was sufficient to keep the cellulose in suspension. The cultures were grown in the dark for a period of two weeks.

At the end of the growth period, the mycelium was removed from the medium by filtration through a coarse stainless steel filter (75 micron pores). The filtrate was examined microscopically for

bacterial contamination and if contaminated was discarded. Culture filtrates not showing contamination were concentrated *in vacuuo* to approximately 50 ml, dialyzed overnight in collodion bags against distilled water to remove salts and other non-collodial material, and filtered through a medium porosity bacterial filter to remove insoluble protein and spores. The filtrate was then concentrated to a final volume of 10 ml.

Usually, several filtrates were prepared at the same time and all were concentrated approximately 200 fold. This concentration was sufficient for 10 microliters of the filtrate to contain enough activity for convenient assay after electrophoresis. The concentrated filtrates were stored at -18° C.

Substrates for Enzyme Action

Carboxymethyl cellulose. Two soluble cellulose derivatives were used in this study. They are products of Hercules Powder Company and are designated "70 premium low" and "70 premium high." The former carboxymethyl cellulose (sometimes referred to as CMC) was used for reducing sugar determinations and the latter for viscosimetric determinations. Respectively, their DS (degree of substitution) is 0.88 and 0.82 and their mean DP (degree of polymerization) is 125 and 200. The degree of substitution refers to the number of substituents per anhydroglucose unit and the degree of polymerization refers to the chain length of the molecule. Therefore "70 premium

low" has an average chain length of 125 glucose units and 88% of the residues have one carboxymethyl group ($-CH_2COOH$). Similarly, "70 premium high" has a mean chain length of 200 glucose units and 82% have a carboxymethyl group. When these studies were initiated, only the former was available and it was used for reducing sugar estimations of cellulase activity. The latter was obtained for viscosimetric estimations because of the higher viscosity of its aqueous solutions. As the rate of enzymatic attack was believed to be independent of chain length, the differences in DP of these two substances were ignored. This factor will be discussed more fully in the later sections.

The increase in reducing sugars was determined by the reductometric copper method and the decrease in viscosity was determined in an Ostwald type viscosimeter. The details of the methods are included in the experimental section.

Soluble and insoluble cellulose dextrins. These compounds are not commercially available and were prepared by the partial hydrolysis of cellulose. The method used was essentially that of Dickey and Wolfrom (28) for producing the homologous series of sugar acetates from cellulose. The procedure consists of simultaneous acetylation and hydrolysis of cellulose with a mixture of acetic anhydride and sulfuric acid to yield a mixture of the acetylated sugars. The free sugars are then obtained by deacetylating the acetates with sodium methoxide.

The detailed procedure is as follows: Ninety grams of powdered cellulose (Solka-floc, a product of the Brown Company, Berlin, New Hampshire) were added slowly to a mixture of 340 ml of glacial acetic acid, 340 ml of acetic anhydride, and 36 ml of concentrated sulfuric acid. The solution was allowed to stand for 72 hours at room temperature, and the resulting dark brown solution was poured into 12 liters of ice water. The white precipitate was collected on a filter and washed with cold water until neutral to litmus. Dickey and Wolfrom (28) had neutralized the acetolyzate with sodium bicarbonate before filtering, but as this step did not materially increase the yield of acetylated dextrins (only 2-3 grams) it was omitted. The precipitate was air dried for several days and then dried completely over $CaCl_2$ *in vacuuo*. Yields of the acetylated dextrins were usually about 135 gm.

The dry acetates were extracted with 2 liters of methanol under reflux for an hour, followed by a second extraction with 800 ml of methanol. Usually 110 to 120 gm of the acetates dissolved and 15 to 25 gm remained undissolved. After cooling the methanolic solution, it was made 0.1 N with respect to sodium methoxide by adding 6.4 gm of metallic sodium to the mixture. In practice the sodium was dissolved in a small quantity of methanol and the methoxide added to the acetate mixture. The flask was stoppered tightly and stirred with a magnetic stirrer. Deacetylation and subsequent precipitation of the insoluble sugars began in a few minutes and was usually complete in an hour; however, the solution was stirred overnight to insure complete deacetylation. The free sugars were collected on a filter and washed

with cold methanol until free of alkali. They were dried in vacuuo over $CaCl_2$. The yield of free sugars was usually 18 to 20 gm. The liquor from the deacetylating mixture contained large quantities of reducing sugars. Chromatographically, these sugars were found to be mostly glucose, some cellobiose, and a sugar that migrated faster than glucose. The latter was probably xylose.

The dry sugars were extracted with successive portions of water. The undissolved residue (usually 3 to 6 gm) was dried in vacuuo over $CaCl_2$, yielding the insoluble dextrins used in this study. The DP range of these dextrins was probably 10 to 30.

The soluble fraction contained large quantities of glucose and cellobiose as well as smaller quantities of soluble higher sugars. The glucose and cellobiose were removed from the mixture by subjecting it to strip paper chromatography on Whatman 3MM filter paper in an isopropanol, water, glacial acetic acid solvent (5:4:1, v/v/v) for 18 hours. A descending system was used. Guide strips were cut from the edges of the sheets (18 x 22 1/2 in) and sprayed with aniline-diphenylamine-phosphate (see Appendix for composition) to locate the sugar bands. The region that contained glucose and cellobiose was cut from the sheet and discarded, and the remaining part of the sheet was extracted with water. These sugars were used in solution and their DP range was probably 4-9. On chromatograms, the trisaccharide appeared to be missing, and the predominant sugars appeared to be the tetra-, penta-, and hexasaccharides. As these sugars were not removed from solution, yields were not determined. Attempts to separate these

soluble sugars on charcoal-celite columns (131, 132) have not been successful in this laboratory. Consequently, the mixture obtained above was used in these studies as the soluble dextrins.

On acid hydrolysis the soluble and insoluble dextrins gave glucose as the only product.

Cellobiose. This compound is available commercially in a high degree of purity. Chromatographically, the cellobiose used in these studies was completely free of glucose and other contaminating sugars.

4-O-β-D-glucopyranosyl-D-sorbitol. This compound, which is the reduction product of cellobiose, was prepared for possible use as a substrate for cellobiase activity. Its non-reducing property would be an asset in such assays. It was prepared by reducing cellobiose with sodium borohydride in aqueous solution. A typical preparation is described below.

To a solution of 3.42 gm of cellobiose (10 mM) in 25 ml of water was added 190 mg (5 mM) of sodium borohydride. The flask was held in boiling water for 5 minutes. At the end of this time the solution gave a negative test for reducing sugars. The solution was cooled and glacial acetic acid was added dropwise to decompose the excess sodium borohydride. Ethanol was added to precipitate the 4-O-β-D-glucopyranosyl-D-sorbitol. The compound was reprecipitated several times in this fashion. Crystallization of the compound was not attempted as other investigators had failed to crystallize it (1). The compound

was dried *in vacuuo* over $CaCl_2$. The yield was about 3 gm and on paper chromatograms the product gave a single spot. The compound was not completely ash-free but was readily hydrolyzed by various enzyme preparations and no further purification was attempted. Sodium borohydride was very satisfactory for the reduction and there was no rupture of the cellobiose molecule with this reagent as had been reported for lactose (49).

β-aryl glucosides. Several β-aryl glucosides from commercial sources were used in this investigation. These compounds and their sources are: (A) salicin (o-hydroxymethyl phenyl-β-D-glucoside), Pfanstiehl Chemical Company, Waukegan, Illinois; (B) phenyl-β-D-glucoside, Nutritional Biochemicals Corporation, Cleveland, Ohio; (C) p-nitrophenyl-β-D-glucoside, California Foundation for Biochemical Research, Los Angeles, California; and (D) 6-bromo-2-naphthyl-β-D-glucoside, Dajac Laboratories, Leominister, Massachusetts.

EXPERIMENTS AND RESULTS

Paper Electrophoretic Studies on Cellulase Homogeneity

Procedures. Where a moving boundry Tiselius electrophoresis apparatus is not available, paper electrophoretic methods have been widely used. While these apparati suffer severe limitations in some respects, they have nevertheless proven to be useful tools in many phases of biological research (14). As their cost is only a fraction of the more expensive moving boundry equipment, smaller laboratories have relied heavily upon paper electrophoretic methods.

Essentially, the method is to suspend a piece of filter paper, moistened with a suitable electrolyte, between two troughs filled with the same electrolyte. Electrodes are placed in the troughs and a potential imposed across the two terminals. Charged molecules will migrate on the paper under the influence of the potential according to their size and charge distribution. After a given length of time, the paper is removed from the apparatus and the distribution of material on the paper is determined by suitable methods.

In all the electrophoresis experiments reported in this section, the following conditions were observed. The apparatus was kept in a refrigerator and the internal temperature of the chamber was about 12° C. All the experiments were carried out on Whatman Number 1 filter paper, and the potential between the two electrodes was maintained at 500 volts (approximately 10 v/cm).

Because there is evaporation from the surface of the paper during the course of the experiment, the resistance of the paper to the flow of current will be decreased. Therefore, either the current or the voltage will vary. All the experiments reported in this section were conducted at constant voltage, simply because the apparatus was equipped with a constant voltage regulator rather than a constant current regulator.

A typical experiment, described in more detail, follows. A strip of Whatman Number 1 filter paper was dipped in the selected buffer until it was thoroughly soaked. The paper was then placed on another strip of filter paper and blotted lightly until the "sheen" was removed. The moist piece of paper was placed on the apparatus (Reco model E-800-2 migration chamber, Research Equipment Corporation, Oakland, California) with the two ends dipping into the troughs containing the buffer. In all the experiments, the troughs contained 350 ml of freshly prepared buffer. After the paper was in place, the enzyme solution was applied to a premarked spot on the paper in 10 ul portions. As rapidly as possible the cover was placed on the apparatus and the desired potential was placed across the two electrodes. At the end of the migration time (usually 6-8 hrs) the current was turned off and the paper was removed and subjected to various tests to locate enzyme activities. These tests are described in full in the later sections. When the apparatus was tested with serum proteins, normal distribution patterns were obtained.

Assay for cellulase. As cellulose is the substrate for cellulase, it seemed probable that the enzyme would be adsorbed on the filter paper. Consequently, it was felt that the enzyme activity would not be quantitatively eluted by water. It was found experimentally that the enzyme hydrolyzed CMC in the presence of filter paper and a method was devised to assay the cellulase on sections of the electropherograms. Experiments indicated that cellulase was about 70% as active in the presence of filter paper as when assayed alone. It seems that part of the activity is so tightly bound to the paper that it cannot act on the CMC. The filter paper itself was largely crystalline and was scarcely attacked in the length of time used for the assay. Therefore, the assay for cellulase was of a semiquantitative nature; nevertheless, it provided figures that were useful for comparison.

The paper, after removal from the apparatus, was cut into 1 cm sections which were placed into test tubes containing the CMC solutions. The substrate was 2 ml of 0.05% CMC ("70 premium low") in 0.1 M acetate buffer, pH 5.0. Incubation was at 50° C. At the end of the incubation time the reaction was stopped by the addition of 2 ml of the Somogyi copper reagent (118, 119). The tubes were covered with marbles and placed in a boiling water bath for exactly 15 minutes. After cooling, 2 ml of the Nelson arsenomolybdate reagent (84) was added, the tubes were shaken vigorously, and the samples were diluted to 10 ml with water. The unhydrolyzed substrate precipitated from solution but settled rapidly to the bottom of the tube. An aliquot of the supernatant was removed and the optical density of the solution

was determined in a spectrophotometer at 505 mu. Blanks for the determination were CMC solution containing filter paper but no enzyme. From a calibration curve previously established with known amounts of glucose, the optical densities were converted into micrograms of glucose. It was found experimentally that the enzyme concentration varied along the migration path and in order to keep the reducing sugar values within the range of the micro Somogyi method the incubation times for different sections of the paper had to be varied. In order to obtain relative enzyme concentrations the reducing sugar values were converted to enzyme units. A preliminary experiment was used to determine the approximate incubation time for the various sections of paper. Standard methods were used for the calculation of the arbitrary enzyme units and a full description is included in the Appendix.

Assay for β-aryl glucosidase. At the time these experiments were carried out, the assumption was made that there was a single β-glucosidase (cellobiase) in the culture filtrates of Myrothecium verrucaria and that the rate of hydrolysis of any β-aryl glucoside would serve as a measure of its concentration. It was not until later that this assumption was shown to be erroneous, and that the filtrate contains cellobiase as well as β-aryl glucosidase. The cellobiase may hydrolyze the β-aryl glucoside, but the reverse is not true. Consequently, these data are reported under the title of β-aryl glucosidase rather than cellobiase.

The substrate used was 6-bromo-2-naphthyl-β-D-glucoside, a compound proposed by Cohen et al (23) for the colorimetric estimation of β-glucosidase. The compound is hydrolyzed by the enzyme to yield glucose and 6-bromo-2-naphthol. The latter compound is coupled to tetrazotized o-dianisidine to give a purple dye which is extracted with chloroform for determination of its optical density in a colorimeter. A direct proportionality exists between the amount of 6-bromo-2-naphthol and optical density over the range of 0-100 micrograms. This procedure was very tedious and a modification of the method was evolved. The bromo naphthol was coupled to diazotized sulfanilic acid rather than the o-dianisidine. The resulting dye, a brominated orange II, is water soluble and this factor greatly simplified the estimation of bromo naphthol. The fastest coupling of the two compounds occurred at high pH's but the color faded rapidly at low naphthol concentrations. It was found that at neutral pH's the coupling was not as rapid but the color was stable for about an hour. Under these conditions the relationship between optical density and concentration deviated from Beer's law and a calibration curve was established with known amounts of 6-bromo-2-naphthol. The 6-bromo-2-naphthol was synthesized by the procedure of Koelsch (63) and was recrystallized from glacial acetic acid before use. The control of pH is essential for reproducible results and in these experiments the coupling was carried out in phosphate buffers at pH 7.3. The method appeared to be satisfactory and, on hydrolysis of the substrate with acid, the amount of naphthol determined

by this method was in good agreement with the theoretical value. For example, hydrolysis of 0.21 uM of substrate gave 0.21 and 0.22 uM of bromo naphthol with this method.

The detailed procedure for estimation of β-aryl glucosidase on electropherograms is as follows: After removal of the strip of paper from the electrophoresis apparatus, it was cut into 1 cm sections and placed into test tubes, each containing 4 ml of substrate (100 ug/ml). The substrate solution was prepared by dissolving 10 mg of the compound in 20 ml of methanol, adding 20 ml of 0.25 M acetate buffer, pH 5.0, and diluting with water to 100 ml. At the time the experiments were conducted, the function of the methanol was thought to be solely that of increasing the solubility of a poorly soluble compound. It will be shown later that the methanol is an active participant of the system and acts as an acceptor for glucose. The tubes with the substrate and paper strips were incubated at 37° C for times that varied according to the activity of the enzyme sample. At the end of the incubation period the samples were autoclaved for 3 minutes at 121° C. This step was necessary because the coupling pH of 7 was not high enough to inactivate the enzyme. After the tubes were cool, the following reagents were added, in order, to the samples: 1 ml of 0.3 M phosphate buffer, pH 7.3, and 1 ml of a 1 mg/ml diazotized sulfanilic acid solution. The latter solution was prepared by dissolving 25 mg of sulfanilic acid in 22.5 ml of water, adding 1 ml of 1.4 N HCl, cooling to 0° C, and then adding 1.5 ml of a 20 mg/ml solution of sodium nitrite. The solution was allowed to stand 10 minutes

before use. The pieces of filter paper were removed from the tubes and the samples diluted to 10 ml. After 15 minutes the optical densities were determined in a colorimeter at 455 mu. These values were converted to micrograms of 6-bromo-2-naphthol from a previously established calibration curve. Relative enzyme concentrations were determined by converting the bromo-naphthol values to enzyme units. The details are included in the Appendix.

The cellulase and β-aryl glucosidase units were arbitrarily defined in such a fashion that both activities could be plotted on a single ordinate. The units have no absolute significance but merely provide figures which are useful in comparing relative amounts of enzyme activity.

Enzyme distribution. In the initial studies, veronal buffer, which has been found satisfactory for serum proteins (14) was used as electrolyte. It was prepared by dissolving 1.86 gm of diethyl barbituric acid and 10.3 gm of sodium diethyl barbiturate in 1000 ml of water. The ionic strength of this buffer is 0.06 and the pH is 8.55. The high pH was desired in order to reduce the tendency for adsorption of cellulases onto the filter paper. With this buffer and Whatman Number 1 filter paper the current increased from about 4 to 10 milliamps over a 6-hour period.

Four of the crude filtrates, prepared as described under Materials, were subjected to electrophoretic migration as described above. Two paper strips (3 x 56 cm) were run for each filtrate. One was assayed

for cellulase activity and the other was assayed for β-aryl glucosidase activity. Initially, the enzyme was placed on the paper halfway between the two electrodes but migration was slight. In all the experiments reported in this section, the enzyme was placed about 15 cm from the center of the paper in the direction of the cathode. Migration of the negatively charged proteins was toward the anode. The results from four filtrates are shown in Figure 1 and the times of migration are shown on each figure. Zero represents the origin or the place where the enzyme was placed on the paper. The patterns were reproducible for each filtrate.

Several attempts were made to locate proteins on the paper. Various indicators, ninhydrin, and the biuret reaction were used, but the concentrations were too low to give positive reactions. Consequently, it has not been possible to correlate enzyme activity with protein concentration.

Electrophoresis at neutral and acid pH's. After the distribution patterns for enzyme activities were established at an alkaline pH, they were determined at a neutral and an acid pH. The experiment at pH 7.0 was carried out in phosphate buffer of ionic strength 0.05 (0.987 gm of $NaH_2PO_4 \cdot H_2O$ and 2.021 gm of Na_2HPO_4 in 1000 ml of water). In calculating the amount of each salt required, the triply charged phosphate ion was omitted from the ionic strength formula since the concentration of this ion at pH 7.0 is nil.

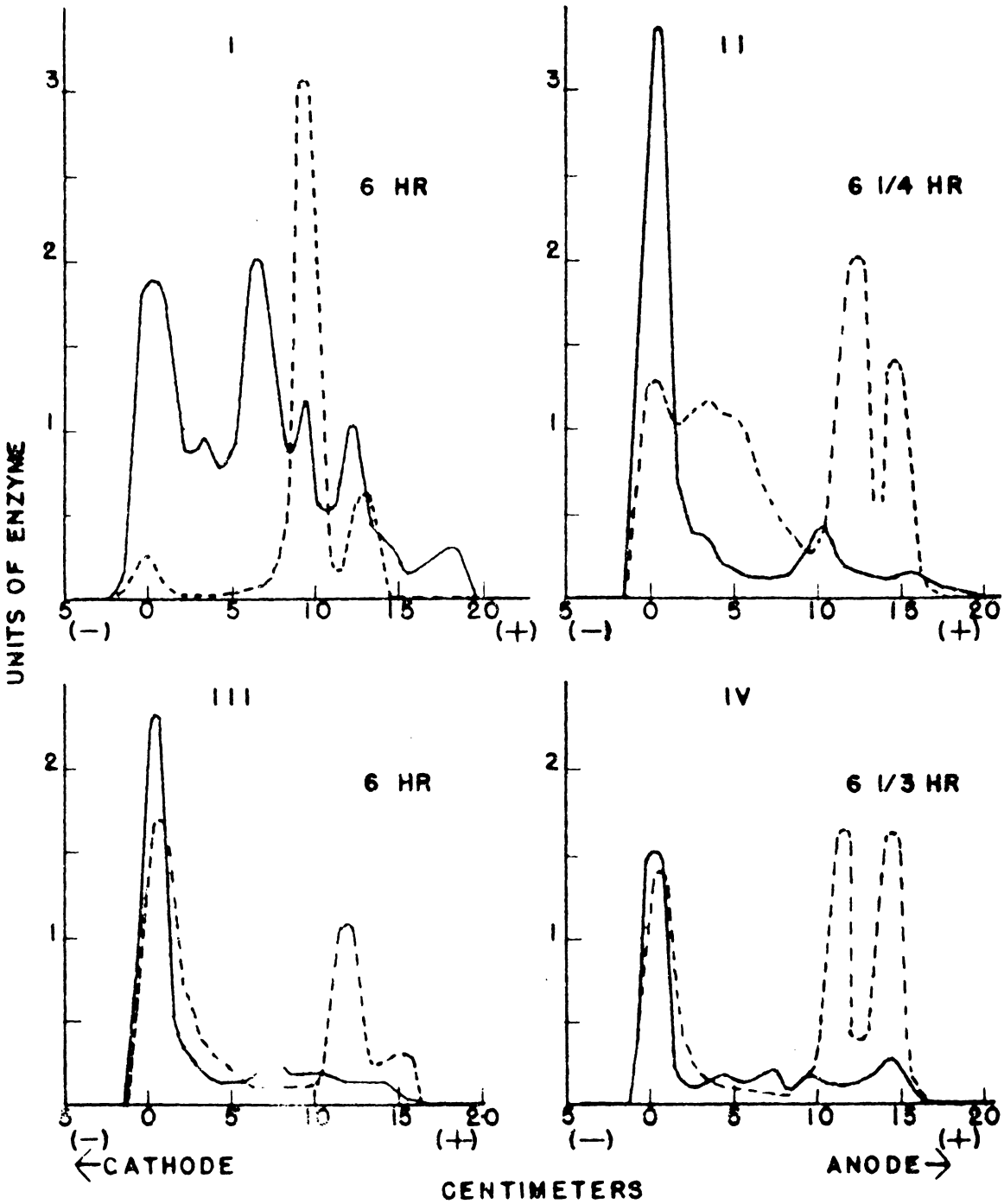


FIGURE I

DISTRIBUTION OF CELLULASE AND β-ARYL GLUCOSIDASE IN PAPER ELECTROPHORESIS OF CRUDE CULTURE FILTRATES OF M. VERRUCARIA

SOLID LINES = CELLULASE
DOTTED LINES = β-ARYL GLUCOSIDASE

With the exception of the buffer, the experiments were carried out in a fashion identical to those listed for the previous experiments. Strips 1 cm wide were cut 20 cm on each side of the origin and assayed for activity. The results for one of the filtrates is shown in Figure 2. This filtrate corresponds to filtrate 3 in Figure 1. The other filtrates gave similar patterns.

