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Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis

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Advances in Chemistry Series

M. Joan Comstock, *Series Editor*

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FOREWORD

ADVANCES IN CHEMISTRY SERIES was founded in 1949 by the American Chemical Society as an outlet for symposia and collections of data in special areas of topical interest that could not be accommodated in the Society's journals. It provides a medium for symposia that would otherwise be fragmented, their papers distributed among several journals or not published at all. Papers are reviewed critically according to ACS editorial standards and receive the careful attention and processing characteristic of ACS publications. Volumes in the ADVANCES IN CHEMISTRY SERIES maintain the integrity of the symposia on which they are based; however, verbatim reproductions of previously published papers are not accepted. Papers may include reports of research as well as reviews since symposia may embrace both types of presentation.

PREFACE

In the decade that has passed since the appearance of the last volume of the Advances in Chemistry Series (Vol. 95) that dealt with the hydrolysis of cellulose, some of the laboratory research described then has advanced to the stage of pilot plant operations and beginning commercial applications. Perhaps even more importantly, the chemical and biochemical techniques applied in this area have become more sophisticated and mechanisms for individual chemical and physical changes of cellulose have been described. The greatest impetus to research in this area is the growing realization that cellulose, both abundant and renewable, may soon be required to serve as the basis for chemical, energy, and food products traditionally based on petroleum or conventional agriculture products. In contrast to the prospects of 10 years ago, there is now intense interest in cellulose as part of biomass, as a pollutant, and as a byproduct of industrial, municipal, and agricultural activities.

The commercial interest in cellulose hydrolysis has created a demand for new approaches leading to improved control and better efficiency of this catalytic process. It has been realized by many workers in the field that deeper insight into the mechanisms of this seemingly simple catalytic event might prove particularly inspiring.

This symposium was designed to focus attention on the state of knowledge regarding the hydrolysis of cellulose. Participants were drawn from among the community of chemists, biochemists, biologists, and engineers. Perhaps it is the advent of serious engineering approaches that most clearly heralds the arrival of new pathways to organic materials from cellulose. The organizers are indebted to the Institute of Paper Chemistry, to the Wood Chemistry Committee of TAPPI, and to Roy Whitney and the American Chemical Society Cellulose, Paper and Textile Division for sponsoring the symposium.

As is so frequently the case, new methods of research bring new workers into the field. It is noteworthy that, of the authors represented in the previous volume, only two participated in the current symposium, one of whom is represented by a chapter in the present volume. It is expected that an even broader array of disciplines will become engaged in this area—certainly molecular biologists, physicists, and economists are already clarifying many points.

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It is our hope that the information contained in this volume will stimulate still another generation of scientists and engineers to realize the potential benefit to mankind of this abundant and ubiquitous resource.

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January 1979

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Perspectives on Preparation of Cellulose for Hydrolysis

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Destruction of fiber and achievement of large surface areas are goals in the preparation of cellulose for hydrolysis to fermentable sugars. Pretreatments and cellulosic raw materials that are appropriate differ from those employed in fiber-conserving pulping systems. Alternative pretreatments to disrupt the lignin–hemicellulose–cellulose complex, remove extractives, and generate large surface areas are discussed. The Purdue processes yield amorphous cellulose by treatment of the raw material with Cadoxen, ferric tartrate, or sulfuric acid as solvents. The Iotech process employs explosive depressurization to remove lignin and to increase surface area while maintaining cellulose crystallinity. Plants with low lignin and extractives content and cell morphology that facilitates downstream processing are preferred in systems designed to optimize the production of fermentable sugars.

Economical hydrolysis of cellulose to obtain fermentable sugars for the manufacture of chemicals and fuels is a major goal of research and development on renewable resources. Enzymatic hydrolysis of lignocellulosic materials proceeds slowly because of steric problems. Acid hydrolysis of lignocellulosic materials leads to loss in yield through degradation. Pretreatment of lignocellulose to remove interfering constituents is a strategy that has been explored repeatedly as a means to alleviate cellulose hydrolysis problems.

Several definitive reviews on pretreatment of lignocellulosic materials for improving cellulose hydrolysis (*1, 2, 3*) appeared a few years ago. More recently, two pretreatment methods (the Purdue process and the Iotech process) have been announced that claim superior perform-

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ance in preparing cellulosic materials for hydrolysis. These processes are somewhat controversial because they resemble well-established analytical techniques and well-known alternatives to xanthation for solubilizing cellulose (*4, 5, 6*). This chapter provides a framework for generating and evaluating pretreatment concepts. The Purdue and Iotech processes can be put into perspective by using this framework.

This monograph is focused primarily on hydrolysis of cellulose. However, the choice of technology for cellulose hydrolysis depends both on the state of the cellulose when it reaches the hydrolysis process and on the fermentation (or other) technology to be applied to the output of the cellulose hydrolysis process. Dr. Humphrey's chapter treats the downstream fermentation technology; this chapter is concerned primarily with preparation of cellulose for hydrolysis.

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Chemicals and Fuels System

An overview of cellulose-based chemicals and fuels systems is presented in Figure 1. In this cyclic system, demand for fuels and chemicals leads to the organization of factors of production (land, labor, capital, etc.) for the generation of biomass for use as a renewable resource. The "plant culture" activity is called "silviculture" if trees are the desired biomass or "agriculture" if herbaceous plants are grown

Numerous materials-handling activities are required to move the biomass in the field to the factory. These activities include harvesting, storage, transportation, and, possibly, preservation activities.

Typically, the raw material for cellulose processing is a lignin–hemicellulose–cellulose (LHC) complex that is not very amenable to hydrolysis. Various pretreatments involving size reduction, separation of constituents of the complex, and processes to increase the accessibility of cellulose to hydrolytic agents may be required. These activities convert a relatively intractable raw material into a cellulosic substrate.

The cellulosic substrate is depolymerized by water (or possibly other agents) so that simple sugars can be obtained. If the pretreatments totally separate cellulose from hemicellulose, the simple sugar would be glucose. Otherwise, the glucose will be mixed with other carbohydrates that may interfere with the fermentation.

Application of microbiological technology to the simple sugars converts them into chemicals and fuels, which can be further transformed and formulated into such products as liquid motor fuels, plastic containers, and tires. These end-use products, when distributed, constitute the supply that consumers use to satisfy demands. Thus the cycle is complete.

The overall system has an impact on cellulose hydrolysis primarily through determining which cellulosic raw materials are available and the

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prices associated with them. That is, the system determines availability, quality, and cost. For example (Figure 1), part of the consumed cellulose becomes a biomass resource through recycling. To the extent that hemicellulose is removed in pulping processes, the system simplifies the biomass resource for hydrolytic processing. Conversely, when paper is coated with olefin polymers, the biomass resource is rendered less suitable for hydrolysis. The cost of the biomass resource depends on its alternative uses, scale of production, and location.

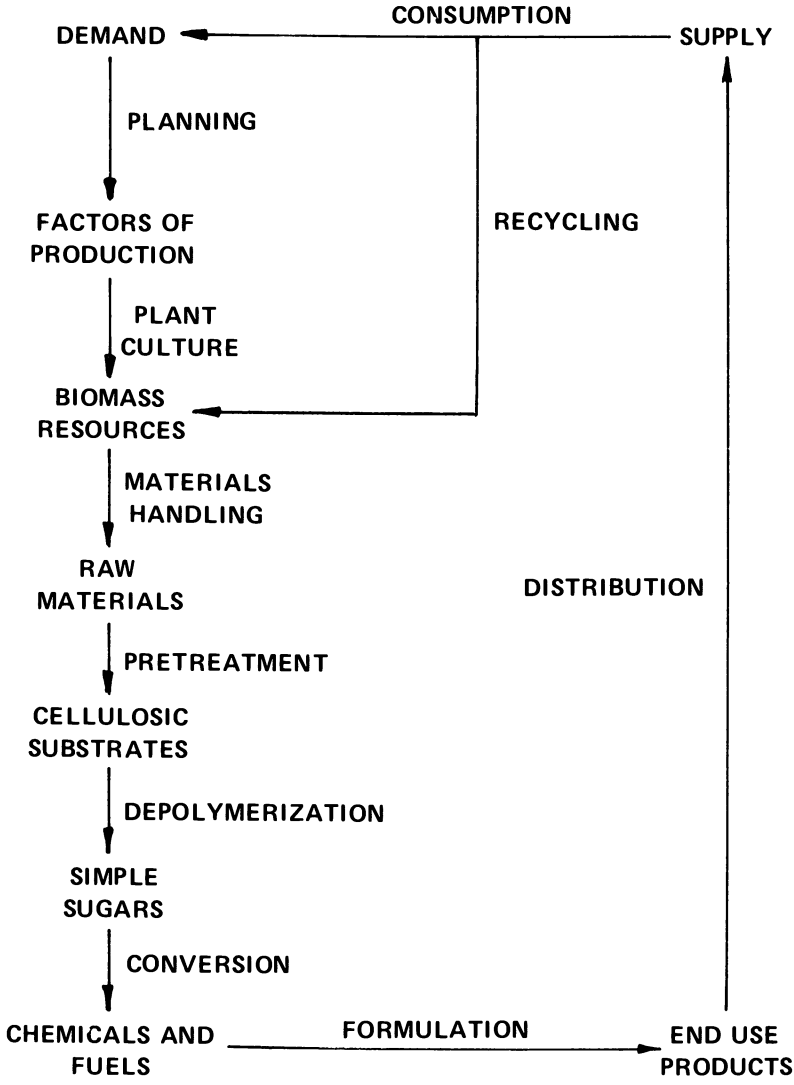

Figure 1. Overview of cellulose-based chemicals and fuels systems

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Composition

The raw material that reaches the factory is a far cry from Avicel or Solka Floc that are used by many experimenters to simulate cellulosic products (Table I). In addition to the fact that the cellulose is tied up in a lignin–hemicellulose–cellulose (LHC) complex, it should be recognized that the terms "lignin' and "hemicellulose" are catchall terms covering a range of compositions and molecular weights.

Table I. Comparison of Selected Biomass Species with Avicel and Solka-Floc (Percentage)[a]

Raw Materials	*Cellulose*	*Hemi-cellulose*	*Lignin*	*Extrac-tives*	*Ash*
Birch Wood	40	36	21	3	< 1
Pine Wood	41	27	27	4	< 1
Bagasse	40	32	20	6	2
Wheat Straw	30	50	13	4	2
Avicel	100	—	—	—	—
Solka Floc	99.5	—	—	—	0.3–0.4

[a] Source: Adapted from Ref. *35*.

In addition to the variations in the LHC composition that occur from species to species, each species has its extractives, which include resins and waxes. These constituents are capable of interfering with cellulose hydrolysis because of their hydrophobic nature. Tannins and other highly reactive materials are constituents of some woody species. When LHC is obtained from nonwoody (herbaceous) species, the range of interfering constituents increases greatly. Sugars, starches, dextran, carotenoids, and many isoprenoids are to be found. Operators of a cellulose hydrolysis process that uses municipal solid waste as its biomass resource may experience seasonal variations in composition and chance inclusion of crankcase oil and other products that inhibit enzymes or kill yeast.

These compositional considerations necessitate attention to raw materials evaluation early in process research and development to (1) establish an in-depth knowledge of the principal raw material, including its composition and history of materials handling prior to arrival at the factory, (2) investigate the effects of seasonal variations and storage under various conditions on composition and on hydrolytic performance, and (3) explore alternative pretreatment processes for upgrading the composition and hydrolytic performance of available raw materials. The resource that is for sale is not pure cellulose; it is a complex mixture containing cellulose: caveat emptor.