The experiments were repeated at pH 5.0 in acetate buffer of ionic strength 0.05 (1.00 ml of glacial acetic acid and 2.673 gm of sodium acetate in 1000 ml of water). The patterns were essentially the same as those at pH 7.0. There was a somewhat greater tendency for the enzymes to move in both directions but neither enzyme activity separated into distinct peaks.

Effect of heat on the activity of cellulase and β-aryl glucosidase. There are sufficient differences between the mobile peaks of cellulase and β-aryl glucosidase to suggest that they depend on different proteins for their activity (Figure 1). However, the peak remaining at the origin contained both activities and the question arose whether there was a single enzyme that was active on both substrates or whether there were at least two enzymes involved. It had been shown the cellulase is relatively thermostable whereas β-glucosidases are readily inactivated by heat (64). It appeared feasible to distinguish between the two activities by running two sets of electropherograms, heating one set and assaying both sets for enzyme activity. Only one filtrate was

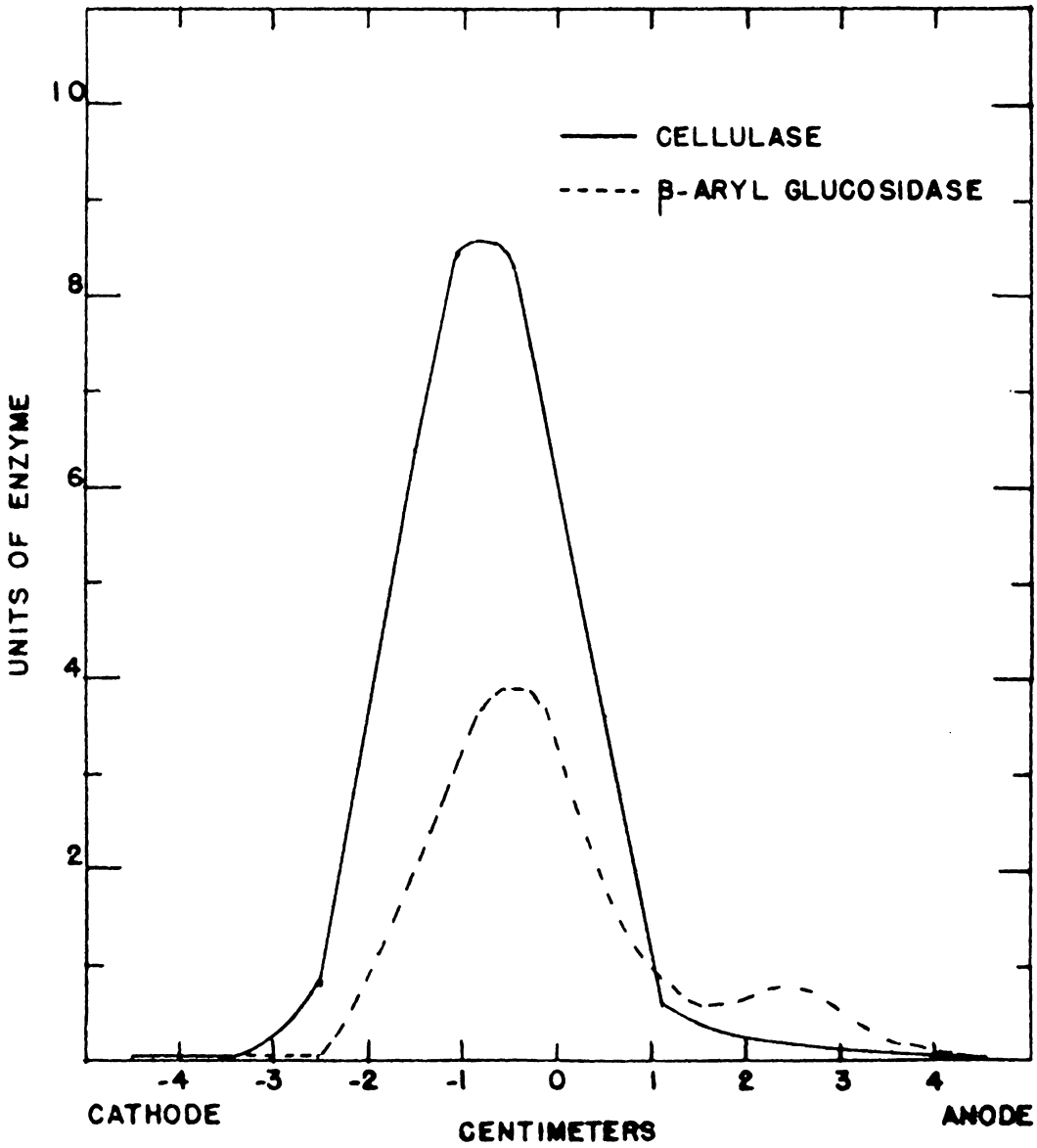


FIGURE 2

DISTRIBUTION OF CELLULASE AND β-ARYL GLUCOSIDASE AFTER PAPER ELECTROPHORESIS AT PH 7.0

subjected to this treatment and it corresponds to Number 3 in Figure 1. The experiments were carried out in veronal buffer of pH 8.55.

At the end of the migration period (6 hr), one set was assayed for cellulase and β-aryl glucosidase as previously described. The other set was cut into 1 cm strips which were placed in test tubes containing 1 ml of 0.05 M acetate buffer, pH 5.0. These tubes were held in a 80° C water bath for exactly 10 minutes. They were removed and immediately cooled in running tap water. To one set was added 1.0 ml of 0.1% CMC in 0.05 M acetate buffer, pH 5.0, and to the other set was added 3 ml of 133 ug/ml of 6-bromo-2-naphthyl-β-D-glucoside in 0.05 M acetate buffer, pH 5.0. The incubation and subsequent assay for activity were identical with the unheated samples. The results of these experiments are shown in Figure 3.

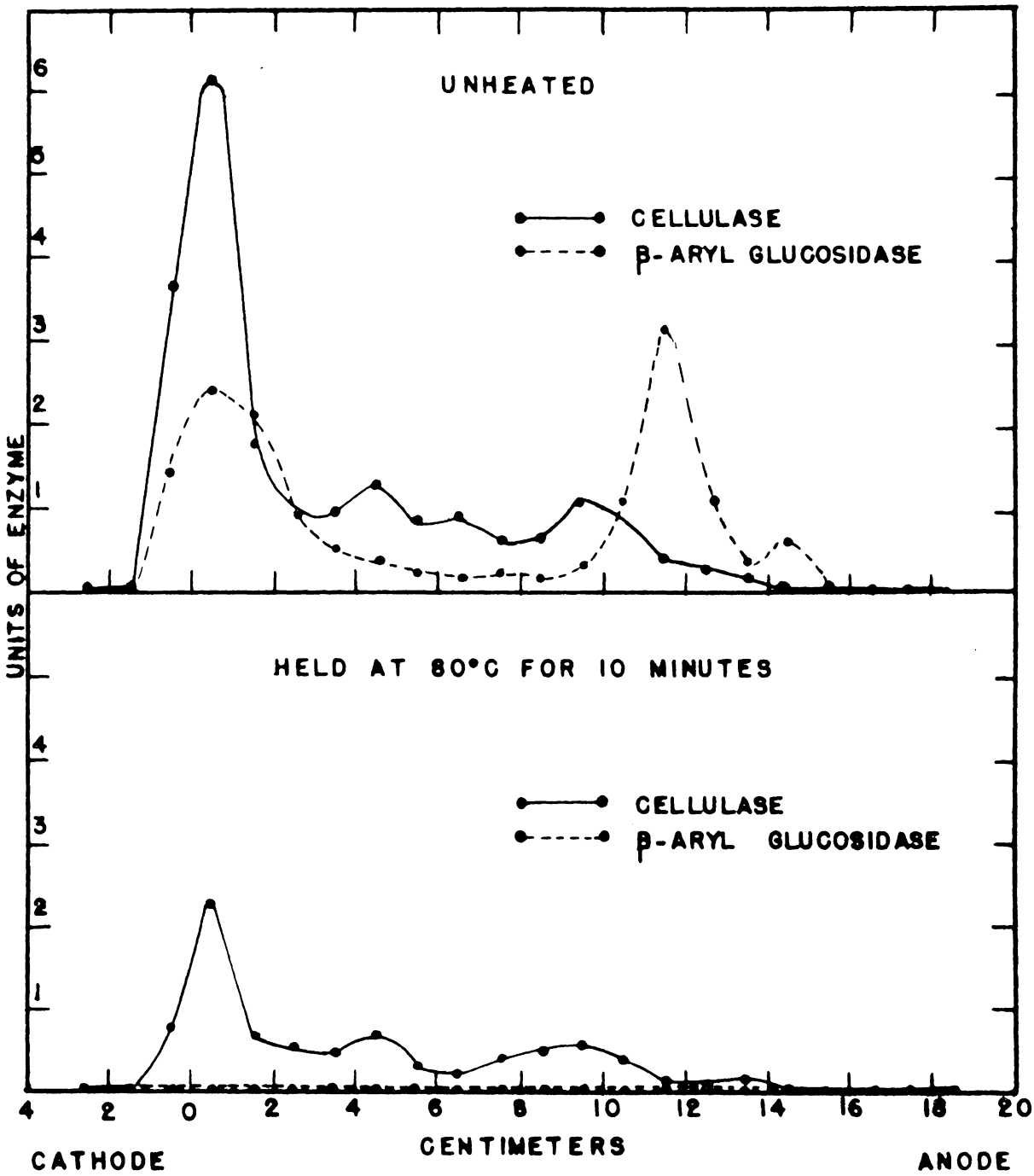


FIGURE 3

EFFECT OF HEAT ON CELLULASE AND
β-ARYL GLUCOSIDASE AFTER ELECTROPHORETIC MIGRATION

Column Chromatography of Crude Cellulase on Cellulose

Procedure. (A) Selection of method. For the separation of cellobiase and transglucosidase from cellulase in culture filtrates of Myrothecium verrucaria, column methods appeared to offer the most promise. Adsorption column chromatography was selected over ion-exchange methods for the following reasons: Ion-exchange methods for the separation of proteins are relatively new but have been successful for the resolution of proteins of molecular weight less than 15,000 (48). According to Whitaker (136) the molecular weight of the Myrothecium cellulase which he isolated was 63,000. It appeared that ion-exchange chromatography of high molecular weight proteins would be a study in itself. On the other hand, adsorption methods for resolving proteins have long been used (103). Cellulose would appear to be the logical adsorbent because cellulase is readily adsorbed on its natural substrate. Reese (105) reported that the cellulase of Myrothecium verrucaria was largely adsorbed by cellulose that had been swollen in 35% NaOH. Reese (105) also reported that both cellulase and cellobiase were adsorbed on cellulose at pH 3.5 but that only the cellobiase was eluted with 0.1% sodium bicarbonate. On the basis of these reports and a few preliminary experiments, cellulose swollen in 35% NaOH was selected as adsorbent and the eluting liquid chosen was phosphate-citrate buffer. Reports on the adsorbability of transglucosidase on cellulose are lacking.

(B) Preparation of alkali-swollen cellulose. Whatman Number 1 ashless chromatographic cellulose powder was used as adsorbent for the column and was prepared as follows: Prior to its use, the cellulose was swollen in 35% NaOH (6 ml/gm powder) which was prepared by dissolving the alkali in freshly boiled distilled water. A stream of nitrogen gas was passed through the NaOH to dispel any oxygen in the flask. The cellulose powder was added, the flask was tightly sealed, and the slurry was allowed to stand 30 minutes. During this time the cellulose suspension turned a yellow color. The cellulose was poured into 4 liters of cold water and was immediately filtered under suction. It was washed on the filter until the filtrate was neutral to litmus and was then resuspended in 0.1 N HCl. It was again washed on the filter until the wash water gave a negative test for chloride. The white powder was air dried and stored until used.

(C) Preparation of column for chromatography. The column (19 x 3.5 cm) was a pyrex test tube which had a small stem sealed to its bottom. A small pad of glass wool was placed on the bottom of the tube, and a water suspension of the swollen cellulose was poured into the column until its height in the column was 15 cm (approximately 18 gm of dry cellulose). Another pad of glass wool on top of the column prevented disturbance of the cellulose during the process of elution. The top of the column was sealed by a rubber stopper, through which a tube led to a reservoir. After the column was packed, it was washed with a liter of distilled water and several hundred ml of 0.02 M sodium citrate (pH 3.0). Several ml of the concentrated

enzyme solution (equivalent to approximately 1 liter of crude culture filtrate) were added and allowed to soak completely into the cellulose. Several ml of the 0.02 M citrate were added to the column to wash the enzyme into the cellulose. The column was fastened to the reservoir and elution was begun.

(D) Elution of the column. The column was eluted by an integrated buffer system which underwent a pH change from 3.0 to 8.5 over a volume of 2000 ml. This change was accomplished by integrating 1 liter of 0.04 M Na_2HPO_4 with 1 liter of 0.02 M sodium citrate (pH 3.0) in a Parr-type mixer (15). A diagram of the entire apparatus is shown in the Appendix (Figure 15). The column was allowed to drip at its own rate and when both reservoirs were empty the column was washed with an additional 500 ml of 0.04 M disodium phosphate to elute as much of the enzyme as possible.

Fifty fractions were collected manually in 50 ml samples and the pH was immediately adjusted to 6.4 with either 3 M sodium hydroxide or 20% phosphoric acid. The end point was determined with a glass electrode and pH meter. The entire elution took about 2 hours and the fractions were stored at -18° C until assayed for various enzyme activities.

pH and the distribution of protein in the fractions. The pH of the 50 fractions was determined in the course of their adjustment to 6.4 The pH elution curve is shown in Figure 4.

The protein in the fractions was determined by a slight modification of the Lowry method (70). The samples (0.5 ml) and water (0.5 ml) were added to 3.0 ml of the alkaline copper reagent. These samples were allowed to stand 10 minutes and then 0.3 ml of the Folin reagent (diluted 2x) was added rapidly. The optical densities were read in a spectrophotometer at 675 millimicrons after they had stood 30 minutes. It was not possible to read the samples at 750 millimicrons as recommended in the original method, because of inherent limitations of the spectrophotometer. Crystalline bovine serum albumin (Armour) was used as standard and there was a linear relationship between optical density and protein concentration over the range 5-100 micrograms. The protein content of the fractions was recorded in micrograms/ml, and the distribution of protein in the 50 fractions is shown in Figure 5.

Hydrolysis of carboxymethyl cellulose. (A) Reducing sugar assay of cellulase. The 50 fractions were assayed for reducing sugar production by cellulase by the Somogyi-Nelson method previously described. The increase in reducing sugars was small and the fractions were reassayed using much higher substrate concentrations. Reducing sugars were determined by the Somogyi (119) titrimetric procedure.

An aliquot of each fraction (0.5 ml) was added to 4.5 ml of 3.33% CMC ("70 premium low") in 0.05 M sodium acetate buffer, pH 5.0. The fractions were incubated at 50° C for 2 hours. Five ml of the Somogyi titrimetric copper reagent was added to the sample. The tubes were

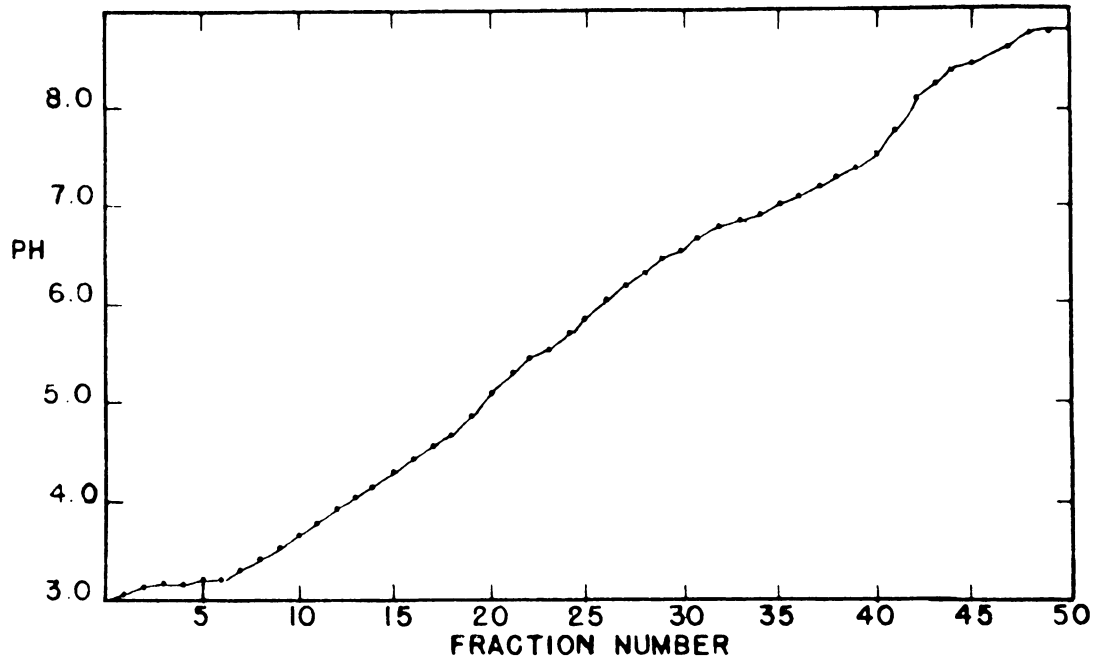


FIGURE 4
PH OF ELUATE FROM COLUMN

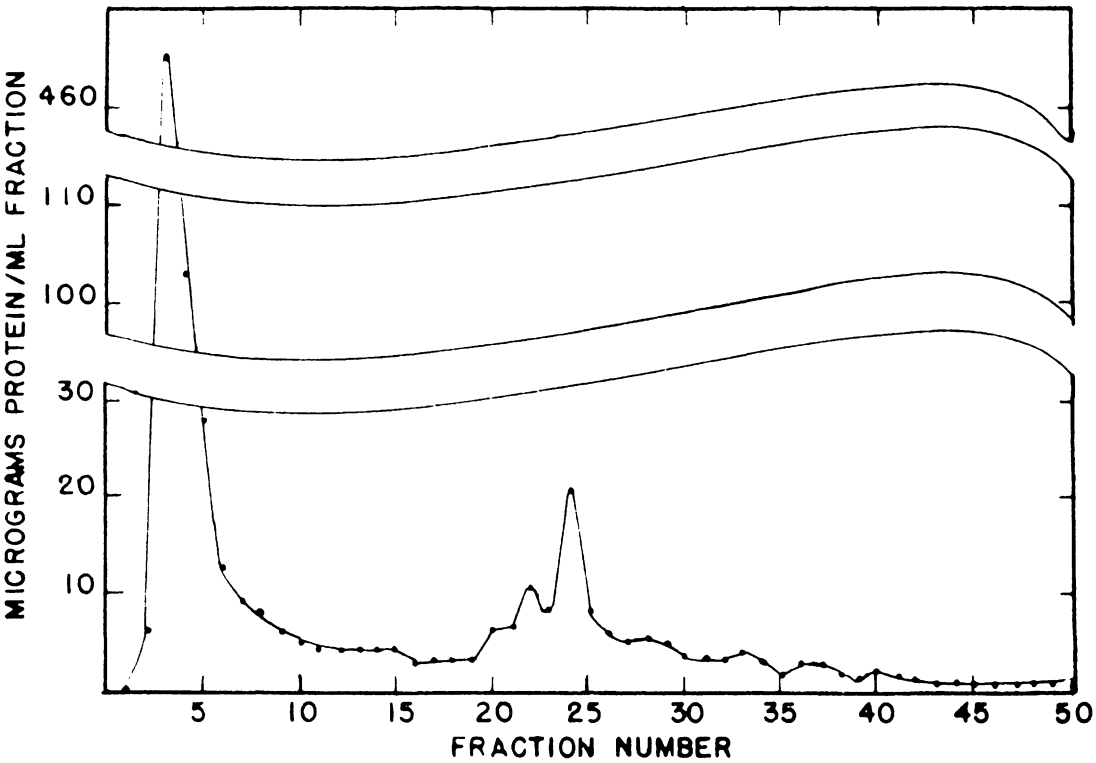


FIGURE 5
DISTRIBUTION OF PROTEIN
IN THE COLUMN FRACTIONS

shaken thoroughly and placed in a boiling water bath for 15 minutes. After cooling, the samples were acidified and the excess iodine was titrated with 0.005 N sodium thiosulfate. Adequate controls of enzyme and substrate were included in the determinations. It was found that several of the samples (especially those in the intermediate pH ranges) had small amounts of glucose and cellobiose in them. Presumably, these sugars came from hydrolysis of the column packing during the process of elution. Corrections were made for the small amounts of these sugars. The volume of thiosulfate was converted to mg glucose using a suitable reference curve prepared with known amounts of glucose.

(B) Viscosimetric assay of cellulase. The fractions were also tested for their ability to decrease the viscosity of carboxymethyl cellulose. A 0.2% solution of CMC ("70 premium high") was prepared by dissolving 400 mg in 100 ml of McIlvaine phosphate-citrate buffer, pH 5.0, (36) and 100 ml of water. The drain time of this solution was determined in an Ostwald type viscosimeter and the solution was diluted with water until the drain time was about 1 minute. The solutions usually required about a 2-fold dilution and the final concentration of CMC was about 0.1%. As only the changes in viscosity are considered, the exact concentration is of little consequence.

Four ml of the substrate solution was placed in a viscosimeter and 0.2 ml of a fraction was added. The solution was mixed rapidly and an initial drain time was recorded. Exactly 30 minutes later a second drain time was determined. The specific viscosity of the

solution was calculated from the drain times of the CMC solutions and water. The reciprocal of the specific viscosity is called the specific fluidity of the solution. The change in specific fluidity is the difference between the initial and final fluidities and was the value recorded. This term is nearly proportional to the amount of hydrolysis of the CMC.

(C) Results. The results of assaying each of the 50 fractions by the reducing sugar and viscosimetric procedures are shown in Figure 6.

Hydrolysis of insoluble cellulose dextrins. Rather than attempt to measure small changes in the reducing power of the insoluble dextrins, only qualitative information was obtained on the hydrolysis of these saccharides. The method adopted was to incubate the dextrins with enzyme and, periodically, to chromatograph the products on paper. The hydrolysis of the dextrins was confirmed by the presence of glucose and cellobiose on the papers.

The insoluble dextrins (105 mg) were suspended in 10.5 ml of 0.1 M sodium acetate buffer, pH 5.0, which was saturated with Dowicide 4 (2-chloro-4-phenylphenol) (126). The latter compound is an antiseptic and was included because of the prolonged incubation periods used in these experiments. The extended incubation periods compensated for the small amounts of enzyme in the fractions. Because the dextrins swell in water they were worked into a smooth suspension with a glass rod. The dextrins (0.2 ml) were pipetted into 50 small screw cap vials and

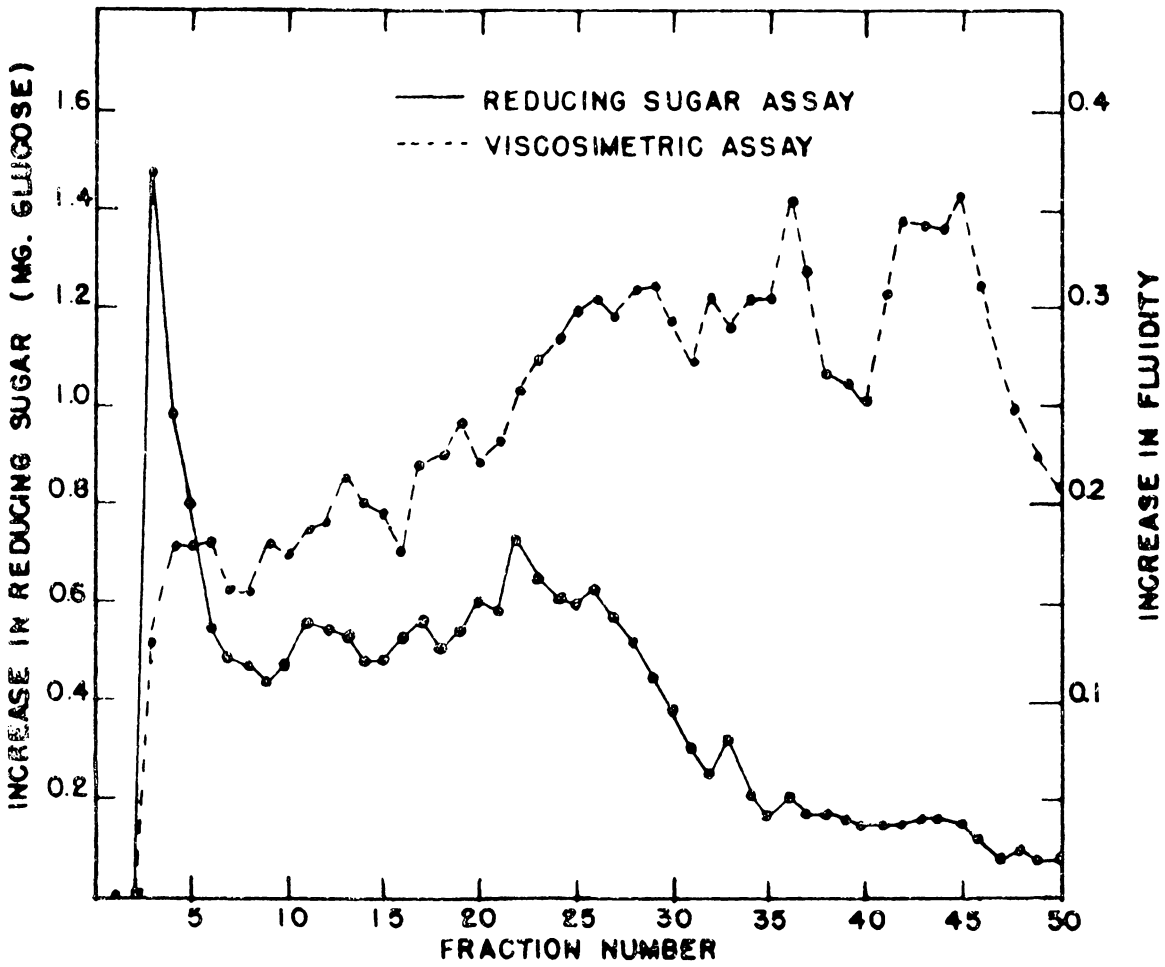
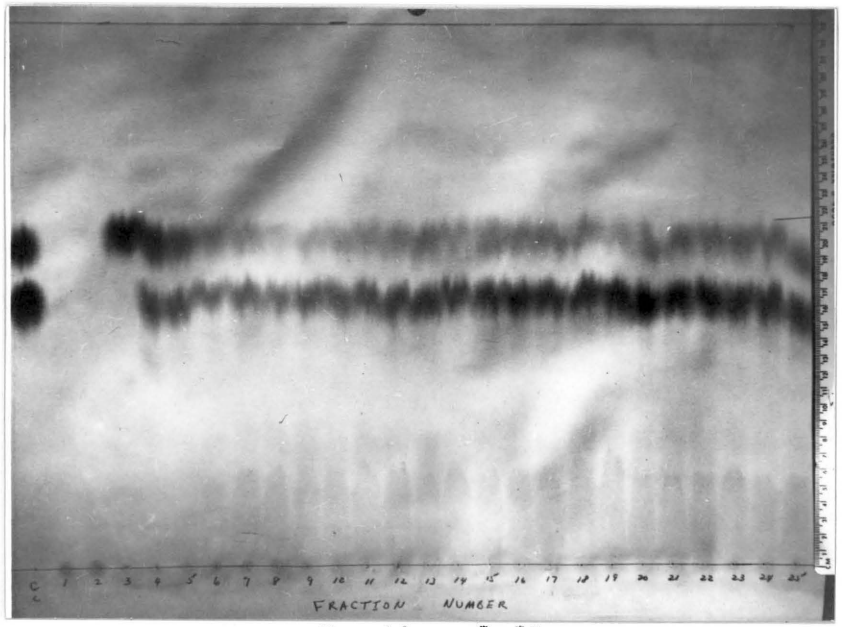


FIGURE 6

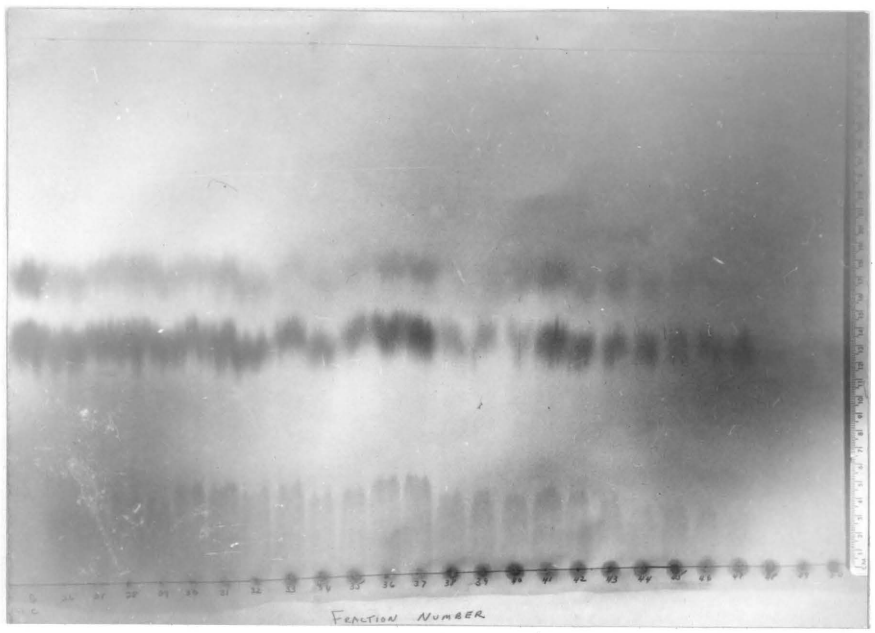
HYDROLYSIS OF CARBOXYMETHYL CELLULOSE BY FRACTIONATED M. VERRUCARIA CELLULASE

0.2 ml of each fraction was added to the corresponding vial. The samples were incubated at 37° C. Periodically, 20 microliter portions of the samples were spotted onto Whatman Number 1 filter paper and chromatographed in an isopropanol, water, glacial acetic acid solvent (6:3:1). The papers were equilibrated 3-4 hours with the vapors of the solvent before the solvent was added to the troughs. Separation was accomplished by a descending system. After 16-20 hours the papers were removed, dried, and sprayed with an amine to locate reducing sugars. Two amine sprays were used in these studies: the aniline-diphenylamine-phosphate reagent of Buchan and Savage (17) and the p-amino hippuric acid-phthalate spray of Sattler (111). The former spray is more sensitive to small amounts of sugar but the entire paper is discolored rapidly by the reagent. The latter spray is less sensitive but the papers may be kept for months with little or no discoloration. The aniline-diphenylamine-phosphate reagent also gives different colors for different types of sugars. These color differences will be discussed later. The composition of the amine sprays is listed in the Appendix.

The results of the hydrolysis of the insoluble dextrins after 4 days are shown in Plate 1. Glucose and cellobiose controls, which were included on each chromatogram, are shown at the far left of the photographs. Fraction 1 which contains no enzyme may be used as control. Fraction 3 had completely hydrolyzed the dextrins to glucose. Glucose and cellobiose were formed in all the other fractions with the exception



Fractions 1-25



Fractions 26-50

PLATE 1

Hydrolysis of Insoluble Cellulose Dextrins

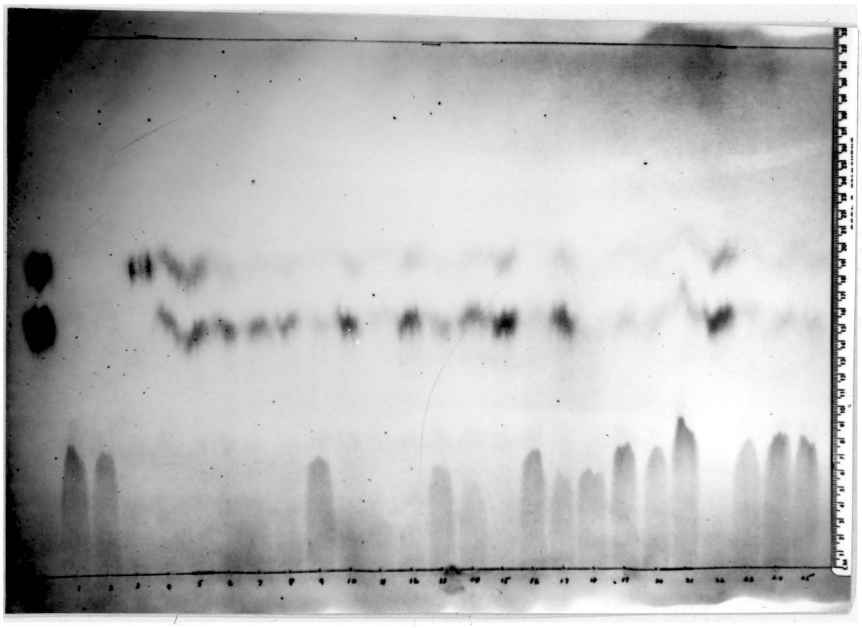
Known glucose and cellobiose are at the far left

of Fractions 48, 49, and 50 which did not seem to have attacked the substrate. Slower moving sugars were present in most of the samples.

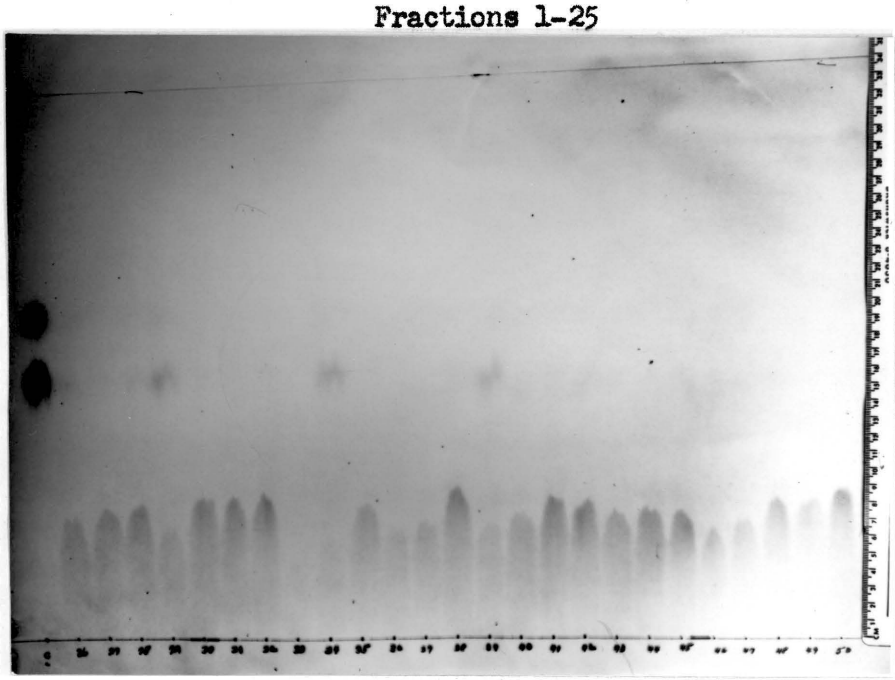
Hydrolysis of soluble cellulose dextrins. The action of the 50 fractions on the soluble dextrins was detected in the same chromatographic fashion as that used with the insoluble dextrins.

Since the soluble dextrins were not isolated from solution, the exact concentration used is not known. However, the concentration was such that 5 microliters gave good sized spots on the chromatograms. An aliquot of the dextrins was adjusted to pH 5.0 with a small quantity of 3 M sodium acetate buffer of that pH. The enzyme mixture consisted of 0.05 ml of the dextrins and 0.05 ml of the appropriate fraction. The final acetate concentration was approximately 0.05 M. The volume of 0.1 ml was incubated in small screw cap vials (2 ml) at 37° C. The samples had either toluene or Dowicide 4 as antiseptic. Periodically, 0.02 ml of the samples were chromatographed in a fashion identical to that described for the insoluble dextrins. The chromatograms for the 8-day hydrolyzates are shown in Plate 2. Fraction 1, with no enzyme, may be used as control. Fraction 3 completely hydrolyzed the substrate to glucose. It is interesting to note that most of those fractions which hydrolyze the dextrins have cellobiose as the predominant product.

Hydrolysis of cellobiose. The hydrolysis of cellobiose was determined by the same chromatographic means listed for the soluble and insoluble dextrins.



Fractions 1-25



Fractions 26-50

PLATE 2

Hydrolysis of Soluble Cellulose Dextrins

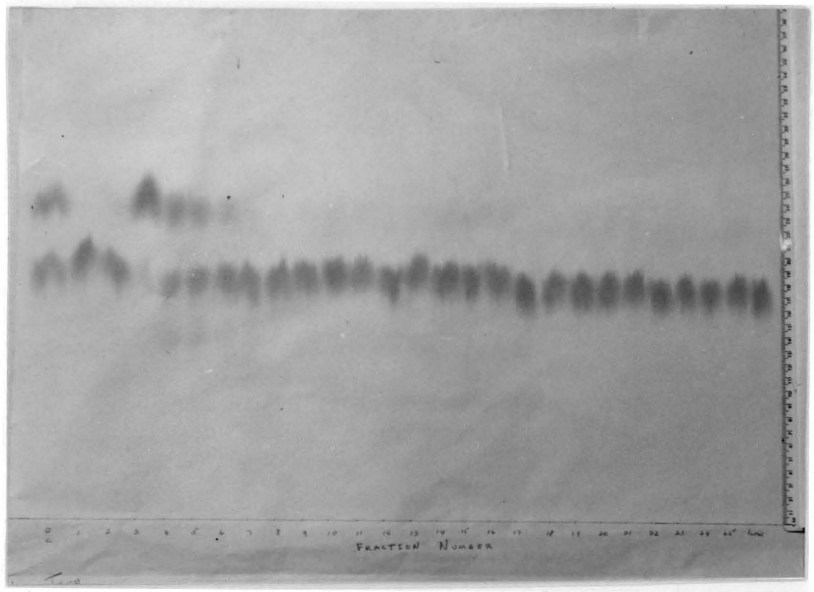
Known glucose and cellobiose are at far left

The enzyme mixture consisted of 0.1 ml of cellobiose substrate (12.5% solution in 0.5 M sodium acetate buffer, pH 5.0), and 0.4 ml of each of the 50 fractions. Incubation was in screw cap vials at 37° C. Periodically, 0.005 ml portions were spotted on Whatman Number 1 filter paper and chromatographed in the same solvent used for the soluble and insoluble dextrins. Toluene or Dowicide 4 served as antiseptic.