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Taxonomic Overview

In general, the route from biomass to glucose follows the pattern shown in Figure 2. The raw materials consist of plant cells that need to be ruptured prior to processing by enzymes or acids. Depending on the pretreatment process selected, interfering constituents of the plant cells may or may not be removed. The LHC complex may be separated into its constituents (lignin, hemicellulose, and cellulose) to a greater or lesser degree. Finally, cellulose fibers are dissociated into cellulose molecules for attack by hydrolytic agents. The hydrolysis proceeds through cello-

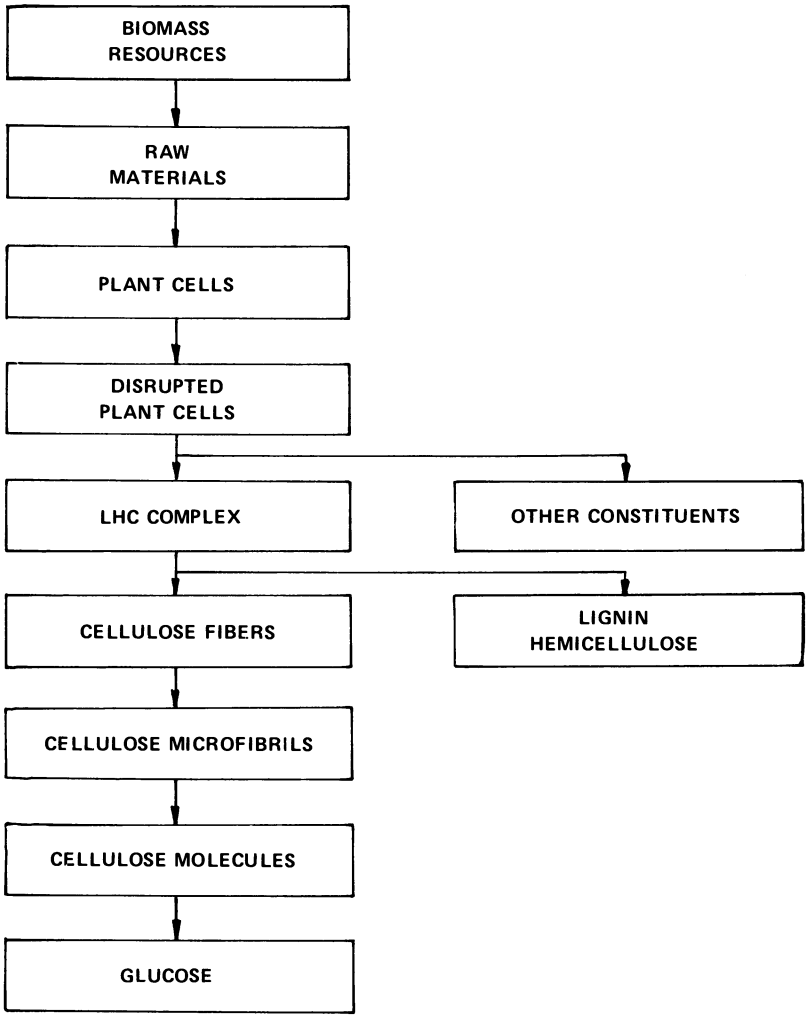

Figure 2. Some steps in conversion of plant constituents into glucose

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dextrins on the way to cellobiose and finally to glucose. The scope of this chapter extends from the biomass raw materials to the cellulose molecules that are ready for depolymerization.

Pretreatment science and technology needs a methodological framework for exposition, evaluation, and planning. The balance of this chapter is devoted to an initial attempt to construct such a framework. The reader is challenged to formulate alternative frameworks and/or expand on the one presented here.

The methodology that was selected for this taxonomy of pretreatment processes is the objectives-tree method developed by Swager (*7*) Lipinsky (*8*), Janstch (*9*), and others (*10*) for presentation of alternatives in a systematic structure.

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Strategic Factors

The overall objective of pretreatment processes can be considered to be "to increase the rate and extent of cellulose hydrolysis." As shown in Figure 3, there are three major approaches to achieve this goal. The one that pertains most to pretreatment processes is "increase accessibility to penetrating agents." However, the other two approaches need to be kept in mind. Improvement of the effectiveness of the hydrolytic agents can simplify the pretreatment process and place less stringent requirements on pretreatment. However, improving the effectiveness of the hydrolytic agent is outside the scope of this chapter.

The approach "select favorable raw material" has a major impact on the selection of pretreatment processes. For example, the poplar responds splendidly to many pretreatments that fail with Douglas fir or pine-based materials (*1*). Specific tissues and cells of a given biomass raw material will respond quite differently. For example, the rind fiber of sugarcane bagasse behaves quite differently from the pith fiber (*11*). In woody species, the selection of tissues low in bark and extractives is an important factor in the ease or resistance to cellulose hydrolysis. Before embarking on development of processes for hydrolysis of a biomass resource, it is highly desirable to exercise discretion with respect to the choice of raw materials at both the species and tissue levels. This idea is all the more important in an initial choice of species and pretreatment process.

In increasing the accessibility to penetrating agents, the selection and breeding of varieties that are better suited to cellulose hydrolysis is an avenue not frequently explored, because improvements in accessibility are generally considered to be in the realm of the chemical engineer. Especially with trees, breeding and selection has been done to improve durability of fiber (*12*). Thus development of successful cellulose hydrolysis, which depends on destruction of fiber, would benefit from specialized breeding and selection to produce less durable fibers.

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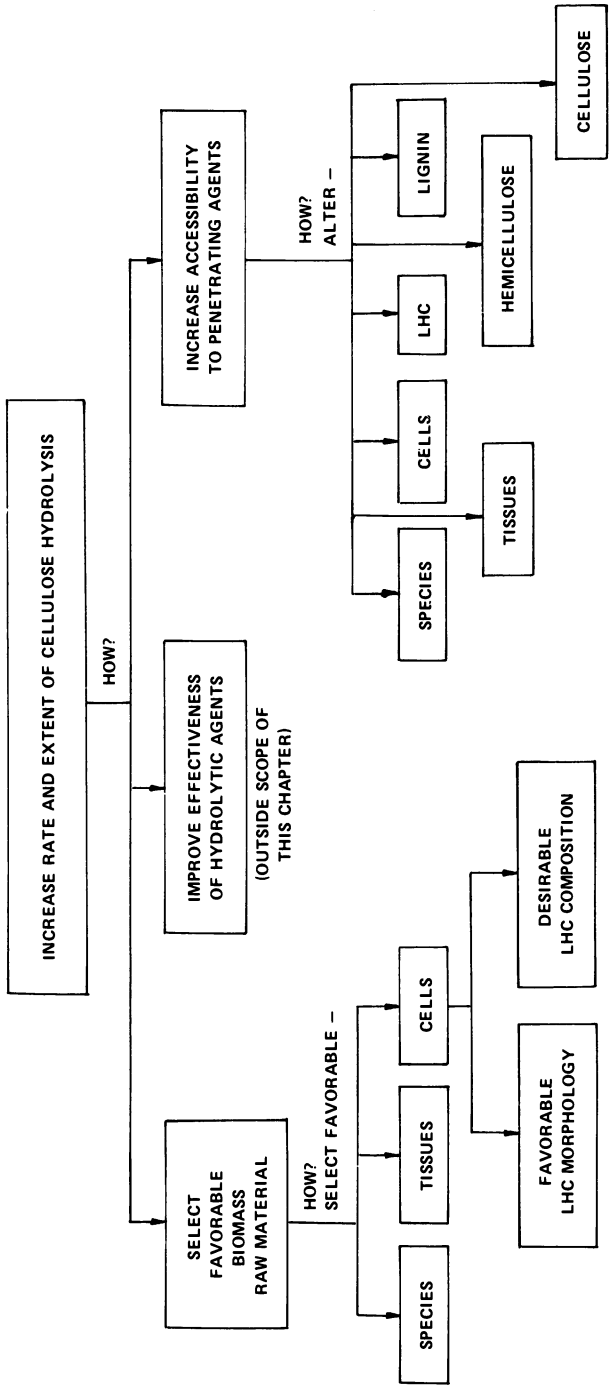

Figure 3. Strategic factors in conception of processes for increasing the rate and extent of cellulose hydrolysis

Processes need to be developed to expose LHC for effective penetration of chemicals that promote separation of LHC or which render cellulose accessible by swelling. These processes can have many objectives: (1) to get at cellulose; (2) to remove hemicellulose from the reaction site before cellulose hydrolysis; (3) to remove lignin from the site; and (4) to bring penetrating or hydrolysis agents into effective contact with cellulose.

Processing Plant Cells

The intact plant cell is a structure that can be manipulated to render LHC vulnerable to attack by acids or enzymes. Especially as energy costs rise, alternative means to process cells should be sought to replace the routine grinding operations that are standard operating procedure. Alternatives in processing plant cells to increase accessibility are arrayed in Figure 4. The main line of development is cell rupture, using ball milling (*13*), two-roll milling (*14*), or other means of size reduction. Location of a cellulose processing unit at the site of a liquid air or liquified natural gas terminal could make available considerable freezing capability for cryogenic grinding options (*15*).

Alternatives to grinding include explosive depressurization, which is involved in the Iotech (*5*) and Masonite (*16*) processes, ultrasonics (*17*), osmotic cell rupture, and conventional chemical pulping techniques. Explosive depressurization appears especially promising because of its effectiveness and relatively low energy consumption.

Cell rupture may not be necessary to achieve effective pretreatment. The effectiveness of irradiation (*1*) with electrons or gamma rays may be due in part to riddling the cells with holes. Depolymerization of the cell membrane to expose the contents is another noncell–rupture approach.

While the cell is intact, it may be desirable to operate on cell constituents that might interfere with hydrolysis. Two approaches to this are solvent pretreatment of the cells and chemical transformation of constituents in the cells.

Processing LHC

Once the protection that the cell structure has provided to LHC has been stripped away, direct contact of chemical reagents with cellulose becomes possible. However, the LHC complex consists of interpenetrating polymers that are highly organized to resist this chemical attack (*2*). The protective agents include the crystallinity of the cellulose itself, the steric effects of lignin and hemicellulose, and the hydrophobic effects of lignin. There may also be protective resins or simple sugars adhering to the LHC that can interfere with hydrolysis.

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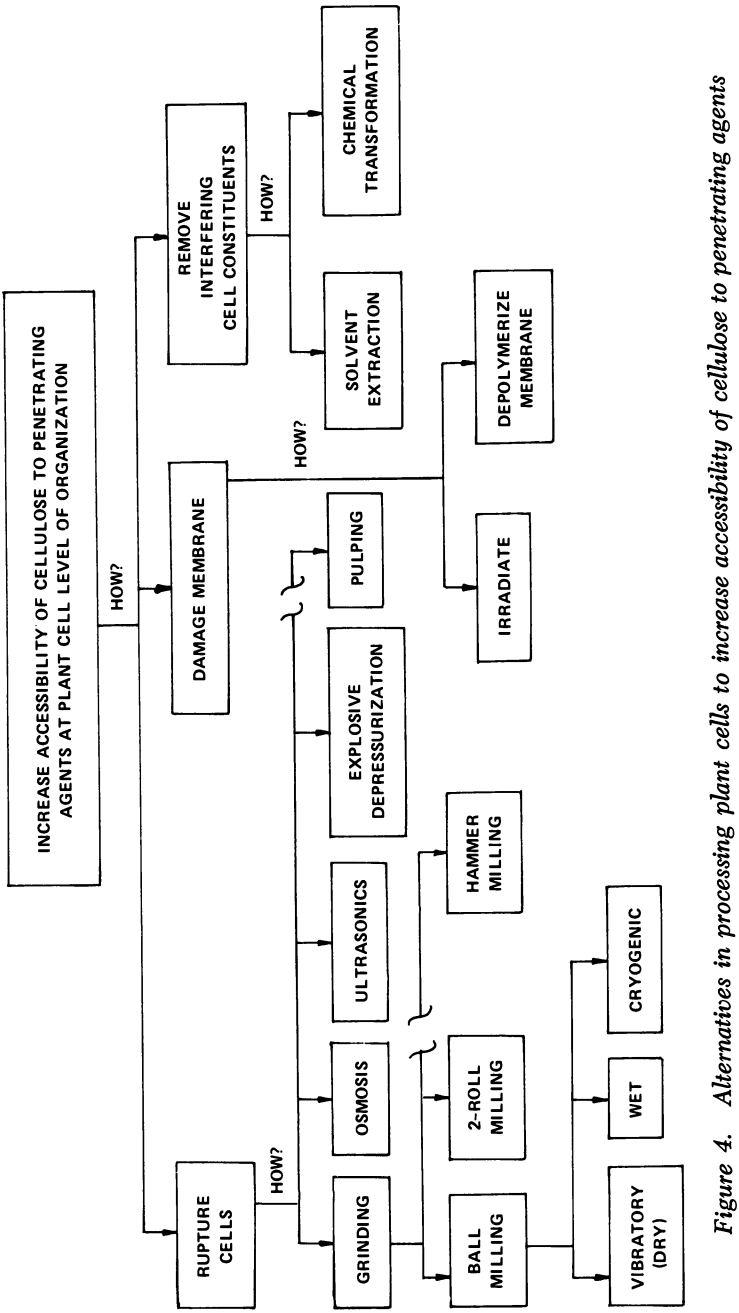