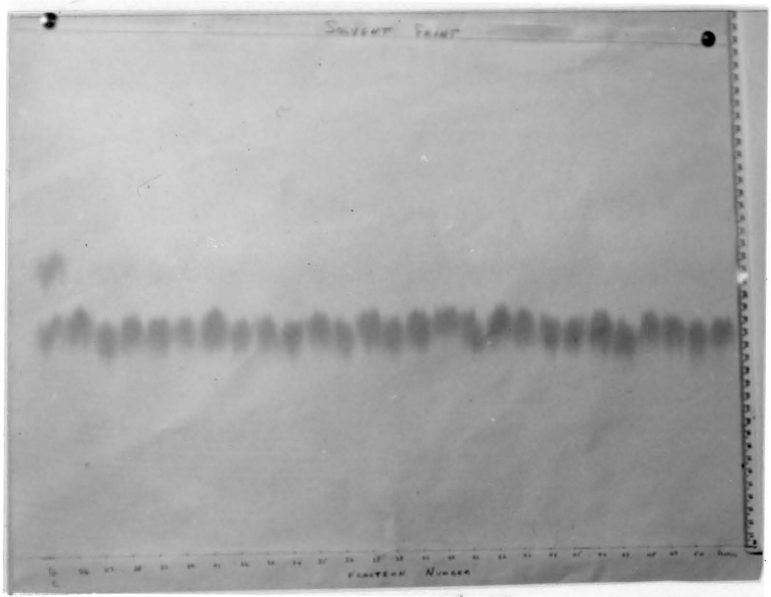
The results of a 300-hour incubation period are shown in Plate 3. It is apparent that not all of the cellulases have cellobiase activity and that all the fractions which liberate glucose have transglucosidase activity also. Higher saccharides were present in Fraction 3 at shorter incubation periods.

Hydrolysis of 4-O-β-D-glucopyranosyl-D-sorbitol. Whitaker (140) used the reduction products of cellobiose, cellotriose, cellotetraose, and cellopentaose as substrates for Myrothecium verrucaria cellulase. The assumption was that the same enzyme hydrolyzed both the reduced compounds and the unreduced sugars. In view of the fact that cellobiase activity was confined to a few fractions of the eluate from the cellulose column, it was decided to test the 50 fractions for their ability to hydrolyze 4-O-β-D-glucopyranosyl-D-sorbitol.

To 0.2 ml of each fraction, 0.1 ml of a 3% solution of the sorbityl compound in 0.3 M sodium acetate buffer, pH 5.0, was added. The samples were incubated in small vials at 37° C. Samples were



Fractions 1-25



Fractions 26-50

PLATE 3

Hydrolysis of Cellobiose

Known glucose and cellobiose are at the far left and a control of cellobiose subjected to the same experimental conditions as the samples, is at the far right

chromatographed periodically in the solvent used for the insoluble dextrins and developed with the aniline-diphenylamine-phosphate reagent.

After 5 days incubation, there were very small amounts of glucose in Fraction 3 and barely detectable traces in Fractions 4 and 5. Only those fractions which liberated glucose from cellobiose, liberated glucose from the reduced compound. The hydrolysis of this compound will be considered further in the section on transglucosidases.

Hydrolysis of β-aryl glucosides. The effect of the 50 fractions on β-aryl glucosides was investigated. Two glucosides, 6-bromo-2-naphthyl-β-D-glucoside and p-nitrophenyl-β-D-glucoside, were used for substrate. The procedure for each is described in detail.

The 6-bromo-2-naphthyl-β-D-glucoside was prepared as described in the section on electrophoresis. Two ml of this substrate (100 micrograms/ml) was placed in each of 50 tubes and 1.0 ml of the respective fractions was added. The tubes were incubated at 37° C for 90 minutes, and the reaction was terminated by adding 1.0 ml of 0.3 M sodium phosphate, pH 7.3, which was 0.02 M with respect to mercuric chloride. The mercuric ions completely inactivated the enzyme and did not interfere with the analysis. Then 1.0 ml of a 0.1% solution of diazotized sulfanilic acid was added and the solutions were diluted to 10 ml. The optical densities of the solutions were determined and the data were recorded as percentage decomposition of substrate.

A 0.00167 M p-nitrophenyl-β-D-glucoside solution in 6.0 M methanol and 0.1 M sodium acetate buffer, pH 5.0, was prepared. To 0.3 ml of this substrate was added 0.3 ml of each of the 50 fractions. The samples were incubated at 37° C for 4 hours. Four ml of 6.25% K_2HPO_4 was added and the samples were diluted to 10 ml. The p-nitrophenylate ion is yellow and its concentration can be measured directly in a colorimeter at 400 millimicrons. The relation between optical density and concentration deviates from Beer's law and for that reason a reference curve was established with known amounts of p-nitrophenol. The p-nitrophenol was recrystallized from hot water before use. The data were recorded as percentage decomposition of substrate.

The patterns of the decomposition of the two β-aryl glucosides by the 50 fractions were identical, and only the pattern for the decomposition of 6-bromo-2-naphthol-β-D-glucoside is reproduced in Figure 7. Relative enzyme concentrations cannot be determined from these curves because of the high percentage of substrate decomposition.

The striking feature of Figure 7 is the fact that the β-aryl glucosidase which chromatographed off the cellulose column in Fractions 20-35 cannot be a cellobiase (see Plate 3). The small peak of activity in Fraction 29 was reproducible with both substrates. It is interesting to note also that there is β-aryl glucosidase in Fraction 2 (Figure 7) but this fraction does not contain either cellobiase or transglucosidase (Plate 3). This fact indicates that there is at least one other non-cellobiase β-aryl glucosidase in Fractions 2-8.

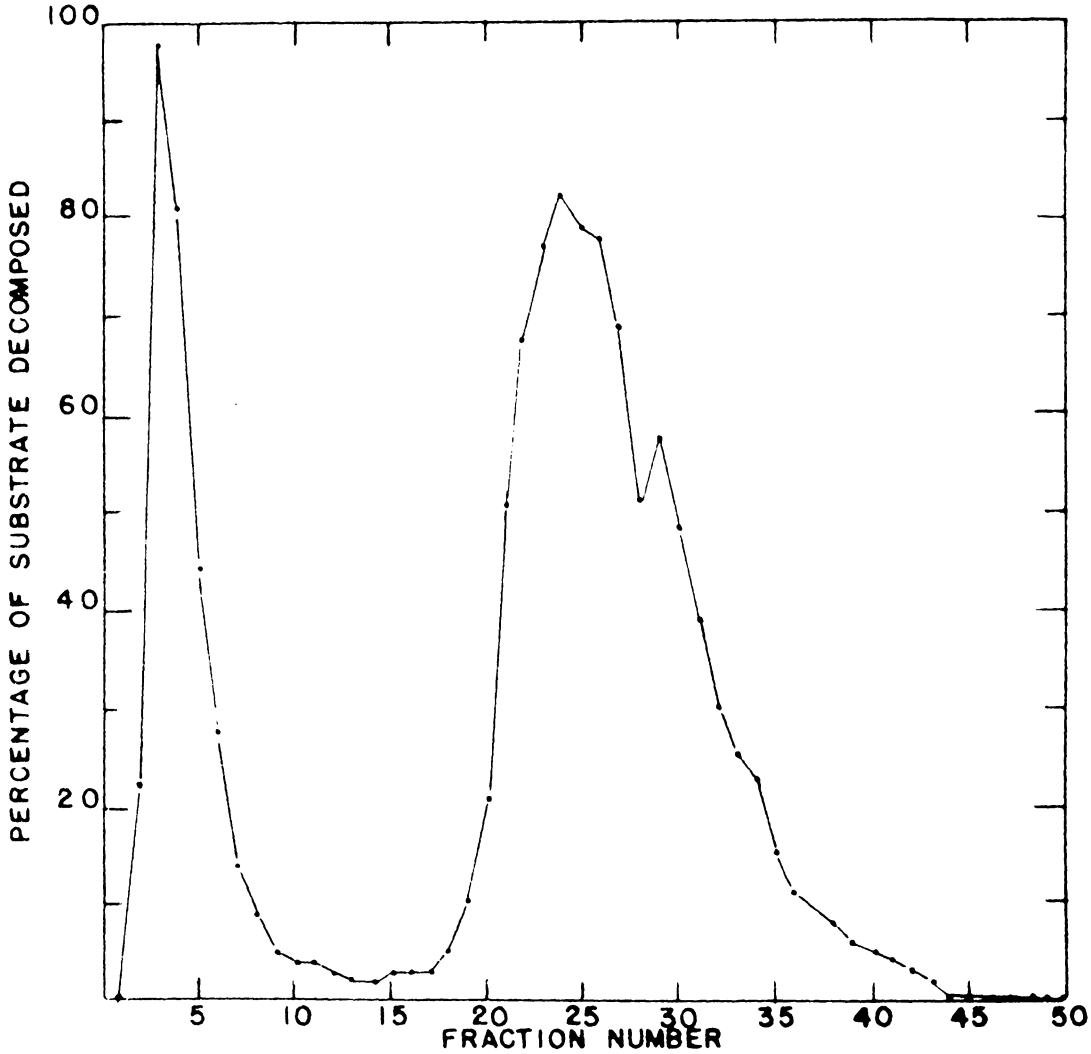


FIGURE 7

COLUMN CHROMATOGRAPHY OF β-ARYL GLUCOSIDASE FROM CRUDE CULTURE FILTRATES OF M. VERRUCARIA

Some Properties of a β-aryl Glucosidase

Electrophoretic studies. It had been established in the paper electrophoresis studies that there were at least three, and possibly four, electrophoretically distinct species of protein contributing to the β-aryl glucosidase activity in culture filtrates of Myrothecium verrucaria. It had been established in the column studies that at least one of these proteins was not a cellobiase by virtue of its complete separation from cellobiase activity of the filtrate. Since an enzyme of this type has not been reported in culture filtrates of this organism, the characteristics of the enzyme were investigated further.

An attempt was made to correlate the enzyme activities which came off the column with the various components shown in electrophoresis. Fractions 3, 23, and 29 were examined for their electrophoretic homogeneity. Fraction 29 was included because the shape of this peak suggested that it might be a separate enzyme from the one in Fraction 23. Twenty ml of each fraction was concentrated in vacuuo to approximately 3 ml and dialyzed 8 hours in collodion bags. They were then lyophillized. The white powder was redissolved in 0.3 ml of distilled water and the concentrate was subjected to paper electrophoresis. An in situ method was employed to locate the position of the enzyme on the paper. Whatman 3MM paper was substituted for the Number 1 paper because it is a thicker paper and more suited for this particular method.

Electrophoresis was conducted in barbiturate buffer (pH 8.55) for 6 hours in a fashion identical to that previously described. The paper was removed from the apparatus, blotted lightly, and sprayed with a solution of 6-bromo-2-naphthyl-β-D-glucoside. The latter solution contained 200 micrograms of substrate/ml in 1 M sodium acetate buffer, pH 5.0, and was 3 M with respect to methanol. The concentrated buffer was necessary to lower the pH from 8.55 to about 5. The strip of paper was then incubated at 55° C in a moist chamber for about 30 minutes. The paper was sprayed with a 5% sodium bicarbonate solution followed by a 0.1% solution of tetrazotized-o-dianisidine (23). The position of the enzyme on the paper was located by the formation of a purple dye from the liberated naphthol. The papers, after having been washed with water to remove excess reagents, left a permanent record of the enzyme position. Crude filtrates were included on the electropherograms for comparison. An example of this technique is shown in Plate 4. Two facts stand out. The β-aryl glucosidase activity of Fraction 3 is virtually identical with the crude filtrate and therefore is still a very complex mixture of different protein species. Fractions 23 and 29 have only a single spot of activity that did not move from the origin. These fractions represent a considerable purification of β-aryl glucosidase because they lack all of the mobile components of the crude filtrate. The activity shown by Fraction 29 is very slight.

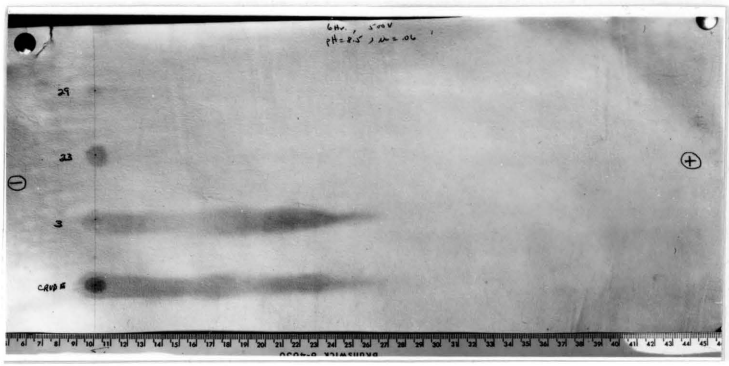


PLATE 4

Paper Electrophoresis of Crude Cellulase and Column Fractions 3, 23, and 29

The origin is at 10 centimeters

The electrophoresis experiment was repeated at pH 5.0 in the acetate buffer previously described and the enzyme position located by the above *in situ* method. Because the pH was already at 5, the buffer concentration of the substrate solution was reduced to 0.1 M.

The patterns for the crude filtrate and Fraction 3 appeared to be identical. There was slow migration from the origin toward both electrodes but there was no separation of activities into distinct bands. Fractions 23 and 29 exhibited a single spot of activity that did not move from the origin.

The experiments were repeated at pH 3.0 in phthalate buffer of ionic strength 0.05 (3.669 gm of phthalic acid and 5.671 gm of potassium acid phthalate in 1000 ml of water). The assay for activity was the same as above except the acetate buffer in the substrate solution was 0.5 M, pH 5.5. Fraction 3 and the crude filtrate appeared to be identical and exhibited slight movement toward the cathode. Apparently, there was some enzyme destruction during the long exposure (6 hr) to the acid pH. Again there was a single spot of activity for Fractions 23 and 29 that did not move from the origin.

It was not possible to tell whether the enzyme in Fraction 29 was different from the one in Fraction 23 on the basis of the electrophoretic studies. Fraction 23 and the adjacent fractions, 22 and 24, were investigated for various properties.

pH optimum. The pH optimum of the mixture of β-aryl glucosidase in crude filtrates had been established at 5.0 in preliminary experiments

and all the assays had been at that pH. The pH optimum of the relatively pure β-aryl glucosidase from the column separation was redetermined, with 6-bromo-2-naphthyl-β-D-glucoside as substrate. The pH range 4.5 to 6.3 was covered with phosphate-citrate buffers (36). The assay medium (in duplicate) consisted of 3.5 ml of 5.7 M methanol which contained 0.5 mg of substrate, 1.0 of the buffer of the desired pH, and 0.5 ml of Fraction 24. Incubation was at 50° C for 30 minutes. After addition of 1.0 ml of 0.3 M sodium phosphate, pH 7.3 (0.02 M with $HgCl_2$), to terminate the reaction and 1.0 ml of 0.1% solution of diazotized sulfanilic acid, the samples were diluted with water to 10 ml. The amount of 6-bromo-2-naphthol was determined and the percentage of decomposition was recorded. The pH activity curve is shown in Figure 8. Replicate experiments gave identical results. The pH optimum of 5.3 shows a slight shift from the optimum exhibited by the mixture in the crude filtrates.

Hydrolase and transferase activity. In all the experiments involving the use of 6-bromo-2-naphthyl-β-D-glucoside, 20% methanol had been included in the reaction mixtures to increase the solubility of the substrate. This procedure had been recommended by Cohen et al (23) in the original description of the method. In the course of this investigation it was found that the methanol was an active participant in the enzymatic reactions. The sequence of events which led to this discovery are related below.

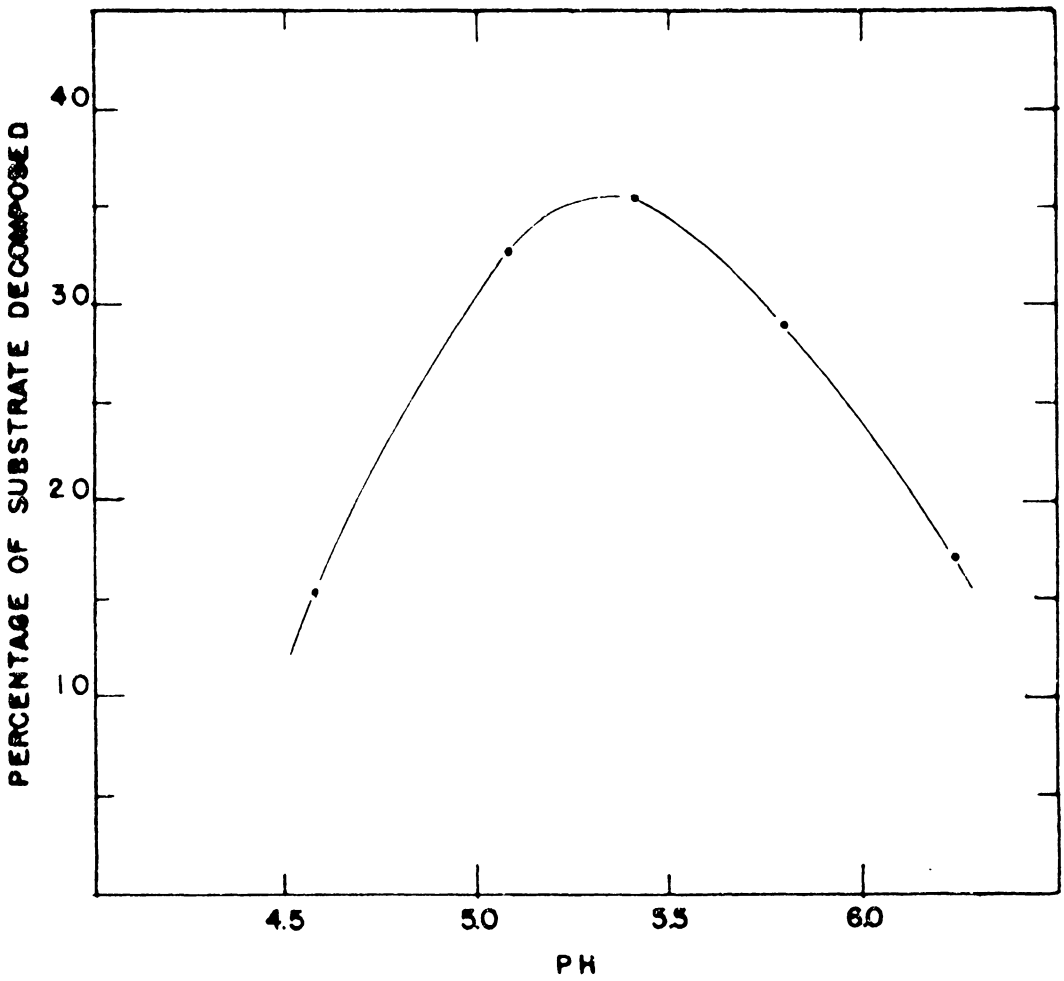


FIGURE 8

INFLUENCE OF PH ON β-ARYL GLUCOSIDASE ACTIVITY

Hestrin et al (46) have pointed out that a measure of the liberation of the aglycone portion of a glucoside molecule is not a reliable method of glucosidase assay unless it can be shown that neither transphosphatation nor transglucosidation is occurring. In other words, it must be verified experimentally that equimolar quantities of the glucone and aglycone moities are liberated before the measure of only one of them can be considered a valid assay for a glucosidase. This condition had not been verified for this particular enzyme and substrate. Therefore, some of the 6-bromo-2-naphthyl-β-D-glucoside was hydrolyzed by a crude filtrate and the hydrolyzate was analyzed for 6-bromo-2-naphthol and glucose (Somogyi-Nelson method). The two compounds were not liberated in equimolar quantities, indicating that the glucose, which was liberated in much smaller quantities than the naphthol, was being transferred to some compound in such a fashion as to mask its reducing group.

Transphosphorylases are well-known enzymes and were suspected of catalyzing the following type of reaction:

Naphthyl-glucoside + H_3PO_4 -----> glucose-1-phosphate + naphthol

This possibility seemed probable in view of the fact that some of the assays had been carried out in phosphate-citrate buffer and most crude filtrates contained some phosphate even after dialysis. A culture filtrate which had been so exhaustively dialyzed that it gave a negative test for phosphate was used to repeat the experiment. The substrate was hydrolyzed in the presence and absence of phosphate and the two hydrolyzates were analyzed for bromo-naphthol and glucose. The results

were identical; that is, in both cases the same amount of naphthol was liberated and the same amount of glucose was transferred. This fact eliminated the possibility that phosphate was serving as acceptor. The methanol which was present in all the reaction mixtures was not yet suspected. However, it was established that the glucose compound which was formed was easily hydrolyzed by acid yielding free glucose.

Enzymes which catalyze the transfer of glucose from one disaccharide molecule to another disaccharide molecule have been known for several years (46), and it was suspected that the substrate itself might be serving as acceptor in the following type of reaction:

2 naphthyl-glucoside ——> naphthyl-glucosyl-glucoside + naphthol

Since naphthol fluoresces under ultra violet light, this possibility was checked by inspecting a chromatogram of the hydrolyzate under ultra violet light. Only two spots that fluoresced were found on the chromatograms and these corresponded to bromo-naphthol and the unhydrolyzed substrate. Thus, the possibility of this reaction was minimized.

Methanol was finally established as the acceptor compound for glucose by hydrolyzing the substrate in the presence and absence of methanol. In the absence of methanol, equimolar quantities of glucose and naphthol were liberated. Further proof that methanol was the acceptor was obtained by chromatographing the hydrolyzates on Whatman Number 1 filter paper in an isopropanol, water, glacial acetic acid solvent (6:3:1) and locating non-reducing carbohydrates with the periodate-benzidine spray reagents of Cifonelli and Smith (22). The composition of these reagents is listed in the Appendix. A compound

with the R_f of a methyl glucoside was present in the reaction mixture which included methanol and was absent in the hydrolyzate without methanol. The total reaction in the presence of methanol is consistent with the following type of reaction:

$$\text{naphthyl-}\beta\text{-glucoside} + \begin{cases} \text{HOH} \longrightarrow \text{naphthol} + \text{glucose} \\ \text{CH}_3\text{OH} \longrightarrow \text{naphthol} + \text{methyl glucoside} \end{cases}$$

The assays which have been used in all the previous sections of this paper actually measured both these activities.

Kinetics of hydrolysis and transfer. After it was established that glucose was being transferred to methanol from one β-aryl glucoside, other β-aryl glucosides were tested.

Phenyl-β-D-glucoside was barely attacked by the enzyme (less than 0.2% decomposition of substrate, as compared to 52% for the naphthyl-glucoside in equivalent experiments). Salicin (o-hydroxymethyl-phenyl-β-D-glucoside) was attacked, but not rapidly enough for convenient assay. For both of these substrates, the phenolic portion of the molecule was estimated with the Folin-Ciocalteu reagent (4). p-Nitrophenyl-β-D-glucoside was readily attacked by the enzyme though less rapidly than the naphthyl-glucoside. However, because of the greater solubility of this compound in the absence of methanol and the greater ease of its analysis, it was used for the remainder of the studies on β-aryl glucosidase.

In regard to the ease of hydrolysis of the glucoside and the aglycone portion of the molecule, the following order was apparent: 6-bromo-2-naphthyl > p-nitrophenyl > o-hydroxymethyl-phenyl > phenyl The significance of this order was not investigated.

It was found that the p-nitrophenyl-β-D-glucoside was alkali sensitive and the Somogyi-Nelson method (an alkaline copper oxidation method) could not be used for the estimation of glucose liberated from this compound. The method of Jones and Pridham (59) was found to be almost ideal for the estimation of glucose liberated from this glucoside. The reagent is a solution of benzidine in glacial acetic acid. The benzidine forms an orange-red color with glucose, whose optical density is directly proportional to the amount of glucose (0.1 - 0.7 micromoles). The acid is too weak to hydrolyze the glucoside, and yet is strong enough to prevent the ionization of p-nitrophenol. The yellow color of the p-nitrophenylate ion would interfere in the analysis. Optical densities were determined in a spectrophotometer at a wave length of 385 millimicrons.

The course of the decomposition of p-nitrophenyl-β-D-glucoside was investigated in the presence and absence of methanol. Two flasks were prepared in the following fashion: One ml of 0.025 M substrate in phosphate-citrate buffer, pH 5.35 (36), was placed in each flask. One ml of water was added to the first and one ml of 60% methanol was added to the second. Each flask received 3.0 ml of Fraction 25, making the final substrate concentration 5 micromoles/ml. Incubation

was at 37° C and samples were withdrawn periodically and analyzed for glucose and p-nitrophenol. For the glucose analyses, 0.1 ml was added to 2.0 ml of glacial acetic acid. When all the samples were collected, 0.9 ml of water and 2.0 ml of 0.4% benzidine in glacial acetic acid were added. The tubes were placed in a boiling water bath for 30 minutes and cooled, and their optical densities were determined at 385 millimicrons. These values were converted to micromoles of glucose from a suitable standard curve of glucose. For the p-nitrophenol analyses, 0.1 ml of the reaction mixture was added to 4 ml of 6.25% K_2HPO_4. When all the samples were collected, they were diluted to 10 ml with water and their optical densities determined at 400 millimicrons. These values were converted to micromoles of p-nitrophenol from a suitable reference curve.

The results of one experiment are shown in Figure 9. In the absence of methanol, equimolar quantities of glucose and p-nitrophenol were liberated. In the presence of methanol, a great deal more of the substrate was decomposed, but it is of interest to note that the curve for glucose liberation was identical in both cases. The liberation of glucose in both cases was linear over the period studied, whereas the liberation of p-nitrophenol in the presence of methanol followed first order kinetics. The first order velocity constants ($k \times 10^3$) for 30, 60, 120, 180, 240, and 300 minutes are, respectively, 2.25, 2.34, 2.25, 2.33, 2.42, and 2.45 min^{-1}.

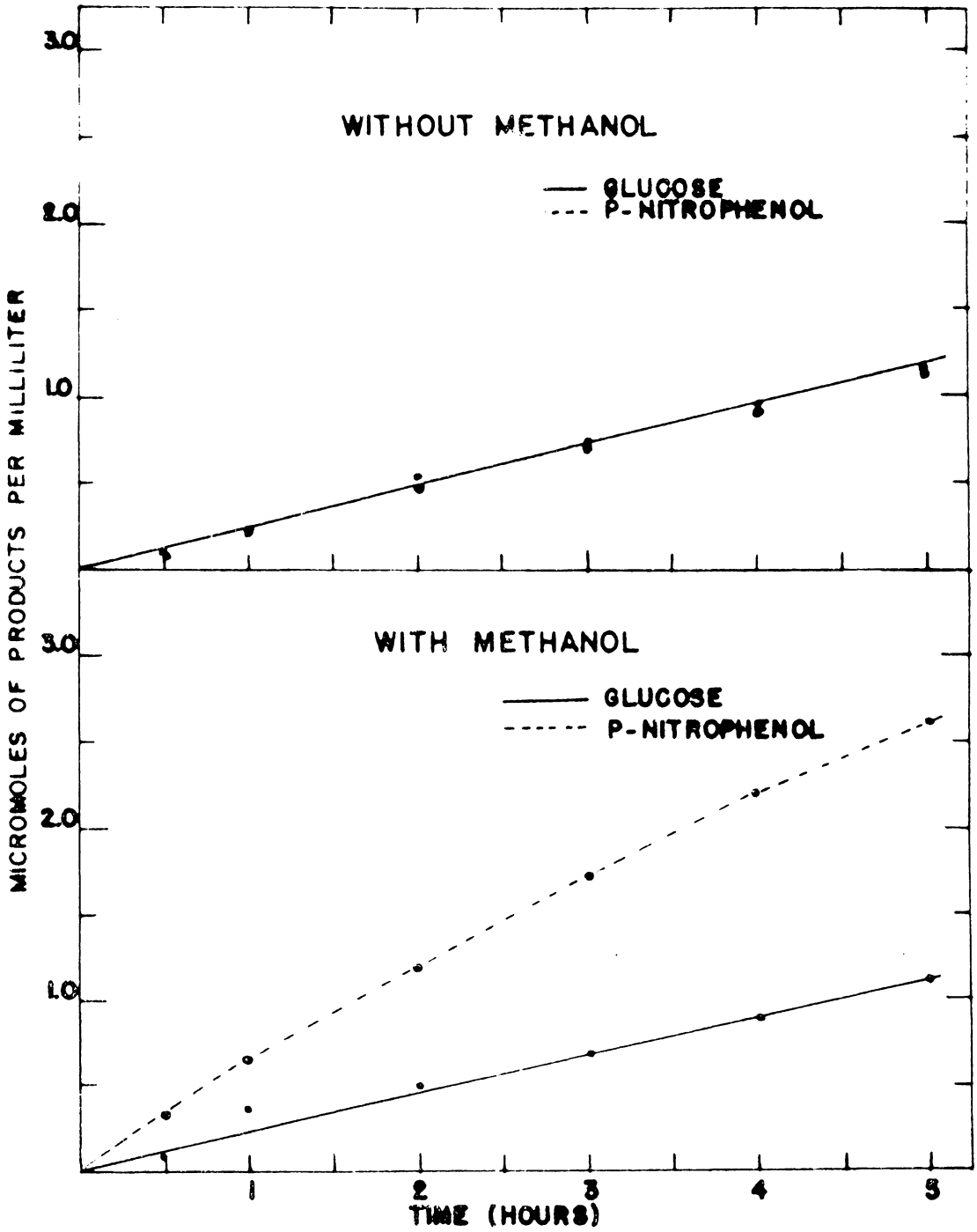


FIGURE 9

DECOMPOSITION OF P-NITROPHENYL-β-D-GLUCOSIDE IN THE PRESENCE AND ABSENCE OF METHANOL

SUBSTRATE CONCENTRATION = 5 MICROMOLES/ML

Effect of methanol concentration. The effect of methanol concentration on the transfer reaction was investigated. Increasing quantities of methanol and water (to 0.6 ml) were added to 0.4 ml of 0.025 M substrate. One ml of Fraction 23 was added to each tube making the final substrate concentration 5 micromoles/ml. At hourly intervals, 0.2 and 0.1 ml were removed for glucose and p-nitrophenol determinations. The determinations were made as previously described. The data from this experiment are recorded in Table 1.

The experiment was repeated with 6-bromo-2-naphthyl-β-D-glucoside. Because of the poorer solubility of this compound the final substrate concentration was 0.5 micromoles/ml. The results of this experiment are shown in Figure 10. The data for the decomposition of p-nitrophenyl-β-D-glucoside included in the figure for comparison were taken from Table 1. The term, % D, refers to the percentage of substrate decomposed and is equal to micromoles of aryl alcohol/micromoles of substrate x 100. The amount of glucose transferred was the difference between the glucose and aryl alcohol. The term, % T, refers to the percentage of glucose transferred and is equal to micromoles of glucose transferred/micromoles of aryl alcohol liberated x 100.

Acceptor specificity of alcohols. The specificity of the enzyme for the acceptor alcohol molecule was investigated in the following fashion: Fourteen compounds were tested for their ability to accept the glucose from the aryl glucoside. These compounds included

Table 1

Kinetics of Hydrolysis of p-Nitrophenyl-β-D-glucoside in Increasing Concentrations of Methanol

Methanol (M)	Time (Hrs)	p-Nitrophenol micromoles/ml	Glucose micromoles/ml	% D	Glucose Transferred micromoles/ml	% T
	1	0.17	0.18	3.9		
0	2	0.33	0.34	7.5		
	3	0.44	0.43	10.0		
	1	0.22	0.25	5.0		
1	2	0.49	0.47	11.2	0.02	4
	3	0.75	0.61	17.0	0.14	19
	1	0.41	0.20	9.3	0.21	51
2	2	0.82	0.38	18.6	0.44	54
	3	1.32	0.58	30.0	0.74	56
	1	0.53	0.20	12.0	0.33	62
3	2	0.96	0.35	21.8	0.61	64
	3	1.39	0.56	31.6	0.83	60
	1	0.56	0.19	12.7	0.37	66
4	2	1.01	0.32	23.0	0.69	68
	3	1.47	0.50	33.4	0.97	66
	1	0.61	0.16	13.9	0.45	74
5	2	1.10	0.26	25.0	0.84	76
	3	1.55	0.42	35.3	1.13	73
	1	0.56	0.16	12.7	0.40	72
6	2	1.14	0.33	25.9	0.81	71
	3	1.61	0.35	36.6	1.26	78
	1	0.57	0.12	13.0	0.45	79
7	2	1.16	0.30	26.4	0.86	74
	3	1.65	0.39	37.5	1.26	77

% D is the percentage of substrate decomposed
% T is the percentage of glucose transferred

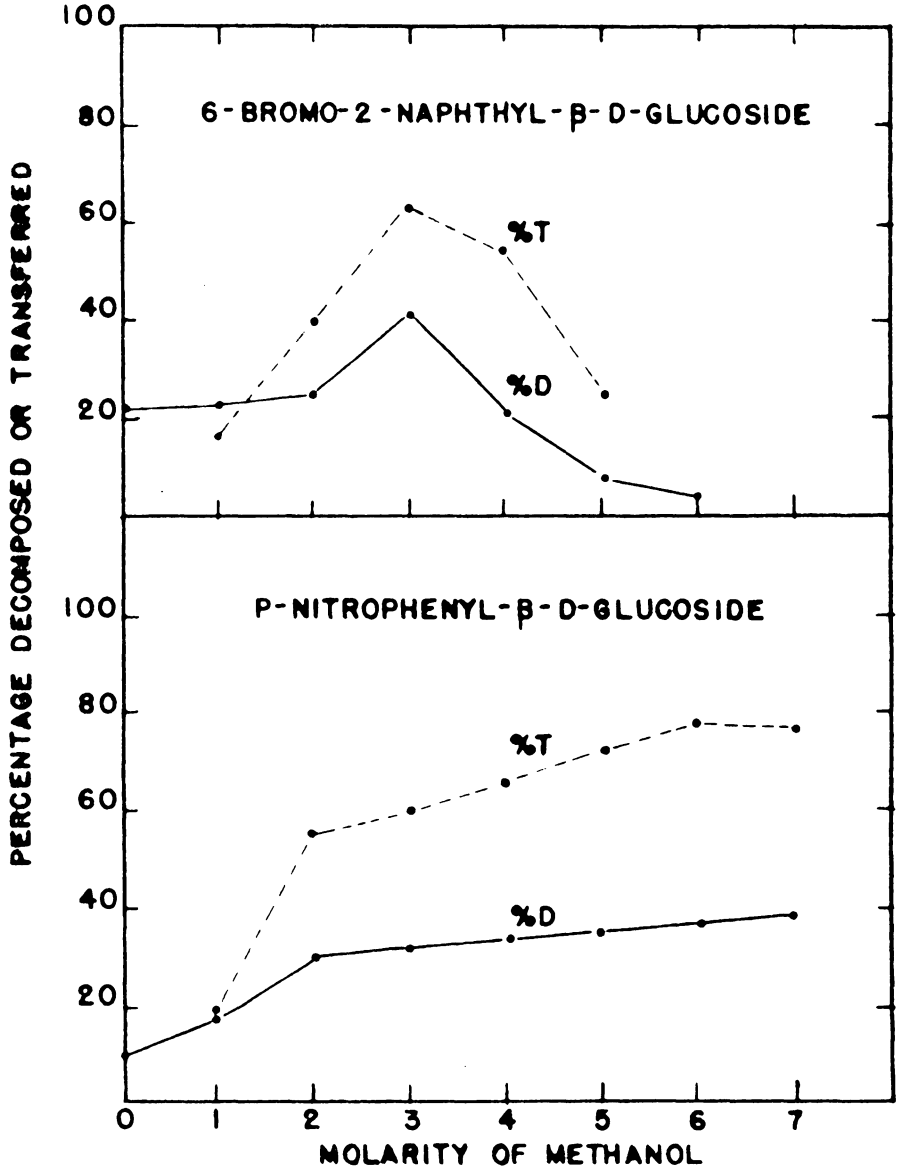


FIGURE 10

EFFECT OF METHANOL CONCENTRATION ON THE DECOMPOSITION OF TWO β-ARYL GLUCOSIDES

%D = PERCENTAGE OF SUBSTRATE DECOMPOSED

%T = PERCENTAGE OF GLUCOSE TRANSFERRED

primary, secondary, tertiary, and sugar alcohols. A control flask with only water as acceptor was run for comparison. The total volume of 2.0 ml consisted of 0.5 ml of 0.02 M substrate in phosphate-citrate buffer, pH 5.35, pure alcohols or concentrated alcohol solutions to give the desired molarity and a volume of 1.0 ml, and 1.0 ml of Fraction 25. Incubation was at 37° C and, periodically, 0.2 and 0.1 ml were removed for glucose and p-nitrophenol analyses, which were made in the fashion previously described. The results of a 3-hour incubation period are shown in Table 2.

Glucose was tested also for its ability to accept the glucosyl residue from the aryl glucoside. The two compounds (0.5 ml of 0.025 M p-nitrophenyl-β-D-glucoside in 0.5 M sodium acetate buffer, pH 5.35, and 5 mg of glucose) were incubated with Fraction 24 (0.5 ml) at 37° C. Periodically, the mixture was chromatographed in isopropanol, water, acetic acid (6:3:1). Duplicate chromatograms were prepared and the first was sprayed with the aniline-diphenylamine-phosphate reagent for reducing sugars. Only glucose could be detected. The other paper was sprayed with periodate followed by benzidine for non-reducing sugars. Only glucose and the unhydrolyzed substrate were found. There were no changes over a 14-day period. The possibility of a disaccharide's formation from the aryl glucoside and glucose is therefore minimized.

Transfer product with methanol. It was indicated previously that the transfer product with methanol was a methyl glucoside. It would be

Table 2

Acceptor Specificity of β-aryl Glucosidase

Acceptor	Molarity of Acceptor	% D	% T
Water	- -	13	- -
Methanol	3.0	25	64
Ethanol	2.0	26	49
n-propanol	1.0	21	42
isopropanol	1.0	16	0
n-butanol	1.0	30	57
tert-butanol	1.0	25	0
n-pentanol	0.2	34	65
tert-pentanol	0.2	19	0
Ethylene glycol mono methyl ether	2.0	21	16
Diethylene glycol	1.0	24	51
Glycerol	1.0	10	0
Sorbitol	0.2	14	0
Mannitol	0.2	14	14
Ribitol	0.2	15	20

% D is the percentage of substrate decomposed
% T is the percentage of glucose transferred

of interest to know whether the configuration is retained or inverted during the reaction. At the writing of this thesis it has not been possible to establish the anomer of methyl glucoside that is formed in the reaction. Quantities of the methyl glucoside, sufficient for an accurate determination of its optical rotation, could not be isolated. Various other techniques have been employed in an effort to determine the configuration.

Cifonelli and Smith (22) reported the separation of the anomers of methyl glucoside on paper chromatograms. The solvent reported by these authors was a tert-pentanol, n-propanol, water mixture (4:1:1). It was found, however, that when known α and β compounds were chromatographed on the same paper with a hydrolyzate containing the unknown methyl glucoside, all three compounds moved with the same R_f. The cause for the discrepancy between the reported and observed behavior of the glucosides has not been established, but Smith (117) suggested that it was probably due to an error in labelling the n-propanol used in the above mixture. He stated that the compound was actually isopropanol instead of n-propanol. However, the mixture, tert-pentanol, isopropanol, water (4:1:1) also failed to resolve the anomers in this laboratory. In any event, it has not been possible to establish the configuration by means of paper chromatography.

Foster et al (31) reported the separation of α- and β-methyl glucosides in borate buffers (pH 10) by paper electrophoresis. These authors used high voltages (900-1500 v) for the separations. Equipment

of this high a voltage capacity has not been available to this laboratory but when experiments were carried out at 750 volts, the α, β, and unknown methyl glucoside migrated with the same velocity. Therefore, it has not been possible to distinguish between the anomers by this procedure.

Barker et al (7, 10) have advocated the use of infrared spectroscopy for the determination of carbohydrate structure. All the α-linked sugars had an adsorption band at a frequency of 842±7 cm^{-1}, whereas β-linked sugars all exhibited a maximum of 981±5 cm^{-1}. There are many bands in common but these two are so characteristic that the authors stated that they could be used as a diagnostic test for the α or β configuration. As small quantities (5-10 mg) of the methyl glucoside could be isolated by strip paper chromatography, this method was investigated further. In cooperation with the Virginia Polytechnic Institute Physics Department the infrared spectrum of known α-methyl glucoside was investigated using a mineral oil "mull" technique and sodium chloride prisms. The "mull" is simply a suspension of the solid compound in mineral oil. At the writing of this thesis, it has not been possible to reproduce the infrared spectrum for the compound that is recorded in the literature. Undoubtedly, the failure is due to inexperience and imperfections in technique which will have to be perfected before reliable information can be expected with the infrared spectrometer.

Designation of the glucosidic bond configuration of the methyl glucoside formed by transferase activity of β-aryl glucosidase will have to await the isolation and purification of sufficiently large amounts for an optical rotation determination.

Transglucosidase

Presence in culture filtrates of M. verrucaria. Prior to the demonstration of higher saccharides as products of certain fractions of the enzyme eluted from a cellulose column (Plate 3), this laboratory had been unable to confirm reports (26, 109) of a transglucosidase in culture filtrates of Myrothecium verrucaria. When crude filtrates acted on cellobiose, the compound was completely hydrolyzed to glucose and no other sugars could be detected on chromatograms. Presumably, the removal of some of the hydrolases from the transglucosidase on the column allowed the products of the latter to accumulate to detectable levels. The fractions which contained the transglucosidase were investigated further.