Figure 4. Alternatives in processing plant cells to increase accessibility of cellulose to penetrating agents

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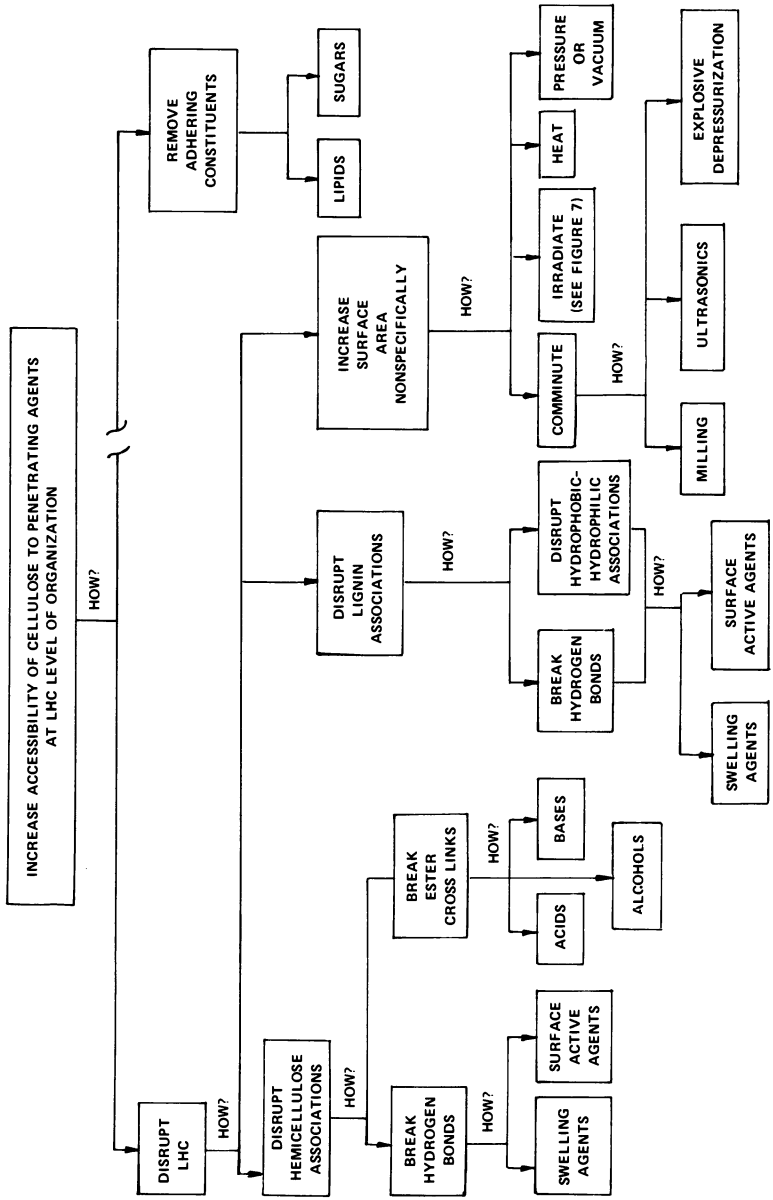

Figure 5. Alternatives in processing LHC to increase accessibility of cellulose to penetrating agents

Alternatives in processing the LHC complex to increase accessibility are displayed in Figure 5. Using this taxonomic description, the reader is invited to supply additional alternatives and or greater detail concerning those presented in the chart.

Removal of non-LHC constituents that are shielding the complex from the hydrolytic agent generally would not be needed if acid hydrolysis is contemplated. However, this factor becomes important with enzymes and especially with microorganisms when highly resinous wood is the biomass resource. For example, Thayer observed surprisingly little microbial digestion of mesquite following a grinding and alkali treatment that is successful with less resinous woods (*18*).

Disruption of the LHC complex can be undertaken relatively nonspecifically by increasing the surface area. The surface area increase can be achieved with methods that are the same as those employed to rupture the cell. Usually, cell rupture is considered only a prelude to achieving enough surface area to disrupt the complex. Increasing the surface area by size reduction usually is combined with some form of chemical attack to disengage any cellulose from LHC or to disengage lignin from LHC. The primary strategies in the removal of hemicellulose from the site of reaction are breaking the hydrogen bonds between hemicellulose and cellulose and breaking ester cross-linking between hemicellulose and lignin. The major lignin strategies are to break hydrogen bonds with the other two constituents and to eliminate the hydrophobic coating effect that lignin has in shielding cellulose.

Swelling agents (e.g., water, ammonia, amines, and alkalis) are the standard means for breaking hydrogen bonds. Ester cross-links generally are broken by chemical reactions involving acids (*1*) or bases (*19*). Very little work has been done on esterification with simple alcohols as a means of breaking ester cross-linking.

The breaking of lignin associations may require the use of solvents with a different solubility parameter from those used as swelling agents for the carbohydrates.

Because LHC is a complex of interpenetrating polymers, hydrogen bonds and ester cross-links can reestablish themselves unless further processing occurs promptly. Therefore, in practice, this disruption of the LHC phase may not be distinguishable from the subsequent stages.

Processing Hemicellulose

Hemicellulose is so vulnerable to acid attack that it does not constitute an accessibility retarding agent for acid hydrolysis. However, it appears that hemicellulose does protect cellulose from enzyme attacks by a shielding action.

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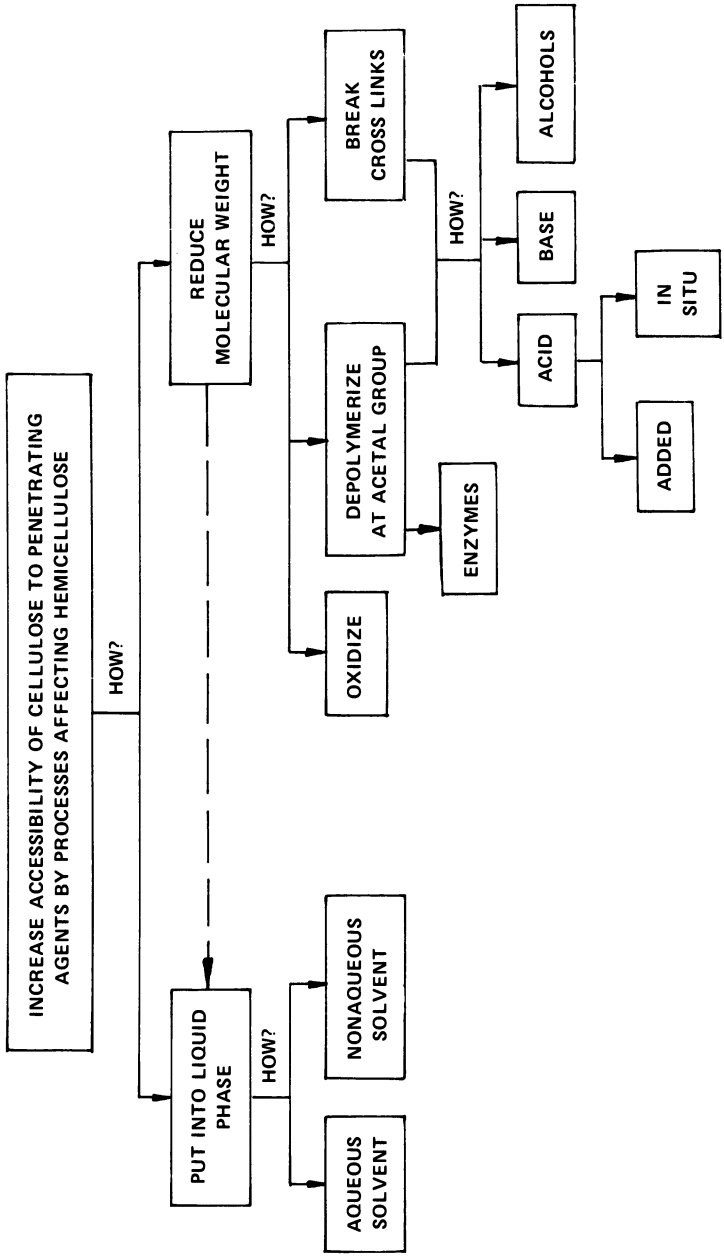

Figure 6. Alternatives in processing hemicellulose to increase accessibility of cellulose to penetrating agents

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The two principal methods for processing hemicellulose to increase the accessibility of cellulose to penetrating agents are to put the hemicellulose into a liquid phase and/or reduce its molecular weight (Figure 6). Such aqueous solvents as alkali solutions solubilize hemicellulose by sodium salt formation and by breaking ester cross-links. Either true solutions or colloidal dispersion may be the mechanism by which the attack the lignin that blocks accessibility to cellulose.

Molecular weight reduction probably plays a role even in pretreatment processes that are not intended to cause this effect (*19*). The most common method of molecular weight reduction is the addition of dilute acid to hydrolyze the acetal groups (*1*). The acid may be generated in situ by the liberation of acetic acid from acetate groups by steam or hot water.

Alternatively, hydrochloric acid or sulfuric acid may be employed. The use of gaseous hydrochloric acid leads to oligomers that cause less of a recovery problem (*20*).

Other methods of molecular weight reduction include pentosanase treatment or formation of low-molecular-weight acetals. Oxidative cleavage of hemicellulose also could lead to reduced molecular weight.

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Processing Lignin

Strategically placed lignin blocks the approach of large molecules, thus protecting cellulose from enzymic attack. That only a fraction of the lignin is strategically placed is demonstrated by the observation that the first 40% of the lignin can be removed from Douglas fir without increasing the enzyme digestibility at all (*1*). Therefore, the problem confronting those who are developing new lignin removal processes is to attack the lignin that blocks accessibility to cellulose.

As shown in Figure 7, there are two strategies in dealing with lignin—put it into another phase, or change its molecular weight. These strategies are the practical expression of the underlying need to strip the lignin coating from the surface of the cellulose microfibrils.

Phase Change. The use of aqueous or nonaqueous solvents to bring native lignin into solution is an appealing approach. However, it requires an extremely effective LHC disruption (Figure 6). Normal grinding will not achieve this degree of disruption but the Iotech process (*5*) or SO_2 treatment do appear to succeed (*1, 21*). Further studies would be required to determine whether it is unchanged lignin that is so readily soluble or whether molecular weight reduction has taken place.

Dispersal of lignin as a colloid is a close relative of true solution as a means of lignin removal. Dispersing agents include alkalis. Effective dispersion may involve molecular weight reduction as a necessary condition.