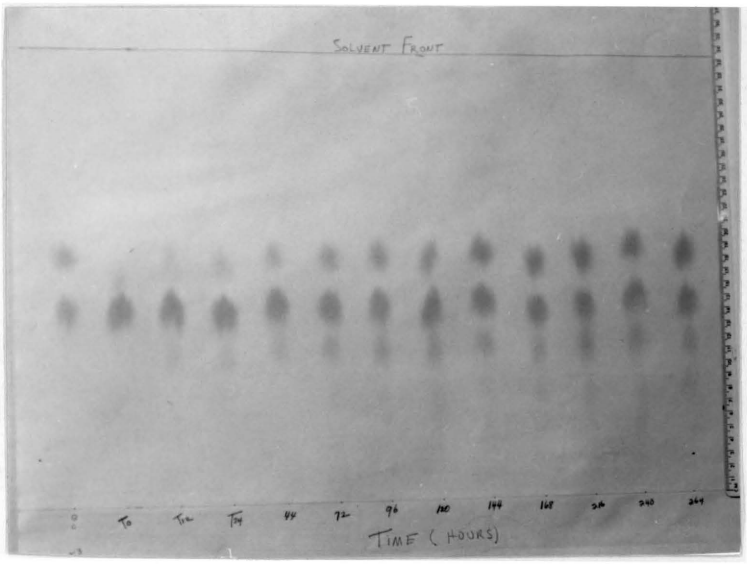
Activity on cellobiose. Most of the transglucosidase activity was in Fractions 3 and 4, with only small amounts in Fractions 5 and 6, and only the former pair was investigated.

Fifty mg of cellobiose was added to each of 3 small vials, which contained 0.1 ml of 0.5 M acetate buffer, pH 5.0. To the first vial was added 0.4 ml of water; to the second, 0.4 ml of Fraction 3; and to

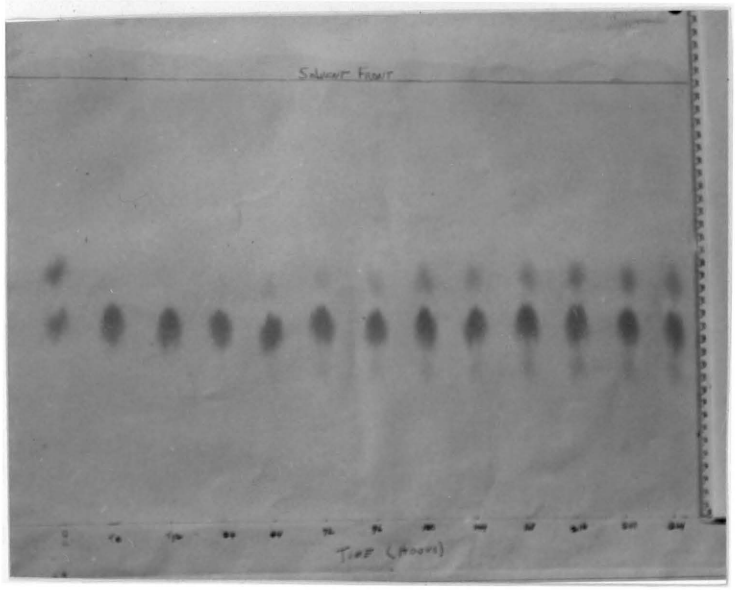
the third, 0.4 ml of Fraction 4. A crystal of Dowicide 4 was added to each vial as an antiseptic. The cellobiose was rapidly dissolved (final concentration 10%) and 3 microliters from each vial were spotted on separate sheets of Whatman Number 1 filter paper (18 x 22 1/2 in). A stream of hot air dried the samples and inactivated the enzyme. The sample with water and no enzyme served as control.

The mixtures were sampled periodically in this fashion for a period of 11 days. They were then chromatographed in an isopropanol, water, glacial acetic acid solvent (6:3:1) for 18 hours. The papers were dried, sprayed with the p-aminohippuric acid-phthalate spray, and developed at 140° C for 10 minutes. The results from Fractions 3 and 4 are shown in Plate 5. The control of cellobiose is not included because there was neither hydrolysis to yield glucose nor spontaneous polymerization to yield other sugars. Two higher saccharides are evident in Fraction 3 and one in Fraction 4. There are suggestions of other sugars in both fractions.

Activity on 4-O-β-D-glucopyranosyl-D-sorbitol. The activity of Fraction 3 on the reduction product of cellobiose was investigated. Fifty mg of this compound was dissolved in 0.25 ml of 0.2 M sodium acetate buffer, pH 5.0, and 0.25 ml of Fraction 3 was added (10% final substrate concentration). The solution was incubated at 37° C with a crystal of Dowicide 4 as antiseptic. After 120 hours, 10 microliters of the solution were chromatographed in the solvent used above.



Fraction Number 3



Fraction Number 4

PLATE 5

Action of Transglucosidase on Cellobiose

Known glucose and cellobiose are at the far left and the times of sampling are, in order from right to left, 0, 12, 24, 44, 72, 96, 120, 144, 168, 216, 240, and 264 hours.

Duplicate chromatograms were prepared; one was sprayed with aniline-diphenylamine-phosphate for reducing sugars, and the other was sprayed with periodate and benzidine for non-reducing sugars.

Glucose was not liberated from the substrate as evidenced by the absence of detectable reducing sugars on the chromatogram. There were three spots on the chromatogram for non-reducing carbohydrates. The first had the R_f of sorbitol, the second the R_f of the unreacted substrate, and the third had an R_f lower than 4-O-β-D-glucopyranosyl-D-sorbitol. There was no spot on the chromatogram that corresponded to glucose. Chromatograms developed by both these techniques are shown in Plate 6.

Products of transfer from cellobiose. Larger quantities of the products of transglucosidase were obtained by incubating 750 mg of cellobiose with 4.0 ml of Fraction 3 and 1.0 ml of 0.25 M sodium acetate buffer, pH 5.0. A crystal of Dowicide 4 served as antiseptic.

The solution was chromatographed periodically and at the end of 14 days there were 3 higher saccharides visible on chromatograms below cellobiose. It has been reported (21) that the aniline-diphenylamine-phosphate reagent gives distinctive colors with the isomeric β-glucosides. This reagent gives a brown color with gentiobiose (β-1,6), a blue color with cellobiose (β-1,4), a light olive brown with laminaribiose (β-1,3), and a pinkish yellow with sophorose (β-1,2). All of the higher saccharides in the enzymatic mixture above gave blue colors with this reagent, suggesting that these sugars are members of the homologous series of cellulose dextrins.

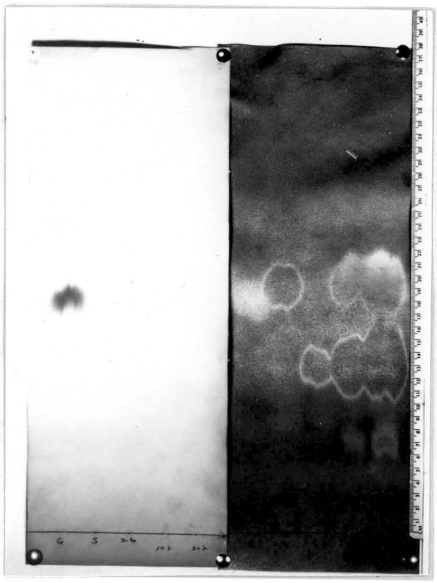


PLATE 6

Action of Transglucosidase on 4-O-B-D-glucopyranosyl-D-sorbitol

The two chromatograms were prepared identically: The one on the left was sprayed with aniline-diphenylamine-phosphate and the one on the right was sprayed with periodate followed by benzidine. The chromatograms have, in order, from left to right, glucose, sorbitol, 4-O-B-D-glucopyranosyl-D-sorbitol, 10 microliters of hydrolyzate and 20 microliters of hydrolyzate. The solvent was allowed to drip off the edge of the paper.

The entire reaction mixture was applied to 10 sheets of Whatman 3MM filter paper (18 x 22 1/2 in) along a line 3 inches from the edge. These strip chromatograms were placed in a chamber and equilibrated with the vapors of the eluting solvent, which was isopropanol, water, glacial acetic acid (6:3:1), for about 4 hours. The solvent was added to the troughs and the papers were irrigated for 20 hours. The solvent was allowed to drip off the edges of the papers. The papers were dried in a stream of warm air, and guide strips were cut from the edges and middle of the sheets. These strips were sprayed with the aniline-diphenylamine-phosphate reagent to locate sugar bands. Three strips, which contained higher saccharides, were cut from the paper and each was eluted with water. The individual sugars were then rechromatographed in the solvent used for the initial separation. A chromatogram of these sugars is shown in Plate 7. Pure glucose and cellobiose samples are included on the chromatogram for comparison. The first two higher saccharides are chromatographically pure, but there are two sugars in the third spot. Because of the small amounts of these two sugars, no attempt was made to resolve them.

Assuming that the higher saccharides are members of the homologous cellulose dextrin series, it is of interest to note that when the R_M values, log $(\frac{1}{R_f}-1)$, (12) are plotted against the probable degree of polymerization of the sugar, a straight line results for the series. Glucose does not fall on the line. An analogous situation is also true for the homologous series of maltodextrins (128).

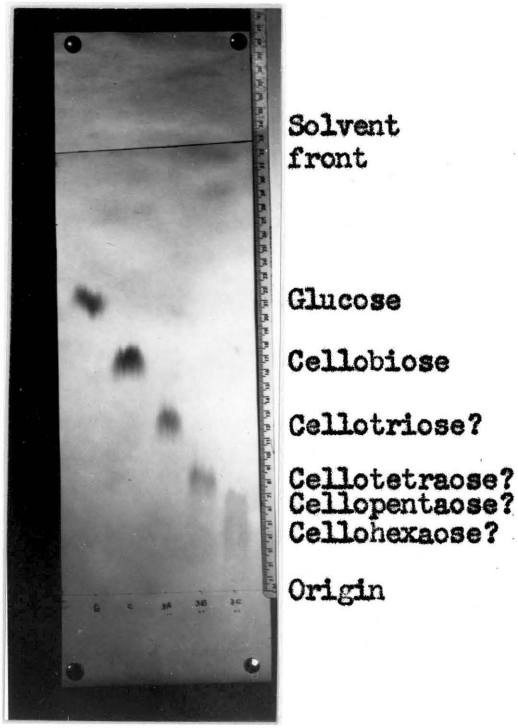


PLATE 7

Products of Transglucosidase from Cellobiose

DISCUSSION OF RESULTS

Heterogeneity of Cellulase

Electrophoretically distinct components. The fact that there are several ionic species of cellulase in culture filtrates of *M. verrucaria* is considered further support of the proposed multienzymatic nature of cellulase. In this respect the paper electrophoresis data presented in this paper complement the starch electrophoresis data (81) reported for *M. verrucaria* cellulase. Both procedures revealed several electrophoretically distinct components with cellulase activity. Correlation of the components shown by the two procedures has not been attempted.

Paper electrophoresis of cellulase has several marked disadvantages. Regardless of the pH at which the experiment was conducted, some of the cellulase remained at the origin. The cause for the failure of the proteins to migrate is unknown. The principal factor is probably strong adsorption of the enzyme onto its natural substrate. Less likely is the possibility that some of the proteins of the cellulase complex are non-ionic. The success of any electrophoresis experiment depends on the detection of small differences in the migration velocity of the components. When the components fail to migrate, there is a greater possibility that there may be several enzymes concealed in the one peak. Another disadvantage is that paper

electrophoretic methods are not readily adaptable to preparative scale work and consequently it has not been possible to test each component for substrate specificity. Nevertheless, in spite of these shortcomings, the heterogeneity of cellulase as revealed by paper electrophoresis is interpreted to mean that the total cellulase activity of culture filtrates of M. verrucaria is dependent on several proteins. It would be premature to call each component a separate enzyme because it is not known what range of properties may exist for a single enzyme or specifically what characteristics differentiate one enzyme from another of related function.

It was concluded that the cellulase and the β-aryl glucosidase remaining at the origin on the electropherograms (Fig. 3) depend on different proteins for their activity, because the β-aryl glucosidase was completely destroyed at 80° C whereas the cellulase was not. The data do not exclude the possibility that β-aryl glucosidase hydrolyzes carboxymethyl cellulose but they do exclude the possibility that the major cellulase hydrolyzes β-aryl glucosides.

It has not been possible to explain the discrepancies which occurred in the cellulase distribution patterns of the various filtrates (Figure 1). Perhaps the particular cultural conditions resulted in the elaboration of different proportions of the components. There is likewise the possibility of selective adsorption of components on residual cellulose of the medium or partial denaturation of certain components in the course of concentration and dialysis. The first

hypothesis seems the most plausible because the cultural conditions of this organism are known to affect the production of various enzyme activities (34).

In any attempt to reconcile the electrophoresis data of this thesis with the electrophoresis data of Whitaker (135) the following facts are important: Whitaker isolated a protein fraction from culture filtrates of M. verrucaria by precipitation with ammonium sulfate, fractionation with ethanol, and precipitation with polymethyl acrylic acid. This protein fraction gave a single component in moving boundry electrophoresis at three acid pH's. There are two possibilities to explain the apparent conflict.

The first possibility is that the enzyme is actually a single protein and represents only one of the components found by starch gel and paper electrophoresis, with the other components having been lost in the process of purification. The second possibility is that in spite of the apparent homogeneity, the fraction is still a very complex mixture of proteins.

In regard to the first possibility, it was mentioned in a previous paragraph that cultural conditions do affect the proportions of enzyme activities elaborated by this organism. Possibly the growth conditions used by Whitaker led to the enrichment of one component and it was this component that he isolated and characterized.

In regard to the second possibility, it has been emphasized by Miller and Blum (81) that moving boundry electrophoresis has a

very short migration path. It is quite possible that closely related ionic species of protein would not have large enough differences in their electrophoretic mobilities to be separated in short migration distances. Also, Whitaker made all his electrophoretic separations at acid pH's. With starch gel electrophoresis, Miller and Blum (81) found that crude cellulase could be resolved into components over a long migration path (50 cm) at pH 7.0. In this investigation there was no separation of cellulase into components at pH 7.0 or below (Figure 2). It is possible that the protein fraction of Whitaker would show multiple components in moving boundry electrophoresis at alkaline pH's.

Miller and Blum (81) have rejected the first possibility and believe that the second is more likely. They believe that the short migration distance in moving boundry electrophoresis simply is not sufficient to effect separation of closely related ionic species of protein.

Gilligan and Reese (34) have emphasized that enzymatic activity is a more reliable determinant of homogeneity than are the physico-chemical methods. A correlation between the components shown in electrophoresis and the substrate chain length specificity is needed. It appears possible that sufficient quantities of the components shown in paper and starch gel electrophoresis could be obtained by preparative scale zone electrophoresis. Starch gel does not appear to be an

ideal supporting medium because of hydrolysis by contaminating amylase in culture filtrates (81), but other media such as agar or silica gel may prove to be of value. The biggest obstacle appears to be the preparation of cellulose of known chain length to use as substrate for characterization of the components.

Substrate specificities of fractions from column. Further evidence of the multiplicity of enzymes present in culture filtrates of M. verrucaria was obtained from chromatography of crude culture filtrates on cellulose columns. It has been possible to correlate various column fractions with a preference for different chain lengths of cellulose.

The results of the viscosimetric and reductometric assay of enzymes which cleave carboxymethyl cellulose (Figure 6) were unexpected and will be discussed in the next section.

A comparison of the hydrolysis of carboxymethyl cellulose (Figure 6), insoluble cellulose dextrins (Plate 1), soluble cellulose dextrins (Plate 2), and cellobiose (Plate 3) offers dramatic evidence that there are multiple components of the cellulase complex whose specificities depend on the chain length of the substrate.

A partial purification of the enzymes has been achieved during elution from the column. Those enzymes which act on the lower members of the cellulose dextrins have been eluted early and those enzymes which act on the higher members of the series have been moved progressively to the later fractions.

Cellobiase is eluted early and is confined to relatively few fractions (Plate 3). An extended hydrolysis period (12 days) was used in order to allow for low enzyme activity in any of the fractions. In all cases, the cleavage of cellobiose was accompanied by the formation of higher saccharides. After 12 days the higher saccharides had disappeared in Fraction 3, although they were present after shorter incubation periods.

Most of the enzymes which hydrolyze the soluble cellulose dextrins are confined to the first 25 fractions. Cellobiose was present also in hydrolyzates of Fractions 29, 34, and 39, indicating some hydrolysis of the soluble dextrins by the enzymes in these fractions and emphasizing the fact that the fractionation is by no means clear-cut.

The enzymes which hydrolyze the insoluble cellulose dextrins are present in Fractions 3 through 47. The difference between the hydrolysis of the soluble and insoluble dextrins is even more marked because of the extended incubation time of the former. With the insoluble dextrins, the formation of glucose and cellobiose parallels the formation of reducing sugar from CMC (Figure 1). Glucose and cellobiose formation, which was rapid in the early column fractions, gradually decreased to nil in the later fractions, indicating that the insoluble dextrins were not available to the enzymes in the last fractions to come off the column.

The enzymes in the last fractions appear to have a preference for longer cellulose chains. Fractions 48, 49, and 50 decrease the

viscosity of CMC but do not hydrolyze the insoluble dextrins. The increase in reducing sugar from CMC is slight in these fractions, indicating only partial degradation of the carboxymethyl cellulose chains.

A summary of the distribution of enzyme activities, with respect to their activities on the various substrates, is shown schematically in Figure 11. Relative activities are represented by the thickness of the bars.

Multiple modes of attack on carboxymethyl cellulose. The results obtained from the reducing sugar and viscosimetric assays of cellulase in the fractions from the column (Figure 6) were totally unexpected. There appear to be two types of enzymes present in the filtrate with respect to the mode of attack. The fractions which came off the column early contain enzymes which rapidly produce reducing sugar but slowly decrease the viscosity, whereas the components which came off the column last rapidly decrease the viscosity but produce very little reducing sugar. This behavior is in marked contrast to intact crude filtrates which have parallel activity on CMC when measured by these two procedures (123). The earlier fractions appear to be enriched with respect to end-cleaving enzymes and the later fractions appear to be enriched with random cleaving enzymes. These two types of enzyme have not been reported together in the same filtrate and furthermore the actual existence of an end-cleaving cellulase has not

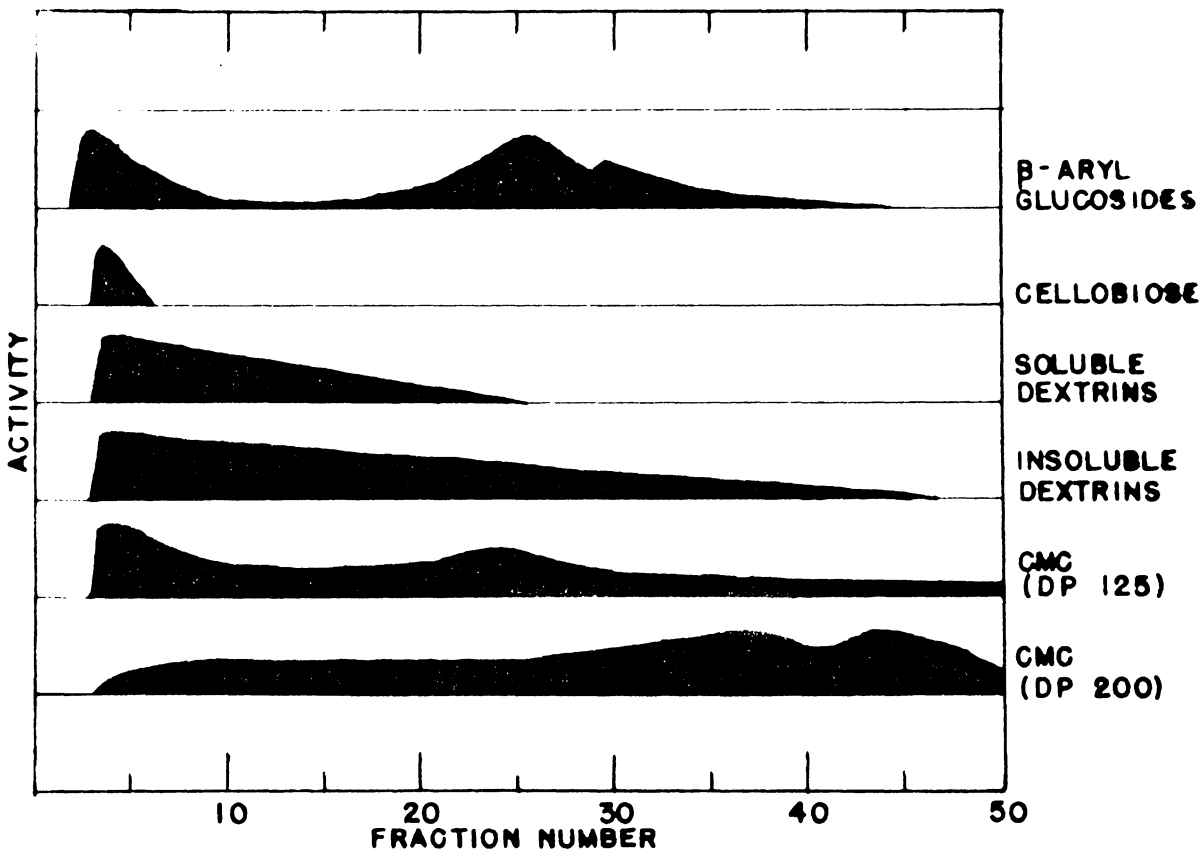


FIGURE 11

SCHEMATIC REPRESENTATION OF ENZYME DISTRIBUTION IN THE COLUMN FRACTIONS

been firmly established. There is an alternate hypothesis which can account for the observed data, without invoking the presence of an end-cleaving enzyme.