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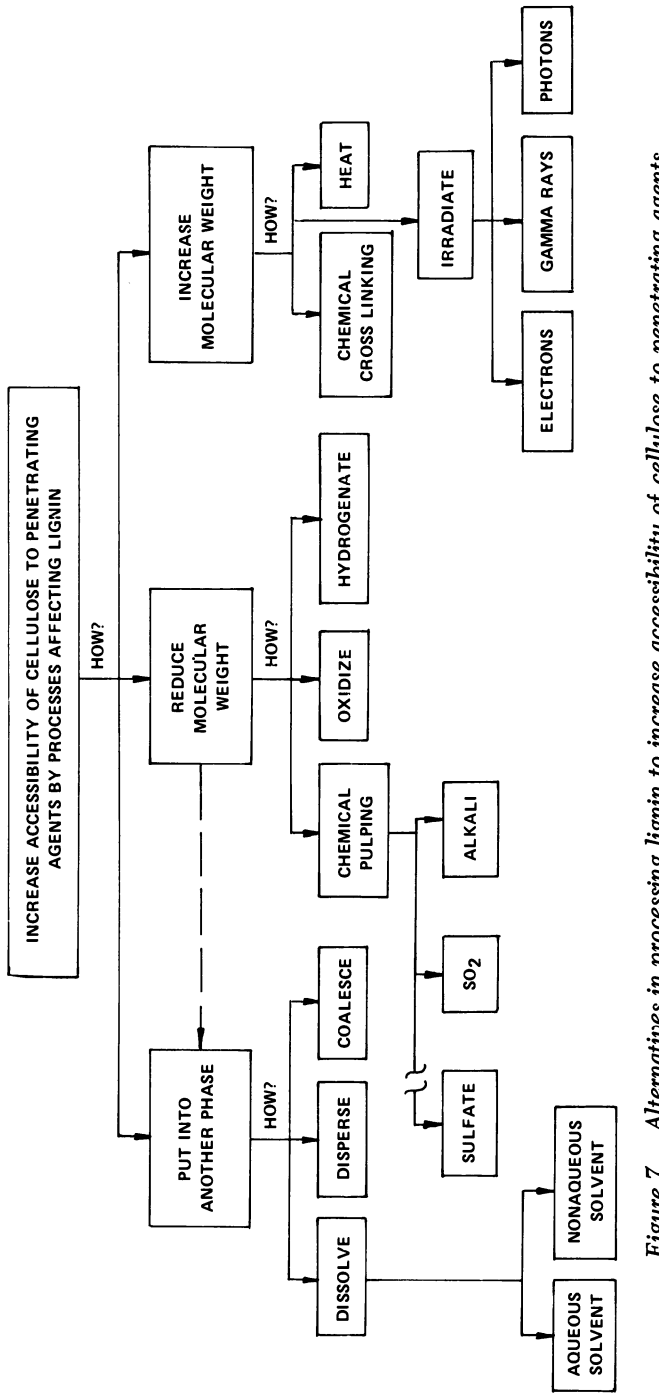

Figure 7. Alternatives in processing lignin to increase accessibility of cellulose to penetrating agents

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It is not necessary for the separate phase to be a solution. There may be phase separation in which the lignin forms a bulk phase of its own by coalescence. Explosive depressurization appears first to coalesce the lignin into separate droplets that are readily soluble in methanol, ethanol, or acetone (*5*).

Molecular Weight Change. It is possible that all successful lignin removal processes function by molecular weight reduction into a range in which the coating capability of the original lignin is lost. The principal means to reduce the molecular weight of lignin-coating cellulosic material are variations on traditional chemical pulping processes, oxidation, and hydrogenation.

Chemical pulping has been developed to remove lignin selectively without disturbing cellulose fibers (*22, 23*). This is not the goal of the lignin removal strategies under consideration. The more the cellulose fiber is damaged during lignin removal, the better the pulping process is as a preparation for cellulose hydrolysis. Specially tailored brutal conditions, which nevertheless are economical, are needed. For example, the Iotech process uses solely steam at conditions that are destructive to the gross fiber structure of the cellulosic material (*5*).

The prevention of lignin self-condensation by addition of chemicals that trap intermediate carbonium ions is under investigation by Wayman's group (*24*).

Oxidation of lignin can occur through chemical or biochemical means (*25*). Oxidation, especially in the presence of alkali, is a promising approach when the oxidizing agent can be air or oxygen obtained by a molecular sieve process (*26, 27*).

Hydrogenation of lignin as a means of increasing the accessibility of cellulose to penetrating agents is more costly than the other approaches, but the lignin byproducts may cover the cost of processing (*20, 28*).

The lignin layer may coalesce when molecular weight is increased, thus removing it from contact with much of the cellulose. It is possible that irradiation, thermal treatment, or even chemical cross-linking would be approaches to using this strategy.

Processing Cellulose

Removal of lignin and hemicellulose is not sufficient to achieve high rates and yields of glucose formation from cellulose. Even cellulosic materials that never had lignin and hemicellulose associated with them (e.g., cotton) are difficult to hydrolyze without pretreatment (*1, 2*). For acid hydrolysis, the problem is that amorphous cellulose goes rapidly through glucose to degradation products, while crystalline cellulose takes

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time to convert to a readily hydrolyzed form (*1*). The ratio of the rates of conversion to glucose and to degradation products can be improved by increasing the susceptibility of cellulose to acid treatment. For enzymatic hydrolysis, the problem is a steric one: the enzyme complex known collectively as "cellulase" consists of several large molecules that must work in sequence. Unless the surface area is extremely large, rates must be slow.

Because cellulose consists of regions of high and relatively low crystallinity, processes to disrupt cellulose operate at two levels: intercrystalline and intracrystalline. The conditions required to swell intercrystalline regions are relatively mild, whereas drastic processes are required to affect the high-crystalline regions. These two areas overlap strongly but need to be distinguished.

Intercrystalline Processes. Normally, the crystalline regions of cellulose can be considered as a source of protection for the amorphous regions. The protection might be reciprocal. Amorphous cellulose exerts a protective defense for crystalline cellulose by providing sites that are relatively readily attacked by enzymes. Unless the gross structure has been greatly increased in accessibility, the glucose formed by action of the enzyme on the amorphous cellulose may diffuse from the reaction site slowly. The presence of the high local concentration of glucose may lead to inhibition of the enzyme complex. Therefore, it is not sufficient for cellulose pretreatment to render the amorphous cellulose readily attackable; successful pretreatment must provide for large open spaces for removal of end products. Similar effects may be observed with concentrated acid hydrolysis in that the initially formed glucose may be undergoing resin formation that blocks the acid from attacking the crystalline regions.

As shown in Figure 8, the intercrystalline regions can be subjected to numerous, alternative, hydrogen-bond-breaking operations. The formation of a true solution of cellulose with aqueous or nonaqueous solvents is the most direct treatment. Because it is also effective for intracrystalline regions, this process is discussed in the next section.

Less thoroughgoing methods include steaming, fine grinding, and thermal and pressure change approaches (*1, 3*). Individually, these alternatives do not appear especially attractive for both technical and economic reasons. However, insufficient attention has been directed to compound processes in which the cellulose is subjected to multiple influences simultaneously. For example, prolonged steaming under moderate pressures is somewhat effective in swelling intercrystalline regions (*9*). However, prolonged processes are costly in equipment and utilities. Therefore, it appears better to process quickly at more drastic conditions. The Iotech process exemplifies this approach.

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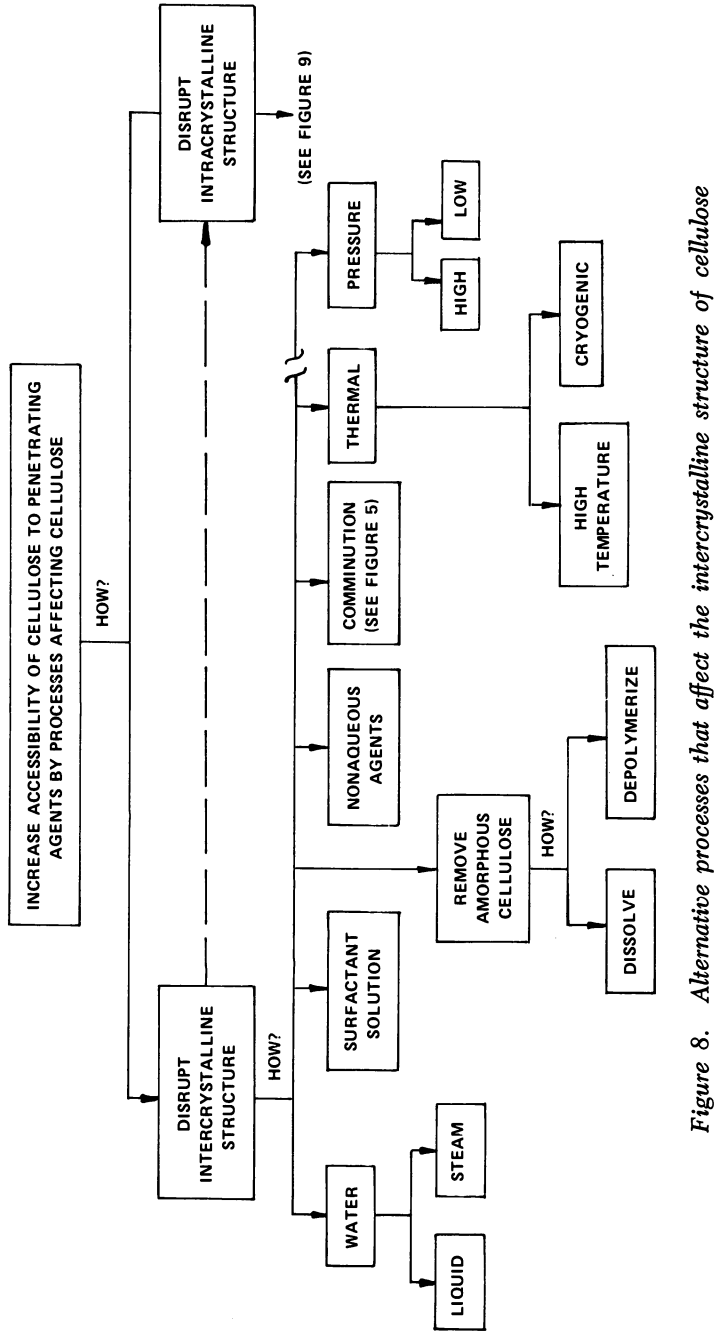

Figure 8. Alternative processes that affect the intercrystalline structure of cellulose

Information concerning the Iotech process has been kindly provided by its inventor, E. A. DeLong (*30*), and his co-workers, R. H. Marchessault (*5*) and L. Jurasek(*6*). Worldwide patent applications have been filed by the Canadian Patents and Development Limited on behalf of Iotech Corporation. The process is said to make use of the explosive depressurization principle which is applied to intact plant cell materials, such as wood chips. Prior to the explosive depressurization, the biomass raw material is subjected to a specific pretreatment with steam and heat under pressure. A unique nonfibrous product results which contains lignin that no longer coats the carbohydrate fraction and is highly soluble. The cellulose contained in the carbohydrate fraction does not lose its crystallinity but has such an enormous surface area compared with the starting material that it is easy to enzyme hydrolyze. Marchessault has shown that degree of swelling is more important than is degree of crystallinity (*31*).

Initial speculations concerning the mechanism of the Iotech process are that the initial steaming treatment puts substantial quantities of water into the intercrystalline regions of the cellulose, while the acetic acid generated by hydrolysis of the acetate groups in the hemicellulose depolymerizes the hemicellulose to a considerable degree. The glass transition temperature of lignin is exceeded. When the pressure is released rapidly, the weakened LHC literally explodes. The lignin coalesces in droplets. Mechanical abrasion of the fibers occurs during the explosive depressurization. This process is effective because of the combination of treatments, no one of which is very effective in itself.

Intracrystalline Processes. Disruption of cellulose's intracrystalline structure occurs by breaking durable hydrogen bonds between cellulose molecules within the microfibril and/or between microfibrils. Pretreatments may be based either on physical or chemical methods (Figure 9). The hydrogen bond breaking may be thought of as a progression in which order is destroyed first in the components of a fiber and only lastly between molecules. However, some processes (e.g., thermal and irradiation processes) may work initially on the molecular level, causing disorder in the microfibril and ultimately in the fiber. Conventional comminution (especially vibratory ball milling and two-roll milling) is relatively effective in improving the accessibility of cellulose to hydrolytic reagents. However, the cost in energy appears to be exorbitant (*12*). However, comminution should not be written off, because judicious use of comminution can expose fresh surface area for the action of swelling agents, solvents, or chemical reactants in compound processes.

As shown in Figure 9, there are numerous alternatives for the breaking of hydrogen bonds by both aqueous and nonaqueous solvents. These procedures had been considered to be uneconomic (*3, 32*), but

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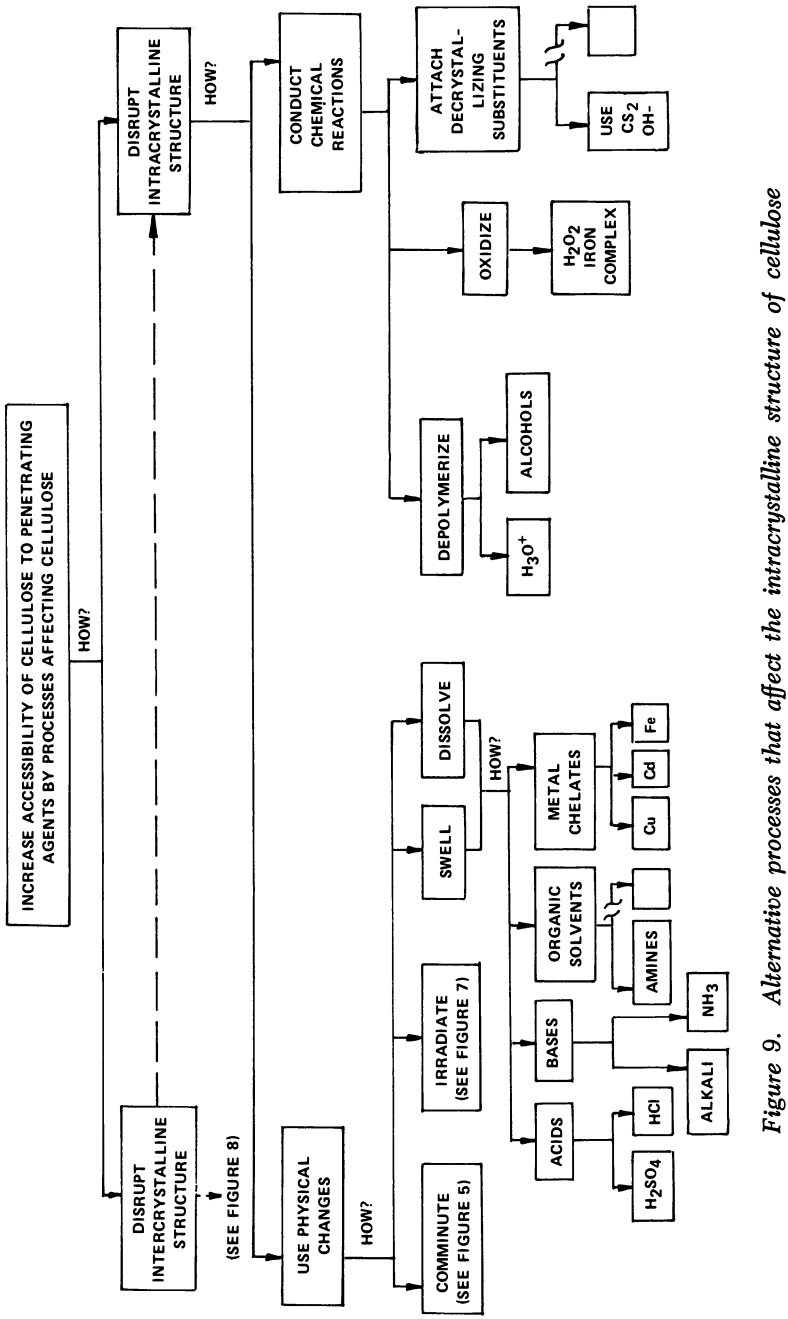

Figure 9. Alternative processes that affect the intracrystalline structure of cellulose

this assumption has been reexamined recently. A Purdue research team has undertaken a vigorous program to explore solvent processing (*4*, *33*). All the solvents they have reported are known to destroy the crystallinity of cellulose. What is new is the attempt to use this established knowledge for development of commercial processes. The methods employed by the Purdue group include (1) the Cadoxen method, which uses a mixture of cadmium salts, ethylenediamine, and alkali, (2) the ferric tartrate method, and (3) the sulfuric acid/methanol method. These systems are known to be effective because they are employed in cellulose analysis. The challenge arises in recovery of the solvent and the coproducts in high enough yields to render the processes commercially attractive. The toxicity of cadmium compounds and the low level of availability of tartaric acid are significant drawbacks for the first two methods. However, other metal chelates may be found that will overcome these objections. In any event, the sulfuric acid/methanol method does not suffer from these drawbacks. The cellulose solvent approach has considerable theoretical significance because both enzymatic and acid hydrolysis processes are greatly improved through this thoroughgoing elimination of crystallinity (*4*, *33*).

The Purdue concepts have been applied to several different agricultural products, such as corn stalks, alfalfa, orchard grass, tall fescue, and sugarcane bagasse. No experiments have been reported on either hardwoods or softwoods. The processes have been explored in two major modes. In the first, the entire agricultural residue is treated with solvent; in the second, a dilute acid pretreatment to remove hemicellulose precedes solvent treatment. The first process is especially desirable for making furfural or fermentation products from hemicellulose as a separate activity. Then, the hemicellulose-free raw material can be converted to substantially pure glucose.

Chemical reaction methods are effective in destroying the crystallinity of cellulose. For example, xanthation can decrystallize cellulose (*34*), but such elaborate processes appear much too costly unless cellophane or rayon fibers are the objectives. One route that might be effective and economical would be treatment of crystalline cellulose with alcohol in the presence of acid catalysts. Low-molecular-weight acetals may be formed, which could plasticize the cellulose at the same time that the degree of polymerization is reduced.

Conclusions

Since the most recent in-depth reviews, the Iotech and Purdue processes have been proclaimed by their advocates as breakthroughs. Wayman's discovery of means to prevent lignin self-condensation during autohydrolysis also is a most significant finding.

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As the taxonomic exercise presented in this review indicates, these processes do address the critical gap in technology for rapid cellulose hydrolysis—achievement of enormous surface area free from lignin coating. The Purdue processes attain surface area by destroying crystallinity; the Iotech process achieves high surface area by destroying fibers but without losing crystallinity. An enthusiast could claim that these processes have solved the cellulose pretreatment problem. Critics can and do argue that these processes are not breakthroughs because explosive depressurization and cellulose solvents have been known for many decades. Debates on novelty are less useful than are results on processing costs and the raw materials scope of these processes. Solvent recovery costs will be crucial in determining whether solvent processes will attain commercialization. Reactor design, steam costs, and extent of hemicellulose degradation will play significant roles in determining the attractiveness of explosive depressurization processes. It is hoped that the approaches sketched in Figures 3 through 9 of this chapter can stimulate invention of numerous pretreatment processes that can meet the wide range of end-use requirements and raw material considerations.

A major opportunity exists for plant breeders, chemists, and chemical engineers to develop new systems for production of fermentable sugars from LHC, instead of making ad hoc adaptations of systems that are optimized for other purposes. Specifically, plant species would be bred or selected for ease of processing of its LHC into cellulose. Attention would be given to low lignin content and plant cell structures that would facilitate the subsequent pretreatment and hydrolysis processes. The durable woods now favored for construction purposes and the strong fiber plants favored for pulp and paper manufacture would not be the most desirable raw materials for these new systems. The new systems need pretreatment processes that destroy fiber structure while freeing the cellulose from the lignin and hemicellulose that otherwise would complicate the course of the hydrolysis reaction. Many elements of desirable systems may be considered failures in agricultural or pulping technology and may need to be rediscovered in this new context.

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2

The Hydrolysis of Cellulosic Materials to Useful Products

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Because cellulose is the most abundant renewable resource, it should have great promise as a source of liquid fuel, food, and chemical feedstocks. Based on present technology, cellulose utilization through hydrolysis processes does not appear economical for the production of sugar syrups or alcohol fuels, particularly if biomass costs are greater than $30/ton. However, there is reason for optimism. If ways can be found to improve yields, specifically to achieve total biomass utilization and to improve the value of the process by-products, then process feasibility may emerge. Two important costs factors are raw materials costs and pretreatment methods to improve yields and biomass utilization. Also, new ways of squeezing the water from the resulting cellulose fermentation products could enhance the process economics.

Cellulose is the most abundant renewable resource available for conversion to fuel, food, and chemical feedstocks. It has been estimated by Ghose (*11*) that the annual worldwide production of cellulose through photosynthesis may approach 100×10^9 metric tons. As much as 25% of this could be made readily available for the conversion processes. A significant fraction of the available cellulose, i.e., $4–5 \times 10^9$ t/year, occurs as waste, principally as agricultural and municipal wastes. Cellulose must be viewed, therefore, as an important future source of fuel, food and chemicals (*see* Table I).

Cellulose hydrolysis and its product, glucose, play a central role in the conversion of renewable resources to foods, fuel, and chemical feedstocks. This is illustrated in Figure 1. Cheap glucose would not only find a demand in the food sweetener market but could serve as a substrate

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Table I. Supply and Demands for the Year 2000

Demand	*10^9 t/year*
liquid fuels	10
food	6
chemical feedstocks	1
total	17
Supply	
potentially available cellulose	25

for the production of fuel, alcohol, and single-cell protein. There is a greater choice of organisms that can grow on glucose compared with other substrates. Also, with glucose substrates there should be less problem with undesirable or toxic residues when using them to produce single-cell protein.

With respect to the hydrolysis step, it can be accomplished by acid, by enzymatic, or by direct microbial attack. Microbial hydrolysis results primarily in the production of cellular biomass or single-cell protein. Acid hydrolysis, while simple and direct, results in a sugar syrup with considerable contamination from the side reaction products. Enzymatic hydrolysis is usually the cleanest hydrolysis process. Unfortunately, it is the most costly of the three to operate.

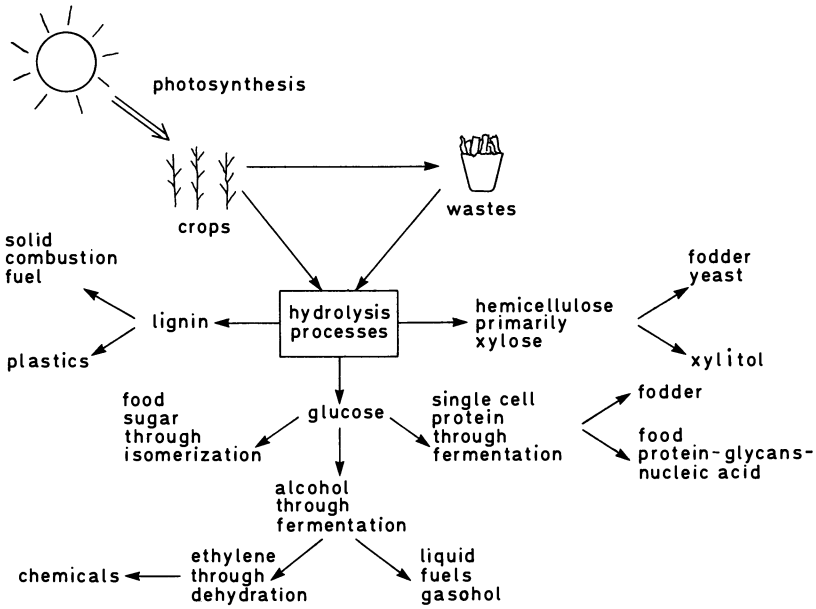

Figure 1. The centrality of cellulose hydrolysis in the conversion processes

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The Nature of Cellulose

For the most part, cellulosic hydrolysis studies have been concerned with pure or at least relatively pure cellulose. However, cellulose in its natural state never occurs in a pure form. It always is associated with lignin and hemicellulose. The portions are indicated in Table II.

Table II. Composition of Cellulosic Materials

Material	*% of Dry Matter*
cellulose	40–60
hemicellulose	20–40
lignin	10–25

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The hemicellulose fraction is a mixture of sugars, primarily pentoses. Depending upon the biomass source, the hemicellulose fraction can be as much as 85% xylan and yields xylose on hydrolysis. Xylose can be used by many microorganisms either fermenting to alcohols or converting to microbial biomass. Xylose can also be hydrogenated to xylitol, a potentially important diabetic sweetener. The lignin, although only on the order of one-fifth of the total biomass, represents approximately 50% of the available combustible energy in naturally occurring sources of cellulose. It seems obvious, therefore, that any cellulose hydrolysis scheme must be prepared to utilize both the lignin and hemicellulose.

Acid Hydrolysis Process

Acid hydrolysis of cellulosic materials has been studied for many years (*13, 33*). Although it is a relatively straightforward process, it has the problems of requiring acid-resistant equipment and yielding a poor grade of sugar (because the product contains many reaction product impurities). However, in terms of practical application, acid hydrolysis of cellulosic material is by far the most commonly used hydrolysis system.

A two-stage acid hydrolysis process is employed in over 40 Soviet wood hydrolysis plants (*15*). These plants have an average annual output per plant of 10,000 t of wood sugar. Most of the output is converted to industrial alcohol and fodder yeast.