It has been established in the previous section that there are cellulases in culture filtrates of M. verrucaria which attack a restricted range of chain lengths of cellulose. The carboxymethyl celluloses used in the two assays were different. The CMC used in the reducing sugar assays had a mean DP of 125 and that used for the viscosity changes had a mean DP of 200. Consequently, there are more of the shorter chains present in the CMC used for reducing sugar assays than for viscosity measurements. The earlier fractions from the column, which have an enrichment of enzymes active on shorter chains, have more substrate on which to act in the reducing sugar determination than in the viscosity determination. Also, the earlier fractions contain cellobiase which rapidly increases reducing sugar. In the viscosimetric assay, there were fewer of the shorter chains for the enzymes in the earlier fractions to hydrolyze because of the higher DP. The hydrolysis of these shorter chains does not markedly lower the viscosity because the longer chains, which are unavailable to the short-chain hydrolyzing enzymes, maintain the viscosity of the solution.

In the later fractions, which have an enrichment of the long-chain hydrolyzing enzymes, there is a reversed tendency. Reducing sugars are produced by the enzymes, but at a much slower rate because there are smaller amounts of the short-chain hydrolyzing enzymes present. It is the latter enzymes which produce end groups most rapidly.

There is a rapid drop in viscosity, however, because the CMC with a DP of 200 is rich in chain lengths suitable for the long-chain hydrolyzing enzymes in the later fractions. Thus, it is possible to account for the types of curves obtained with the two assays, assuming only random cleaving enzymes of varying chain length specificity.

There is other experimental evidence to support random cleavage for all the enzymes in the fractions. Examination of Plate 1 reveals the presence of higher sugars in almost every fraction. An end-cleaving enzyme would not give rise to the observed sugars. These sugars are not apparent in Fractions 3 and 4, because of the higher activity of these fractions. However, the same sugars were present in these fractions when sampled at a shorter incubation period.

The demonstration of different ionic species of cellulase by paper electrophoresis and the fractionation of enzymes on a cellulose column to give fractions, whose activities have been correlated to the chain length of substrate, conclusively establish the multiple nature of the cellulase of M. verrucaria.

Formation of Glucose

Independence of cellobiose formation. According to Reese (69), the glucose found in enzymatic hydrolyzates of cellulose arises as the result of a cellobiase. However, Whitaker (135) believes that the enzyme is capable of splitting single glucose molecules from the

cellulose chain and that the presence of glucose in enzymatic hydrolyzates does not depend on prior cellobiose formation. The view of Whitaker is confirmed by data presented in this thesis. When various fractions from the cellulose column acted on insoluble cellulose dextrins (Plate 1), glucose and cellobiose were formed in large quantities. However, the majority of these fractions was unable to hydrolyze cellobiose (Plate 3). If cellobiose were the end product of cellulase action, then cellobiose would accumulate in most of these fractions and glucose would not be formed because of the absence of a cellobiase.

It is of interest to recall, however, that when the enzymes present in the fractions acted on the soluble dextrins (Plate 2) the predominant product was cellobiose and only in a few cases was glucose formed. It appears that the ability to cleave single glucose molecules from the chains is a property of a particular enzyme and substrate, and that not all the enzymes in culture filtrates have this ability.

It is not possible to offer a complete explanation for the observation that cellobiose but not glucose (94, 106) inhibits the enzymatic hydrolysis of cellulose. It seems probable that the inhibition is related to the specificity of the enzyme, particularly the specificity for the β-1,4 linkage. Cellobiose is the smallest member of the series with this linkage and could conceivably block some of the reactive sites on the enzyme surface. Glucose does not possess the linkage; consequently, the enzyme no longer has an affinity for it. It blocks none of the reactive sites and thus does not inhibit the reaction.

Significance of Intermediate Dextrins

Support of random cleavage hypothesis. Previous reports (43, 64) on the presence of intermediate dextrins in enzymatic hydrolyzates have been rejected (109) because of the known presence of reverting activity in the crude enzymes used.

It has been established in this thesis that slower moving sugars do accumulate to detectable levels in hydrolyzates of insoluble cellulose dextrins by fractions from the cellulose column (Plate 1). It has been established also that transglucosidase occurs only in Fractions 3, 4, 5, and 6 (Plate 3). Therefore, higher saccharides in other fractions could not arise as the result of the action of transglucosidase on cellobiose. The sugars contain no sulfur and therefore could not be sulfate esters formed in the hydrolytic preparation. Furthermore, the insoluble dextrins are completely hydrolyzed to glucose by Fraction 3, demonstrating the susceptibility of the entire molecule to enzymatic cleavage.

The conclusion that these higher saccharides represent intermediate dextrins, which are formed as the result of the random cleavage of the cellulose molecule, is inescapable.

β-aryl Glucosidase

Electrophoretic behavior. In the initial studies on electrophoresis, it was established that the β-aryl glucosidase consisted of a minimum of 3 electrophoretically distinct species of protein. Two of these were mobile and migrated several centimeters while the third failed to move (Figure 1).

There were two peaks of β-aryl glucosidase activity that were eluted from the cellulose column (Figure 7). The first peak was a highly impure mixture of enzymes. In Fraction 3, there were several electrophoretically distinct species of protein (Plate 4) with β-aryl glucosidase activity. In addition to the many protein species of β-aryl glucosidase, Fraction 3 possessed many other enzyme activities. It contained cellulase, cellobiase, and transglucosidase. It was found also to contain an α-glucosidase which hydrolyzed maltose. It is probable that this fraction contained other enzymes whose presence remained unknown simply because it was not profitable to test for their presence. Fraction 3 contained the bulk of the protein that was eluted from the column (Figure 5). It is not known whether any of the various activities belongs to the same protein, whether each activity is represented by a single separate protein, or whether each activity is represented by several ionic species of protein in a fashion similar to the β-aryl glucosidase. In any event, Fraction 3 is still a very complex mixture.

In the past, the hydrolysis of β-aryl glucosides has been used as a measure of cellobiase activity (105). Since Fractions 3 and 4 possessed both cellobiase and β-aryl glucosidase activity (Plate 3 and Figure 7) it seemed probable that one or more of the β-aryl glucosidases in these fractions was a cellobiase. This possibility is minimized by the fact that Fraction 2 possessed β-aryl glucosidase activity (Figure 7) and was devoid of both cellobiase and transglucosidase activity (Plate 3).

The electrophoretic behavior of the β-aryl glucosidase in Fraction 23 is of interest. This enzyme did not migrate in paper electrophoresis at three widely separated pH's (8.55, 5.0, and 3.0). One of these pH's could conceivably be the isoelectric point of the enzyme, but in that event the enzyme should be a charged molecule at the other two pH's and should migrate accordingly. The failure to migrate at any of the three pH's minimizes the possibility that any of the values might be the isoelectric point.

There are other possibilities that may account for the failure of the enzyme to move under a potential of 500 volts. The enzyme may have been tightly bound by adsorption to the paper or there may have been a reaction between the enzyme and the paper to form covalent bonds. It does not seem probable that adsorptive forces could be strong enough to overcome a potential of this magnitude, especially since cellulose is not a substrate of the enzyme, nor does it seem probable that the protein and the paper are sufficiently reactive to form covalent bonds. Both of these possibilities are minimized by the fact that the enzyme was eluted from a cellulose column without either strong adsorption or chemical reaction between the protein and the cellulose.

Still another possibility is that the protein is non-ionic. It could be a cyclic protein without any ionizable groups. Examples of cyclic proteins with enzyme activity are not known.

All of the possibilities for an explanation of the failure of the β-aryl glucosidase to migrate in paper electrophoresis seemed

equally remote and a positive conclusion was not possible on the basis of the available data.

Acceptor specificity. It has been shown in this thesis (Table 2) that all of the primary alkyl alcohols tested had a marked influence on the rate of decomposition of β-aryl glucosides. All of the primary alkyl alcohols increased the rate of substrate decomposition over that of the water control. Secondary and tertiary alcohols and glycerol had no effect on the course of the reaction, but the sugar alcohols, mannitol, and ribitol, served as acceptors for glucose, although sorbitol did not.

It is of interest to compare the acceptor specificities of this β-aryl glucosidase with those of the β-aryl glucosidase from almond emulsin (25). The emulsin enzyme used all of the primary alcohols tested as acceptor but did not transfer to tertiary alcohols. It did transfer glucose to isopropanol, glycerol, and sorbitol. Thus, it appears that the alcohol acceptor specificity varies considerably with the source of the enzyme.

It is interesting to examine the data in Figure 9 and Table 1 more closely. In the absence of methanol equimolar quantities of p-nitrophenol and glucose were liberated, but in the presence of methanol excess p-nitrophenol was liberated and a great deal more of the substrate was decomposed. In both cases, however, the amount of glucose liberated was approximately the same. Thus, the hydrolase and

transferase activities appear to be independent functions. Presumably, a hydrolase is liberating equimolar quantities of glucose and p-nitrophenol whether methanol is present or not. With methanol, there is an additional reaction which liberates p-nitrophenol and transfers the glucose to methanol. This hypothesis implies that two enzymes are involved. However, it may only involve two reactive sites on the same protein. The protein surface may have reactive sites which are specific for the acceptor. Some of the sites may be specific for water and function in the presence or absence of alcohols, whereas some of the sites may be specific for alcohols and inactive in their absence.

Preference for alcohol as acceptor. The preference for alcohol over water as acceptor is demonstrated in Table 2. In all cases where alcohol served as acceptor there is an increase in decomposition of the substrate. If the substrate, enzyme, and buffer are ignored, a 3 M methanol solution is approximately 6 mole percent methanol. In other words, 6 percent of the total number of molecules are methanol. In spite of the preponderance of water molecules, 64 percent of the glucose was transferred to methanol. The preference is demonstrated even more forcefully in the case of n-pentanol. A 0.2 M n-pentanol solution is only 0.4 mole percent, yet 65 percent of the glucose was transferred to the alcohol.

It is not yet possible to offer a rational explanation for the preference of the enzyme for alcohol over water as acceptor for glucose.

There were marked differences in the rates of hydrolysis of 6-bromo-2-naphthyl- and p-nitrophenyl-β-D-glucoside in the presence of increasing concentrations of methanol (Table 1 and Figure 10). With the naphthyl glucoside there is a optimum at 3 M methanol, above which the enzyme appears to be rapidly inactivated. However, with the p-nitrophenyl glucoside the enzyme has a marked tolerance for the alcohol and is most active at 7 M methanol (approximately 28 percent). It is not known whether the difference implies two or more enzymes or whether the substrate and products of the two compounds influence the stability of the enzyme in the presence of high concentrations of methanol.

Function in culture filtrates. At the present time it is not possible to assign any positive function, regarding cellulose decomposition, to the non-cellobiase β-aryl glucosidase found in culture filtrates of M. verrucaria. As far as is known, this is the second report of such an enzyme in culture filtrates of a cellulolytic organism. Jermyn (56, 57, 58) has isolated a similar enzyme from filtrates of Stachybotrys atra, and has studied its properties extensively. However, he did not report whether it would transfer glucose from glucosides to alcohols.

It is possible that this enzyme has other functions in culture filtrates that are currently unknown. Aromatic glucosides are common

constituents of plant material and in its natural habitat, the soil, M. verrucaria would be in contact with these materials in decaying plants. Presumably, there the function of the β-aryl glucosidase would be to liberate glucose from β-aryl glucosides for utilization by the living organism. It is possible that the enzyme is simply a constitutive enzyme that has been retained by the organism, even though it has been cultured for years in the absence of aromatic glucosides, and has no direct connection with the extracellular decomposition of cellulose itself. Further studies are necessary to clarify the role played by this enzyme in culture filtrates of cellulolytic organisms.

It has not been possible to assess the significance of the transferase activity of the enzyme. This property of glucosidases has been known for many years. It was demonstrated for α-glucosidases in 1913 (16) and for β-glucosidases in 1936 (25). Even so, the significance of the reaction in natural processes is very obscure. It is possible that the primary acceptor is water and the ability to transfer to alcohols is pure coincidence.

Transglucosidase

Relation to cellobiase. It has not been possible to separate cellobiase from transglucosidase in the course of this investigation. All the fractions from cellulose chromatography which hydrolyze cellobiose also transfer glucose to form higher saccharides. It appeared possible that they were the same enzyme.

However, Aitken et al (2) have shown that transglucosidase is present in M. verrucaria culture filtrates on occasion when cellobiase is absent. This report indicates that the two are separate enzymes. On the basis of this report, it seemed probable that only transglucosidase was present in Fractions 3, 4, 5, and 6. All of the glucose that was formed could be coming from transfer action and none from hydrolase activity. This possibility is eliminated, however, because in extended hydrolysis periods (Plate 3) glucose is the only product. With shorter incubation periods, higher saccharides were present in Fraction 3 but in time were hydrolyzed to glucose, indicating the presence of hydrolases. Fraction 3, therefore, contains both cellobiase and transglucosidase.

Probable mechanism of transfer. The data from the transfer experiments with 4-O-β-D-glucopyranosyl-D-sorbitol indicate that it is the non-reducing moiety of the molecule that is transferred, and the reducing function of the compound is not absolutely essential for activity. Assuming that the new linkage will be β-1,4 the reaction is:

2 Sorbityl-β-glucoside ——> Sorbityl-β-cellobioside + sorbitol

These data do not exclude the possibility that with cellobiose, the reducing portion of the molecule may be transferred.

Role in culture filtrates. It has not been possible to assign any significance to the presence of transglucosidase in culture filtrates of Myrothecium verrucaria, in relation to cellulose decomposition. It is important to know of their presence, however, inasmuch as they could profoundly alter the products of cellulose decomposition. In any experiments seeking the end products of cellulase action, the presence or absence of transglucosidase must be ascertained.

SUMMARY

The cellulolytic enzymes in Myrothecium verrucaria culture filtrates have been investigated by means of paper electrophoresis and cellulose column chromatography.

Multiple peaks of cellulase were exhibited in paper electrophoresis, indicating that several proteins are contributing to the total cellulase activity of culture filtrates.

The cellulase was partially fractionated on an alkali-swollen cellulose column by elution with buffers of increasing pH and ion strength. Effluent fractions exhibited specificities in regard to substrate chain length, when tested for their ability to hydrolyze carboxymethyl cellulose of two degrees of polymerization, soluble and insoluble cellulose dextrins, cellobiose, and two β-aryl glucosides.

Fractions with cellobiase exhibited transglucosidase activity also. Fractions without cellobiase or transglucosidase accumulate glucose, cellobiose, and higher saccharides when hydrolyzing water insoluble cellulose dextrins. The formation of glucose in the absence of cellobiase indicates that cellobiose is not an obligatory glucose precursor. The formation of higher saccharides in the absence of transglucosidase indicates that these sugars are true intermediate dextrins and not reversion products.

A β-aryl glucosidase, which is not a cellobiase, was separated from cellobiase in culture filtrates and some of its properties were studied.

A transglucosidase was partially separated from cellulase activity. It synthesized, from cellobiose, what appeared to be the homologous series of cellulose dextrins through the hexasaccharide.

All of the data presented in this thesis are consistent with the hypothesis of random cleavage of glucosidic linkages in cellulose by several enzymes having different specificities with regard to chain length.

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APPENDIX

Carbohydrate Analytical Methods

Somogyi iodometric reagent. The Somogyi copper reagent has the following composition (119):

$CuSO_4 \cdot 5H_2O$	8.0	gm
Na_2CO_3 (anhyd.)	30.0	"
Rochelle salt	30.0	"
Na_2SO_4 (anhyd.)	180.0	"
NaOH	1.6	"
KIO_3	0.785	"

The rochelle salt and sodium carbonate were dissolved in previously boiled distilled water, the NaOH was added and the copper sulfate, which was dissolved in a small amount of hot water, was added. The solution, after boiling, was united with a hot solution of the sodium sulfate, and diluted to 975 ml with hot water. The potassium iodate was added to the cooled solution and it was diluted to 1000 ml.

The other reagents necessary for the Somogyi iodometric procedure are:

2.0 N sulfuric acid
1% starch solution
0.1 N sodium thiosulfate solution
0.005 N sodium thiosulfate solution - prepared as needed from the 0.1 N solution
2.5% potassium iodide solution
0.0100 N potassium iodate

For sugar determinations, 5 ml of the copper reagent was mixed with 5 ml of the sample and the tube was held in a boiling water bath for 15 min. The sample was cooled and 1.0 ml of 2.5% KI was added. Two ml of the 2.0 N sulfuric acid was added and the excess iodine was titrated

with 0.005 N thiosulfate. The 0.005 N thiosulfate was standardized against 0.0100 N potassium iodate. The copper reagent was calibrated with known amounts of glucose.

Somogyi colorimetric reagent. This copper reagent (119) was formulated for use with the Nelson arsenomolybdate reagent (84) (below). The composition of the reagent is:

$CuSO_4 \cdot 5H_2O$	4.0 gm
Na_2CO_3 (anhyd.)	24.0 "
$NaHCO_3$	16.0 "
Rochelle salt	12.0 "
Na_2SO_4	180.0 "

The carbonate and tartrate were dissolved in hot water. The copper sulfate was dissolved and the bicarbonate followed. The sodium sulfate which had been dissolved in 500 ml of hot water and boiled was united with the first solution. The solution was cooled and diluted to 1000 ml.

Nelson arsenomolybdate reagent. Ammonium molybdate (25 gm) was dissolved in 450 ml of distilled water and 21 ml of concentrated sulfuric acid was added. Three grams of $Na_2HAsO_4 \cdot 7H_2O$ which had been dissolved in 25 ml of water was added and the solution was held at 37° C for 24 hours. It was stored in a brown bottle.

For sugar determinations, 2 ml of the Somogyi reagent was added to a 2 ml sample and held in a boiling water bath 15 min. The sample was cooled and 2 ml of the Nelson reagent was added. The sample was diluted to 10 ml and the optical density of the solution was determined at 505 millimicrons. The reagent was calibrated with known amounts of

glucose.

Benzidine reagent. The reagent (59) was 0.2% (w/v) benzidine in glacial acetic acid. For sugar determinations, 1 ml of the sample was heated with 4 ml of the reagent in a boiling water bath for 30 min. After cooling to room temperature, the optical densities of the samples were determined in a spectrophotometer at 385 millimicrons and converted to micrograms of glucose from a reference curve established with known amounts of glucose.

Carbohydrate Spray Reagents

Aniline-diphenylamine-phosphoric acid. This spray (17) was composed of 5 volumes of a 4% solution of aniline in ethanol, 5 volumes of a 4% solution of diphenylamine in ethanol and 1 volume of 85% phosphoric acid. The authors recommended that the diphenylamine be freshly prepared before use. It was found that isopropanol could be substituted for the ethanol with equally good results. The papers were developed at 80° C for 10 min.

p-Amino hippuric acid-phthalic acid. This reagent (111), a solution of 300 mg of p-amino hippuric acid and 3.0 gm of phthalic acid in 125 ml of n-butanol, was used in the investigation. In the original procedure ethanol was used as solvent, but butanol permitted the use of either a dipping or spraying technique without dislocating sugars on the paper. The results with both alcohols were identical and the papers were developed at 140° C for 15 min.

Periodate-benzidine. This reagent (22) is specific for the α-glycol structure; consequently, it can be used for any carbohydrate (reducing or non-reducing) that contains this structure. The reagents are a saturated solution of potassium periodate (diluted three-fold before use) and a solution of benzidine (1.86 gm benzidine in 50 ml of 50% ethanol, 10 ml of acetone and 5 ml of 0.2 N HCl).