The major soluble components of acid hydrolysates are sugars, such as xylose, glucose, and cellobiose; furfurals, such as furfuraldehyde and hydroxymethyl furfural; and organic acids, such as levulinic acid, formic acid, and acetic acid (*13*). When natural sources of cellulose are acid-hydrolized, numerous products can result, largely because of the hemicellulose materials. These make it difficult to produce a relatively pure sugar product and limit the utility of the acid hydrolysis process.

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In order to minimize the product contamination, the acid hydrolysis process is usually performed in two stages. The first stage involves contact with dilute acid (1% sulfuric) at relatively low temperatures (80–120°C) and short times (30–240 min). The purpose of this stage is to extract the hemicellulose fraction, mostly as pentoses. The second stage is performed with stronger acid (from 5–20% sulfuric) and higher temperatures (approximately 180°C). The purpose of the second stage is to hydrolyze the cellulose to glucose. The overall objective is to optimize conditions such that the glucose yield is maximized and the secondary product contamination is minimized.

Cellulosic materials are quite variable from source to source, not only in cellulose, hemicellulose, and lignin content but also in the crystallinity of the cellulose. As a consequence, each natural substrate would be expected to have its own unique set of process conditions to optimize glucose yield and minimize secondary product contamination. The next section on kinetics of acid hydrolysis will examine this point.

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Acid Hydrolysis Kinetics

A considerable amount of experimentation has been done on the kinetics of acid hydrolysis of pure cellulose substrates. Little experimentation has been done on natural cellulosic materials. Typical examples of kinetic studies of acid hydrolysis of cellulose can be found in the papers of Saeman (*33*) and Grethlein (*13*). These researchers depict the acid hydrolysis process as a pseudo-first-order sequential process, with the rate constants as a function of the acid concentration raised to a power, i.e.,

$$\underset{(C_x)}{\text{cellulose}} \xrightarrow{k_1} \underset{(C_1)}{\text{glucose}} \xrightarrow{k_2} \underset{(C_o)}{\text{decomposed glucose}} \tag{1}$$

where

$$\frac{dC_x}{dt} = -k_1 C_x \tag{2}$$

$$\frac{dC_1}{dt} = +Y_1 k_1 C_x - k_2 C_1 \tag{3}$$

$$\frac{dC_o}{dt} = +Y_2 k_2 C_1 \tag{4}$$

and where

$$k_1 = K_1 (A)^m \exp(-E_1/RT) \tag{5}$$

$$k_2 = K_2 (A)^n \exp(-E_2/RT) \tag{6}$$

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In these expressions, C_x = concentration of cellulose, C_1 = concentration of glucose, C_o = concentration of decomposed glucose products, k_1 and k_2 are the rate constants for the respective reactions, Y_1 and Y_2 are the stoichiometric coefficients, and A is the acid concentration.

Grethlein (*13*) and Saeman (*33*) have estimated values of the various constants for acid hydrolysis of Solka Floc and Douglas Fir. These are given in Table III.

Table III. Acid Hydrolysis Constants for Various Cellulosic Materials

Constant	*Douglas Fir*	*Solka Floc*
K_1, min^{-1}	1.73×10^{19}	1.22×10^{19}
K_2, min^{-1}	2.38×10^{14}	3.79×10^{14}
E_1, cal/g · mol	42,900	42,500
E_2, cal/g · mol	32,800	32,700
m	1.34	1.16
n	1.02	0.69

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The fact that these constants are quite similar for Douglas Fir and Solka Floc is remarkable since both behave quite differently when enzymatically hydrolyzed. It would be expected that each cellulosic material would behave differently. For most cellulosic materials, there is a unique temperature at a fixed acid concentration and reaction time that gives optimal glucose yield. This is illustrated in Figure 2, which summarizes the glucose yield data of Grethlein (*13*) for acid hydrolysis of Solka Floc as a function of temperature for a fixed residence time (0.22 min) at various acid concentrations (0.5, 1.0, 1.5, and 2% sulfuric acid) and solids concentrations (5–13.5% cellulose). Since 0.22 min approaches a practical minimal contact time that can be achieved, due to mass-transfer lags in economically sized cellulosic substrates, it is doubtful that greater than 50–60% conversion of cellulose to glucose is practically realizable without appreciable secondary product formation. More work is needed on short-time, high-temperature, and high-concentration acid hydrolysis. It would appear that acid hydrolysis, because of the glucose breakdown products, will be limited in its applicability. Conversion of the glucose syrup to fuel ethanol is the only use envisioned at the present time.

Enzyme Hydrolysis Process

There are many sources of cellulolytic enzymes; however, the fungus *Trichoderma viride* has proved to be the most effective source to date. The microbiology (*25, 32*) and enzyme kinetics (*24, 30*) studies on this organism have been pioneered by workers such as Reese and Mandels at

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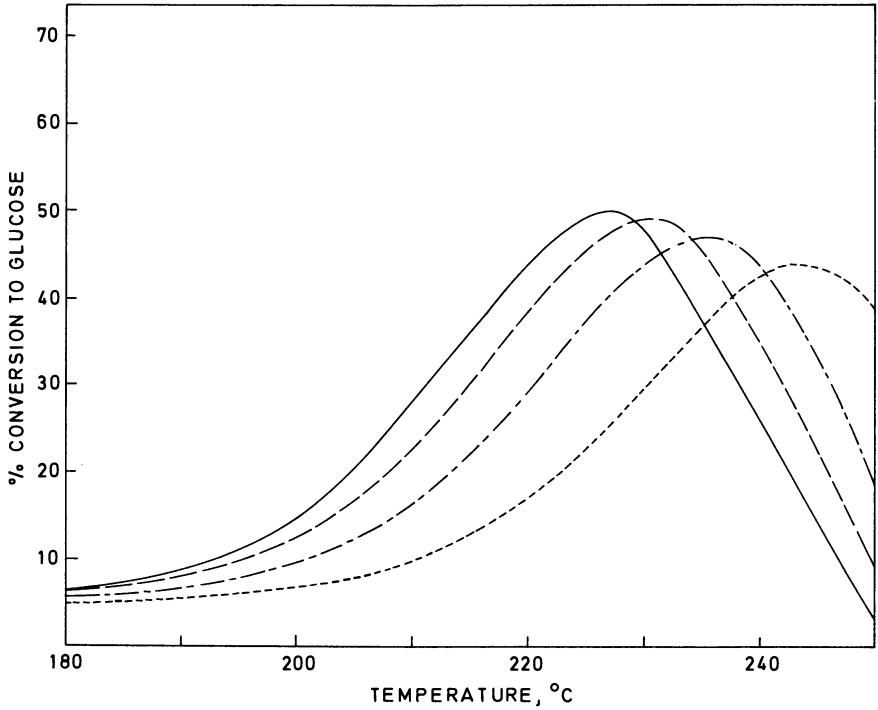

Figure 2. Acid hydrolysis of cellulose; yields as a function of temperature: (——) 2% acid, (— —) 1.5% acid, (— — —) 1% acid, (– – –) 0.5% acid.

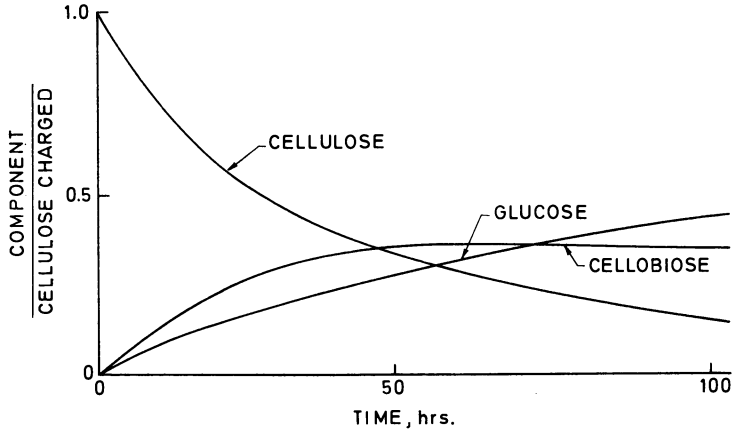

Figure 3. Typical enzymatic digestion of cellulose

the U.S. Army Natick Laboratories. With proper pretreatment, such as ball milling, a cellulase preparation from *T. viride* is able in a reasonable time (< 100 hr) to break down completely pure cellulose substrates. This is not true for natural sources of cellulose such as bagasse or wood chips. With these substrates, it is usually difficult to obtain much more than 50% conversion. The problem is that natural sources of cellulose are protected from enzymatic hydrolysis by associated hemicelluloses and lignin. Either the removal of lignin and hemicelluloses from the native cellulose or the addition of hemicellulases is necessary to improve the glucose yield in enzymatic hydrolysis of naturally occurring cellulosic materials.

In a typical enzymatic hydrolysis of a 5% suspension of ball-milled newsprint, a sugar syrup containing 1.6% glucose, 1.4% cellobiose, and 0.2% xylose is readily obtained. Figure 3 is illustrative of kinetic results obtained with *T. viride* cellulase at 3.5-filter-paper-units/mL (FP units/mL) strength in a 5% suspension of ball-milled newsprint.

There are two reasons for the measurable cellobiose concentration in the *T. viride* cellulase hydrolyzed syrups. The most likely is that *T. viride* has rather poor β-glucosidase activity so that cellobiose accumulates. Evidence of this is that additions of β-glucosidase to the *T. viride* cellulase improves its activity. A second reason is that the β-glucosidase enzyme is strongly glucose inhibited. Hence the rate of cellobiose hydrolysis slows down as the glucose concentration rises, allowing cellobiose to accumulate.

At least three major cellulase components are involved in cellulose hydrolysis (*6, 8, 19, 39*). These are endo-β-glucanases, exo-β-glucanases, and β-glucosidase (cellobiase). The most widely accepted model for the enzymatic hydrolysis of pure cellulose is depicted in Figure 4. Crystalline cellulose (C_x) is attacked by endo-β-glucanases to give amorphous cellulose and some oligosaccharides. These materials are attacked in turn by *exo*-β-glucanases to give glucose (C_1) directly and by the cellobiosyl-hydrolases to give cellobiose (C_2), which, in turn, is hydrolyzed by β-glucosidase to give glucose. The latter is thought to be the dominant mechanism by which *T. viride* cellulases produce glucose. Consequently, the overall reaction may be depicted as a two-step reaction of cellulose going to cellobiose and then to glucose. Both steps are inhibited by their products.

The problem of modeling the hydrolysis kinetics is complicated by the fact that cellulose is a solid substrate; consequently, the reaction can be surface limited (*5, 12*). Furthermore, some sites are more susceptible to hydrolysis—e.g., the amorphous regions as well as specific regions of the crystalline cellulose such as edges, corners, and dislocations. Several investigators (*17, 20, 36*) have suggested that the kinetic model should be based on a "shrinking site model" in which the number of susceptible

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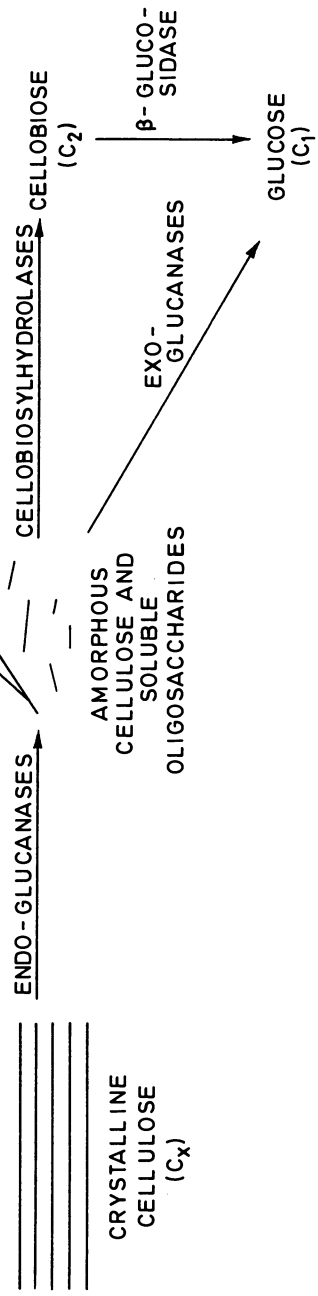

Figure 4. Model of enzymatic hydrolysis of cellulose

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sites is proportional to the surface area. Further, the enzyme must first be adsorbed at these sites in order to function. Therefore, the kinetic model should include an adsorption isotherm to relate the soluble enzyme concentration (E_X) to the adsorbed enzyme concentration (E_{ads}) (*4*). An example of such a kinetic model is outlined in the next section.

Shrinking Site Hydrolysis Model

In evolving the shrinking site model, it will be assumed that the disappearance of cellulose can be represented by the following mechanisms:

$$C_x \xrightarrow[E_x \leftarrow]{I_2} C_2 \xrightarrow[E_2 \leftarrow]{I_1} C_1 \tag{7}$$

where C_x is the solid cellulose substrate concentration and C_2 and C_1 are the cellobiose and glucose concentrations. The disappearance of cellulose can then be given by

$$\frac{dC_X}{dt} = -KE_{ads}C_x\left[\frac{I_2}{I_2 + C_2}\right] \tag{8}$$

where

K = reaction rate constant
E_{ads} = grams of absorbed enzyme/gram of cellulose
I_2 = cellobiose inhibition concentration, g/L

If one assumes that a Langmuir-type adsorption isotherm can be used to relate the concentration of adsorbed cellulase on the cellulose to the free cellulase in solution, i.e.,

$$E_{ads} = E^\circ_{ads}\left[\frac{E_x}{\alpha + E_x}\right] \tag{9}$$

and

$$E^\circ_{ads} = K'\eta 4\pi R^2 n \tag{10}$$

where

E°_{ads} = saturation concentration of cellulase/gram of cellulose
K' = no. adsorption sites/gram of cellulose
η = no. adsorption sites/unit surface area
R = mean mass radius/cellulose particle
n = no. cellulose particles/gram of cellulose

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In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

and noting that

$$C_x = \frac{4}{3}\,\pi R^3 \rho N \tag{11}$$

or

$$R = (3C_x/4\pi\rho N)^{1/3}$$

ρ = grams/milliliter of cellulose

N = no. cellulose particles/liter solution

then

$$\frac{dC_x}{dt} = KK'\eta 4\pi n\,(3/4\pi\rho N)^{1/3}\,C_x^{4/3}\left[\frac{E_x}{\alpha + E_x}\right]\left[\frac{I_2}{I_2 + C_2}\right]$$

$$= -K_x C^{4/3}\left[\frac{E_x}{\alpha + E_x}\right]\left[\frac{I_2}{I_2 + C_2}\right] \tag{13}$$

Using Equation 13 as a starting point, it is now possible to construct a kinetic model for the enzymatic hydrolysis of cellulose. This model is summarized in Table IV. Six kinetic parameters are required to define the system. With the proper selection of parameter values, the data depicted in Figure 3 can be duplicated by the model. Wilkie and Yang (*37*) have suggested that a simple distribution coefficient is adequate for expressing the adsorption isotherm of C_x and C_1 activity on a −20-mesh Wiley-milled newsprint (*see* Figure 5). These data were obtained at a relatively low enzyme activity where a linear isotherm would be expected to apply. The distribution coefficient was very small (D = 0.04 FP units/mL/FP units/g solids), which suggests that the cellulase enzyme binds rather tightly to the solid cellulose. It also suggests that simple countercurrent

Table IV. Enzymatic Cellulose Hydrolysis

$$C_x \xrightarrow[E_x]{I_2} C_2 \xrightarrow[E_2]{I_1} C_1$$

Cellulose: $$\frac{dC_x}{dt} = -K_x C_x^{4/3}\left[\frac{E_x}{\alpha + E_x}\right]\left[\frac{I_2}{I_2 + C_2}\right]$$

Cellobiose: $$\frac{dC_2}{dt} = +\frac{dC_x}{dt} - \left[\frac{K_2 E_2 C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right]$$

Glucose: $$\frac{dC_1}{dt} = +\left[\frac{K_2 E_2 C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right]$$

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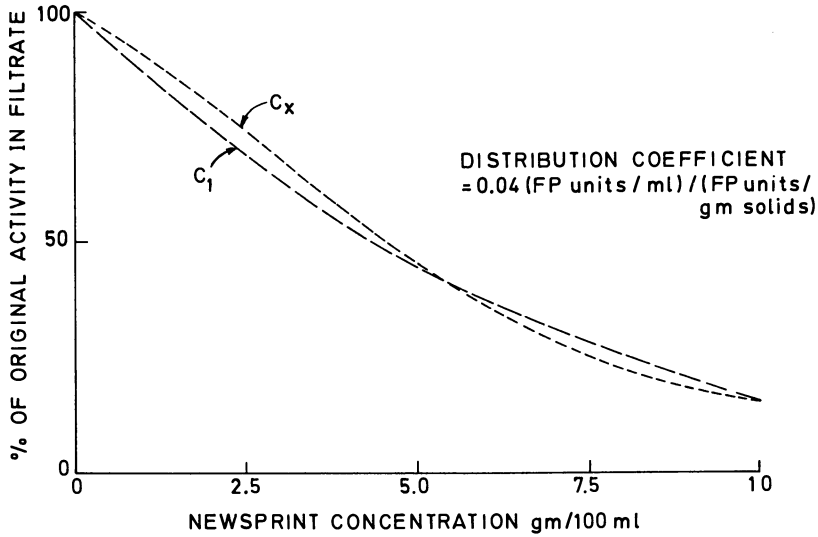

Figure 5. Data of Wilke and Yang on adsorption of C_1 and C_x cellulase activity (in terms of FP units) on −20-mesh Wiley-milled newsprint. Distribution coefficient = 0.04 (FP units/mL)/(FP units/g solids).

adsorption of the cellulase from the sugar syrup exiting from the hydrolyzer will permit a high degree of cellulase recovery and reuse.

Moreira in his PhD thesis work attempted to verify the shrinking site model by estimating the adsorbed protein on the solid cellulose as a function of cellulose digestion and time (*28*). The data suggest that a Langmuir-type adsorption equilibrium occurs and that as the cellulose hydrolysis occurs, a condition is reached where all the adsorption sites are saturated. Then, as the cellulose hydrolysis proceeds, adsorbing sites disappear and enzyme is ejected form the shrinking cellulose surface and goes back into solution.

Microbial Hydrolysis of Cellulose

Microbial hydrolysis of cellulose can often be very direct, fast, and complete. Even though a microorganism may produce primarily an extracellular cellulase, hydrolysis is usually faster in the presence of organisms than just the cellulase-containing solution alone. The reason for this is that cellulolytic organisms grow on the cellobiose or glucose, thus continuously removing them from solution and relieving their inhibitory effects. This high hydrolysis rate is illustrated in Figure 6, where data for the growth of a *Thermoactinomyces* sp. at 60°C on Avicel is given (*2*). Note that the maximum rate of cellulose hydrolysis, occurring at

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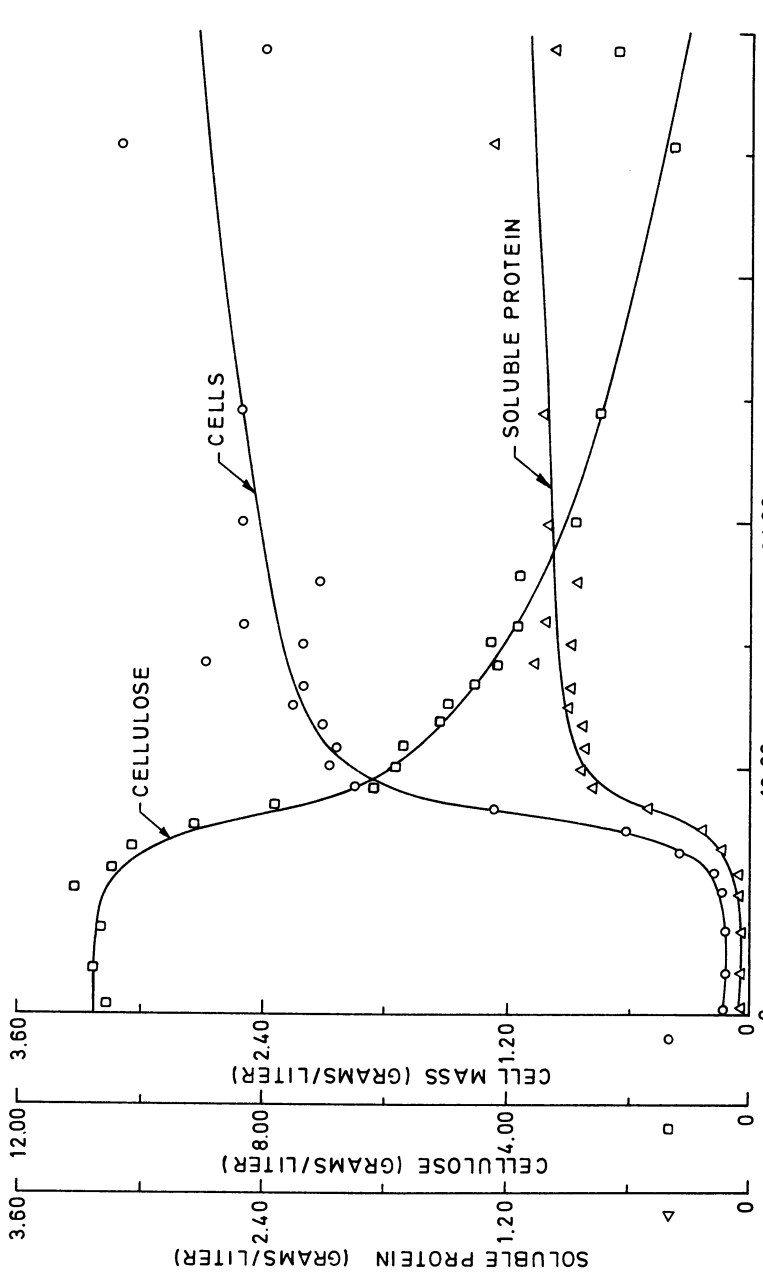
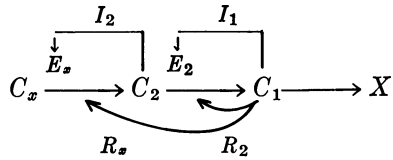


Figure 6. Growth of Thermoactinomyces *on Avicel*

8–10 hr into the fermentation, is equivalent to a volumetric cellulose hydrolysis rate of nearly 2 g/L·h. This kind of volumetric rate is considered excellent for most fermentative processes.

In modeling a microbial process for the hydrolysis of cellulose, one must account for the generation of the enzymes as the organism grows and the repression of the enzyme production due to glucose. One model that has appeared in the literature (*20*) for this process is given in Table V. This model is viewed as a rather simplistic expression of what actually

Table V. Cellulose Fermentation: Shrinking Site Model (Microorganism grows only on glucose)



Cellulose: $$\frac{dC_x}{dt} = -K_x C_x^{4/3}\left[\frac{E_x}{\alpha + E_x}\right]\left[\frac{I_2}{I_2 + C_2}\right]$$

Cellobiose: $$\frac{dC_2}{dt} = +\frac{dC_x}{dt} - \left[\frac{K_2 E_2 C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right]$$

Glucose: $$\frac{dC_1}{dt} = +\left[\frac{K_2 E_2 C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right] - \left(\frac{dX}{Y\,dt} + mX\right)$$

Cells: $$\frac{dX}{dt} = +\frac{\mu_{max}\, C_1 X}{K_s + C_1}$$

Enzyme$_x$: $$\frac{dE_x}{dt} = Y_{E_x/X}\left(1 - \frac{C}{R_x + C_1}\right)\frac{dX}{dt}$$

Enzyme$_2$: $$\frac{dE_2}{dt} = Y_{E_2/X}\left(1 - \frac{C_1}{R_2 + C_1}\right)\frac{dX}{dt}$$

Typical Values for Parameters

K_x	= 0.084 (g/L)$^{-1/3/hr}$	m	= 0.01g/g-hr
α	= 0.30 g/L	μ_{max}	= 0.25 L/hr
I_2	= 1.0 g/L	K_s	= 0.1 g/L
K_2	= 10.0 g/L-hr	$Y_{E_x/X}$	= 0.01 g/g
K_M	= 4.0 g/L	$Y_{E_2/X}$	= 0.03 g/g
I_1	= 1.0 g/L	R_x	= 0.01 g/L
Y	= 0.5 g/g	R_2	= 0.01 g/L