The paper was sprayed lightly with the periodate and after 6 min. was sprayed with benzidine. The sugars are located by the presence of white spots on a blue background.

Cultural Methods

Composition of stock culture medium. Stock cultures were maintained on agar slants of the following composition:

KH_2PO_4	0.5 gm
$MgSO_4$	0.2 "
$NaNO_3$	2.0 "
Agar	17.0 "
Distilled water	1.0 liter

After the slants had hardened, a strip of sterile filter paper was placed aseptically on the agar surface. Prior to their sterilization, the paper strips were moistened with 0.01 N NaOH. The cultures were transferred monthly and stored at room temperature.

Composition of mass culture medium. The medium used for the production of active culture filtrates was essentially that of Saunders, Siu, and Geneset (112). It had the following composition:

KH_2PO_4	0.20 gm
K_2HPO_4	0.15 "

$NaH_2PO_4 \cdot H_2O$.....2.00 gm
Na_2HPO_4.....1.50 "
NH_4NO_3.....0.60 "
$NaNO_3$.....3.80 "
$MgSO_4 \cdot 7H_2O$.....0.30 "
yeast extract (Difco)....0.075"
cellulose (Solka-floc)...4.00 "
distilled water.....to 1.0 liter

The yeast extract replaced the minute quantities of trace elements present in the original medium.

Determination of Enzyme Units

Cellulase units. An active culture filtrate was arbitrarily taken as a reference standard and the activities of the samples were related to this one. It was found experimentally that the Enzyme-times-Time relationship holds completely for cellulase. For example, 1 ml of enzyme produced 478 micrograms of reducing sugar in 24 min. and 0.2 ml produced 475 micrograms of reducing sugar in 120 min. In both cases, Enzyme-times-Time is a constant, that is, 1 x 24 and 0.2 x 120 are both equal to 24. Since this relationship is valid, a universal type of curve was prepared for the determination of cellulase units.

Graded amounts of the reference filtrate (0.1 to 0.8 ml) were added to 8 test tubes. The correct volume of water was added to each to make the total volume 1.0 ml. To each tube was added 1.0 ml of 0.1% CMC ("70 premium low") in 0.2 M acetate buffer, pH 5.0. The samples were incubated at 50° C for 5 min. The reaction was stopped by adding the Somogyi copper reagent and the amount of reducing sugars was determined by the Nelson-Somogyi method.

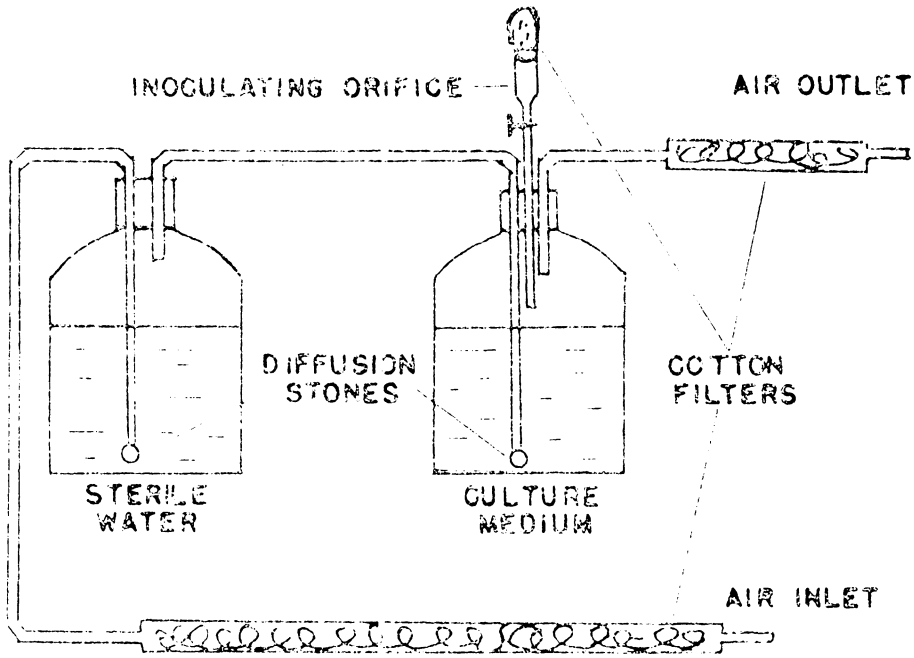

FIGURE 12

DIAGRAM OF MASS CULTURE APPARATUS

The amount of reducing sugar (as glucose) that was liberated was then plotted on the ordinate and the ml of enzyme on the abscissa (Figure 13). The reference filtrate was arbitrarily defined as containing 20 units/0.1 ml. The abscissa was then re-plotted in terms of Enzyme-times-Time values (5 x 20, 5 x 40, etc.). Since the Enzyme-times-Time relationship is valid, this curve can be used for any concentration of enzyme for any length of incubation. Assume that an unknown sample of enzyme, under the standard conditions, produced 55 micrograms of glucose in 40 min. Reading on the ordinate (Figure 13), 55 micrograms of glucose is equivalent to an ET value of 200. Therefore, Enzyme x Time equals 200 or Enzyme x 40 equals 200. The sample contained 200/40 or 5 units. This type of calculation was used for all the cellulase units.

<u>β-aryl glucosidase units</u>. The conversion of the micrograms of 6-bromo-2-naphthol to β-aryl glucosidase units was much simpler than in the case of cellulase units. Over the entire range of enzyme concentration tested under the conditions specified, the amount of 6-bromo-2-naphthol liberated was directly proportional to enzyme concentration (Figure 14). A β-aryl glucosidase unit was defined as that amount of enzyme which liberated 20 micrograms of 6-bromo-2-naphthol in one hour under the conditions specified (4 ml of 100 micrograms/ml substrate, pH 5.0, and 37° C temperature). Since the enzyme concentration is directly proportional to product formation, a reference curve is not necessary for the estimation of β-aryl glucosidase units. Assume that

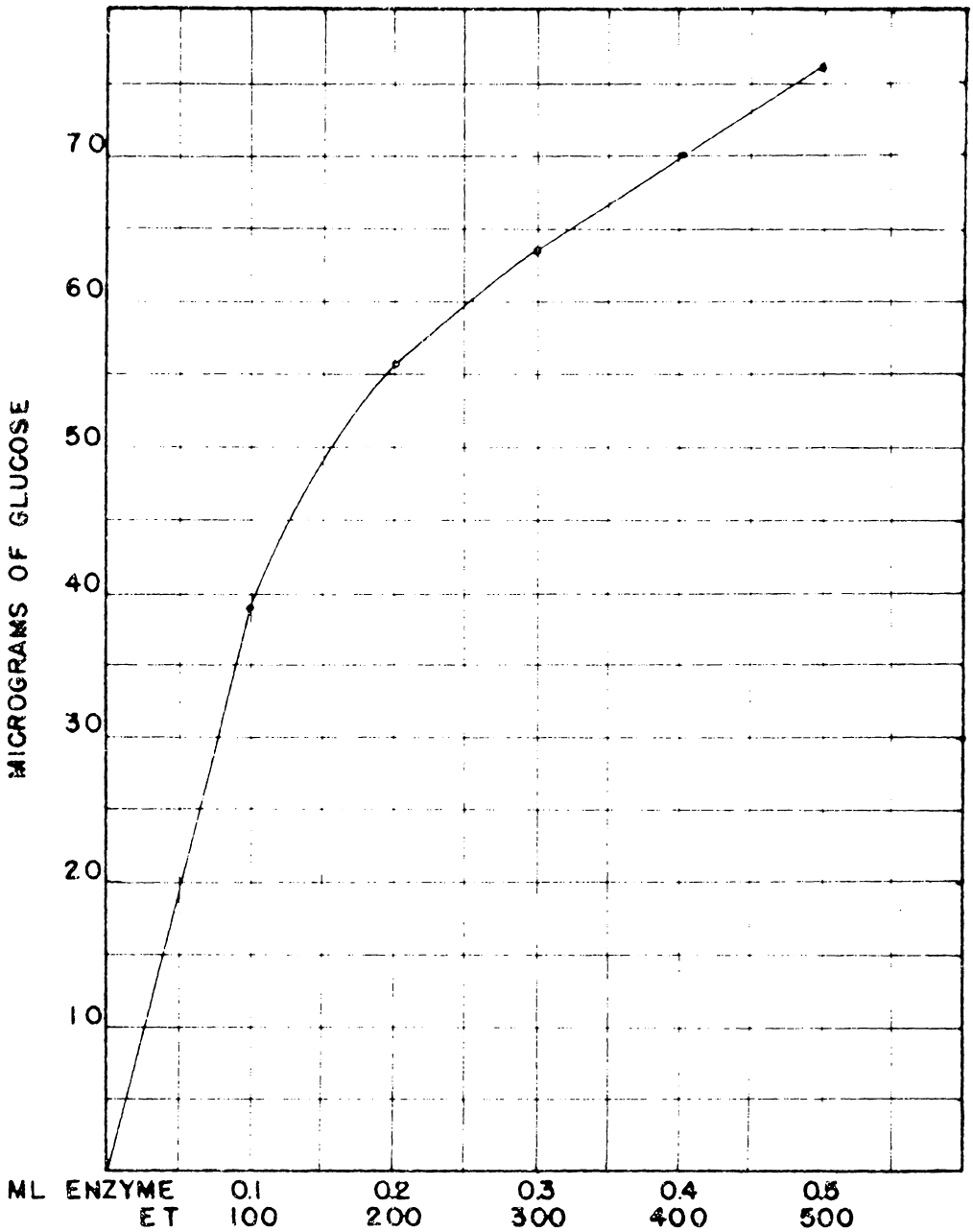

FIGURE 13

REFERENCE CURVE USED FOR
ESTIMATION OF CELLULASE UNITS

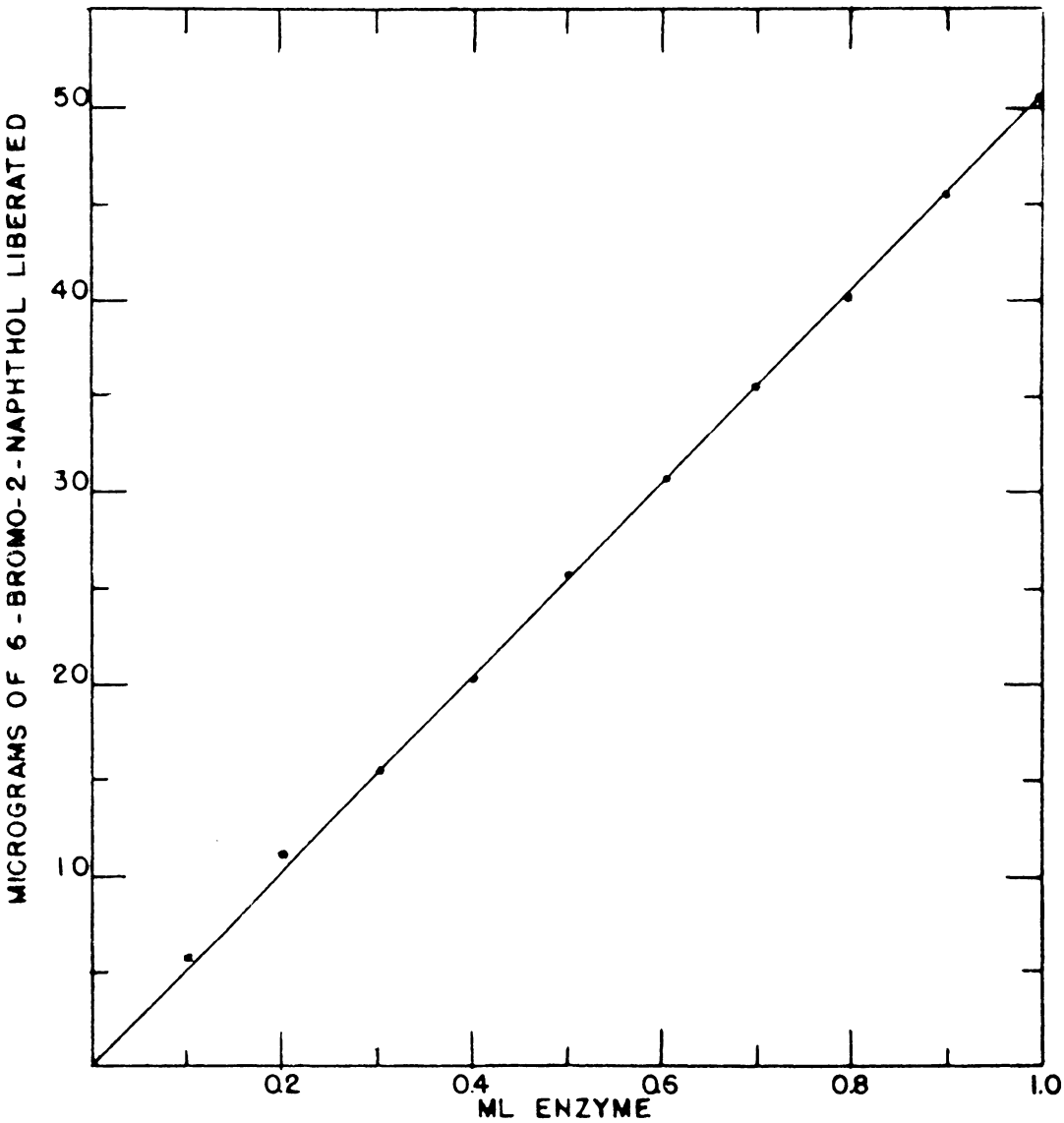

FIGURE 14

RELATION BETWEEN PRODUCT FORMATION
AND β-ARYL GLUCOSIDASE CONCENTRATION

an unknown solution of β-aryl glucosidase liberated 40 micrograms of 6-bromo-2-naphthol in 120 min.; then, the sample contained 60/120 x 40/20 or 1 unit.

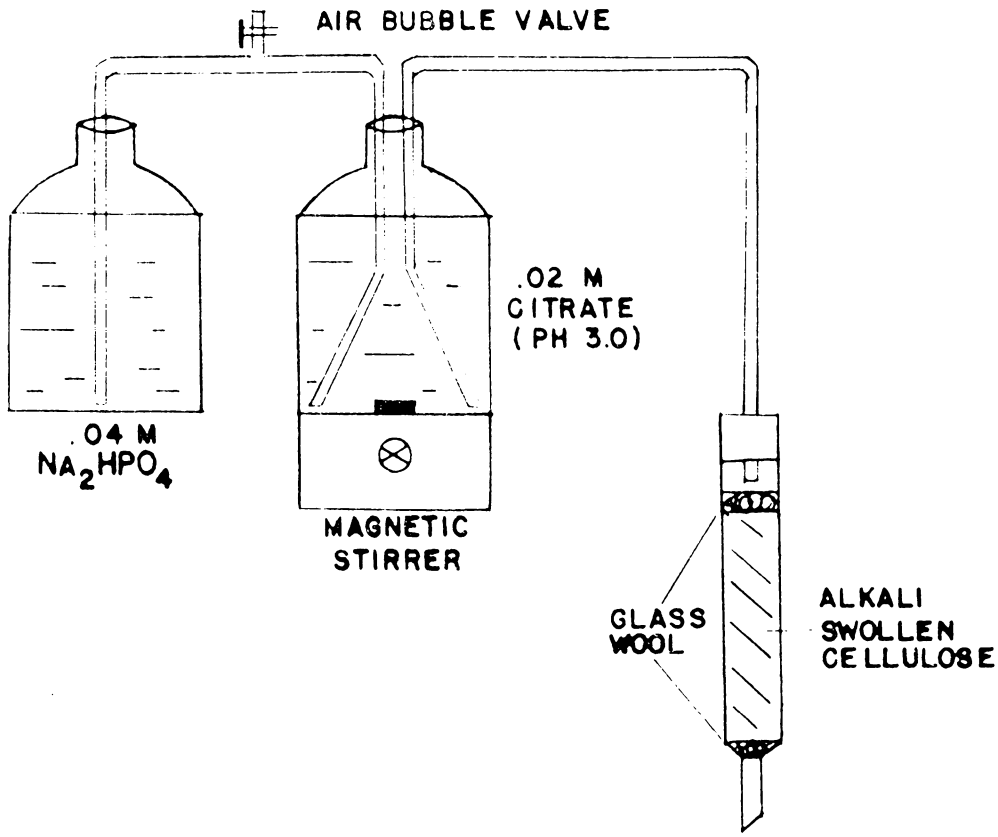

FIGURE 15

DIAGRAM OF GRADIENT ELUTION DEVICE

ABSTRACT OF THE DISSERTATION

CELLULOLYTIC ENZYME SYSTEMS OF *MYROTHECIUM VERRUCARIA*

by

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in

Biology

Major in Biochemistry

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Blacksburg, Virginia

CELLULOLYTIC ENZYMES OF MYROTHECIUM VERRUCARIA

Abstract

Crude culture filtrates of Myrothecium verrucaria were investigated in an attempt to gain information on the following: (A) the multiplicity of the cellulolytic system, (B) the formation of glucose in the course of cellulose hydrolysis, (C) the significance of intermediate dextrins in the course of cellulose hydrolysis, (D) the properties of a β-aryl-glucosidase present in filtrates of this organism, and (E) a transglucosidase which synthesized higher saccharides from cellobiose.

In paper electrophoresis at pH 8.55 in veronal buffer of ionic strength 0.05 and a potential gradient of 10 volts/cm, concentrated culture filtrates exhibited multiple components with cellulase activity. The electrophoretic pattern for each filtrate could be reproduced; however, different filtrates gave different proportions of the components. The presence of electrophoretically distinct components with cellulase activity indicated the multiplicity of the cellulolytic system.

At pH 7.0 and lower there was no separation of the cellulase in the filtrates into the components shown at 8.55.

The β-aryl-glucosidase also exhibited several peaks on paper electrophoresis, indicating that it, too, was dependent on several proteins for its activity. With the exception of a peak of activity that remained at the origin, the mobile peaks of cellulase and β-aryl-glucosidase did not coincide, indicating that the two separate activities are

not dependent on the same proteins for their respective activities. The activities remaining at the origin were also dependent on different proteins because the β-aryl-glucosidase was destroyed by heat whereas the cellulase was not.

The concentrated culture filtrates were subjected to column chromatography on alkali-swollen cellulose. The enzymes were eluted with phosphate-citrate buffers of increasing pH and ion strength. Effluent fractions were collected and assayed for their ability to hydrolyze carboxymethyl cellulose of two degrees of polymerization, insoluble cellulose dextrins, soluble cellulose dextrins, cellobiose, 6-bromo-2-naphthol-β-D-glucopyranoside, and 4-O-β-D-glucopyranosyl-D-sorbitol.

The results not only indicated a multienzymatic nature of cellulase but also that the components of the cellulase system were specific for a particular range of chain lengths of cellulose. Some of the fractions hydrolyzed carboxymethyl cellulose (DP 200) but did not hydrolyze the insoluble dextrins, the soluble dextrins or cellobiose; some hydrolyzed both the carboxymethyl cellulose and the insoluble dextrins but did not hydrolyze the soluble dextrins or cellobiose; and some hydrolyzed the carboxymethyl cellulose, the insoluble and soluble dextrins but not cellobiose. There were a few fractions which hydrolyzed the carboxymethyl cellulose, soluble and insoluble dextrins and cellobiose. Cellobiase was confined to a relatively few fractions and all the fractions with cellobiase activity also exhibited transglucosidase

activity.

Cellulase fractions which were void of cellobiase and transglucosidase activity accumulated glucose, cellobiose, and higher saccharides when hydrolyzing insoluble cellulose dextrins. The formation of glucose in the absence of cellobiase indicated that the cellulase is capable of removing single glucose units from the end of the chain and cellobiose is not an obligatory glucose precursor. The formation of higher saccharides in the absence of transglucosidase indicated that these sugars were true intermediate dextrins, arising as the result of random cleavage of the cellulose molecule, and were not the synthetic products of a transglucosidase.

A β-aryl-glucosidase, which was not a cellobiase, chromatographed off the cellulose column. Some of the properties of this enzyme were studied. It was found to hydrolyze several β-aryl-glucosides with varying ease. In the presence of various alcohols it transferred the glucose moiety of the glucoside to the alcohol. With methanol the product was methyl glucoside, but it was not possible to establish the configuration of the linkage that was formed.

The transglucosidase synthesized, from cellobiose, several higher saccharides which appeared to be the homologous series of cellulose dextrins through the hexasaccharide.