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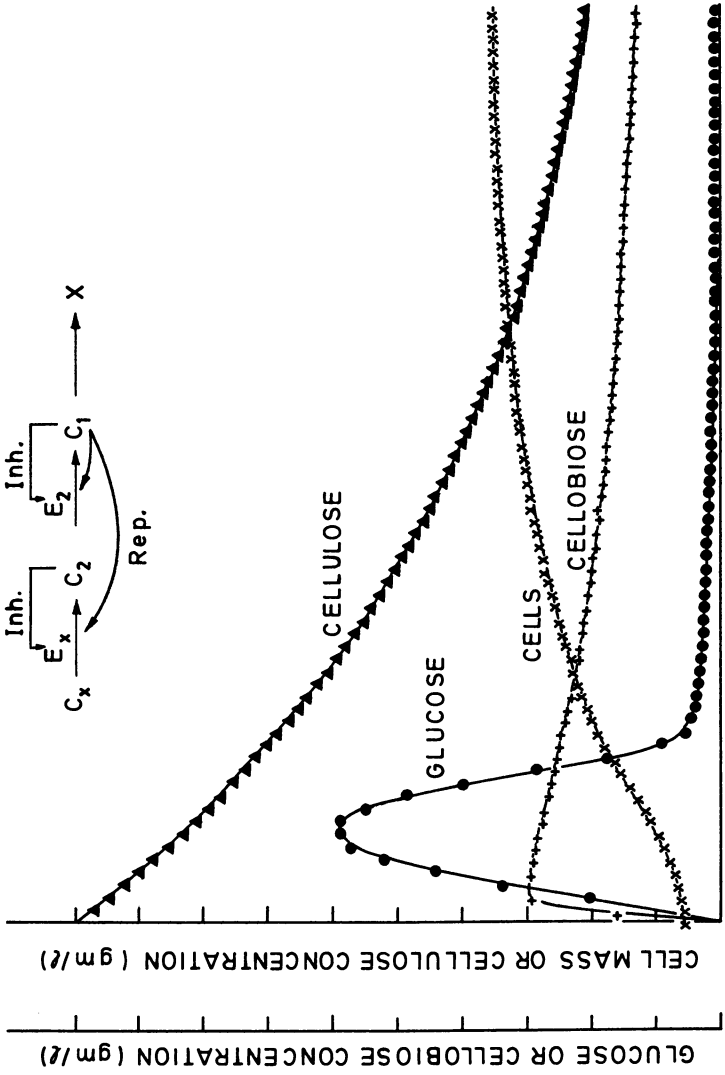

Figure 7. Shrinking site model for cell growth on cellulose

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occurs. Even such a simplistic model has 14 constants or parameters that must be evaluated. Parameter identification of this number of constants for a single process is most difficult. However, the model does predict the behavior of a typical batch fermentation of cellulose (*see* Figure 7). The model clearly indicates that relatively little benefit is obtained from microbial cellulose digestion beyond 50% conversion unless the cellulose costs are high and one is concerned with the cellulose-to-cell protein ratio and wants to minimize this ratio in the final product.

The model in Table V assumes that the microbial cells excrete an extracellular endoglucanase, which is adsorbed on the solid cellulose and initiates the hydrolysis process, which ends in glucose that is taken up by the cells. The work of Binder and Ghose (*3*) suggest that this may not be so in some cases of cell growth on cellulose. For example, they found *T. viride* cells had essentially a constant adsorption-distribution coefficient for cotton cellulose and could adsorb several times their own weight of cellulose (*3, 4*) (*see* Figure 8). They found the process of adsorption of cellulose on cells to be rather slow. In contrast, the adsorption process for soluble enzyme on solid cellulose substrate is relatively fast. Therefore, the model depicted in Table V is believed to be more consistent with observed results than the cell-cellulose adsorption model proposed by Binder and Ghose (*3*).

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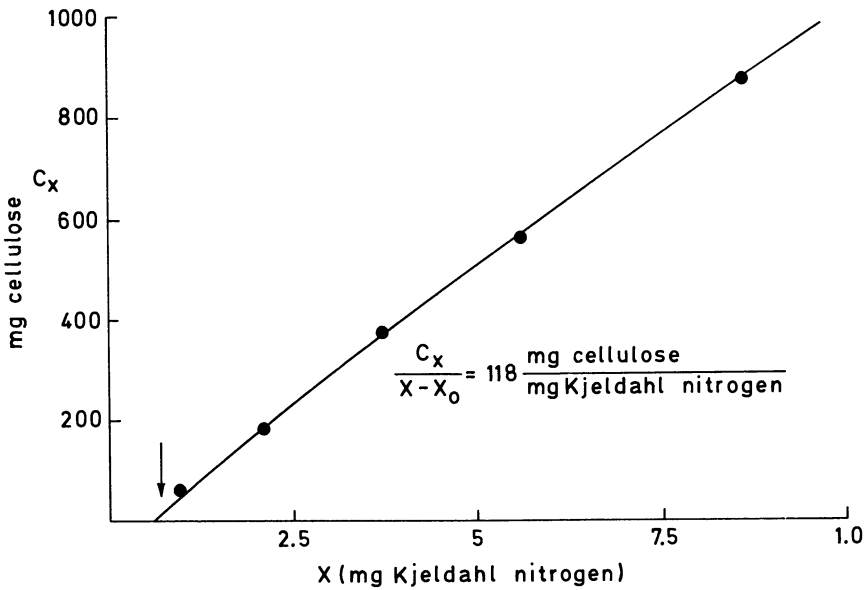

Figure 8. Data of Binder and Ghose on adsorption of powdered cotton cellulose by cells of T. viride. Note: T. viride *is approximately 8.0% Kjeldahl nitrogen on a dry basis. Therefore, the* T. viride *cells are capable of adsorbing 9.4 times their weight of cellulose.*

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In studies of cellulose utilization by some species of cellulolytic organisms, it has been observed that growth on cellulose occurs at a faster rate than that on glucose (*16, 21*). Also, when cellobiose is used as the substrate, it has been noted that glucose accumulates in appreciable quantities (*2*). Various mechanisms have been offered to explain this phenomenon. However, it is now known that many cellulolytic organisms prefer to grow on cellobiose. Indeed, they have the capability of phosphorylating cellobiose and taking up this organic molecule directly without first having to hydrolyze it. Production of glucose is a secondary reaction. Only when cellobiose has been fully utilized will the organism turn to utilizing the glucose. Figure 9 is illustrative of the typical kind of data one gets when such an organism is grown on cellobiose. Note the manner in which glucose accumulates and then is utilized. Such behavior is similar to diauxic growth.

Table VI presents a kinetic model for preferred growth of a microorganism on cellobiose (C_2) but having an extracellular β-glucosidase (E_2) that is glucose inhibited and in which the glucose uptake is repressed. This model readily fits the cellobiose data shown by the solid lines in Figure 9 (*20*).

This behavior is one explanation of why organisms can grow so well on pretreated or ball-milled cellulose. The growth rates are only limited by the number of active sites or surface area available to them for attack per unit concentration of cell biomass or enzyme. An organism (X) will grow at a near-maximum growth rate until such time that the maintenance demand of that organism (m) is not being met by the rate of carbonaceous energy supply. Growth then begins to slow down and finally stops when the energy supply is less than the maintenance demand, i.e.,

$$-\frac{dC_x}{dt} = \frac{1}{Y_{X/C_x}} \cdot \frac{dX}{dt} + mX \tag{14}$$

and

$$\frac{1}{Y_{X/C_x}} \cdot \frac{dX}{dt} > 0$$

as long as

$$-\frac{dC_x}{dt} > mX$$

When

$$-\frac{dC_x}{dt} = mX$$

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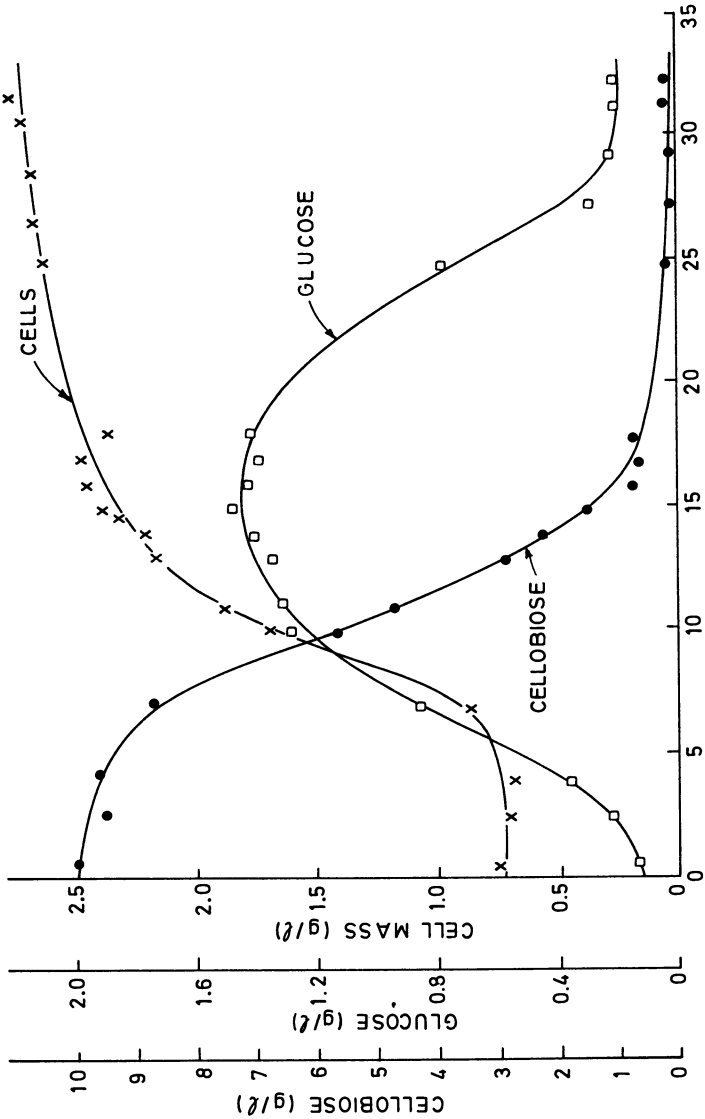
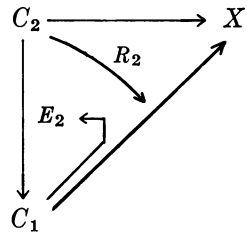


Figure 9. Growth of Thermoactinomyces *on 1% cellobiose (55°C, 500-mL shake flask)*

Table VI. Growth of *Thermoactinomyces* on Cellobiose



Cellobiose:

$$\frac{dC_2}{dt} = -\left[\frac{K_2E_2C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right] - \left[\frac{dX}{Y\,dt} + mX\right]_{\frac{dC_2}{dt} > 0}$$

Cells:

$$\frac{dX}{dt} = \frac{\mu_{max_2} C_2X}{K_{S_2} + C_2} + \left(1 - \frac{C_2}{R_2 + C_2}\right)\frac{\mu_{max_1} C_1X}{K_{S_1} + C_1}$$

Glucose:

$$\frac{dC_1}{dt} = +\left[\frac{K_2E_2C_2}{K_M + C_2}\right]\left[\frac{I_1}{I_1 + C_1}\right] - \left(1 - \frac{C_2}{R_2 + C_2}\right)\frac{\mu_{max_1} C_1X}{K_{S_1} + C_1}$$

Enzyme$_2$:

$$\frac{dE_2}{dt} = Y_{E_2/X}\,\frac{dX}{dt}$$

Table VII. Growth Parameters for *Thermoactinomyces* on Various Substrates

Substrate	μ_{max} (hr^{-1})	m (*g substrate/ g cell · hr*)	Y (*g cell/ g substrate*)
Cellulose	0.4–0.5	0.02–0.08	0.40–0.5
Cellobiose	0.2	0.027	0.27
Glucose	0.48	0.038	0.44
Oxygen/glucose	—	0.032	1.03

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growth ceases, and when

$$-\frac{dC_x}{dt} < mX$$

growth ceases and/or cell lysis occurs.

Typical growth parameters for a *Thermoactinomyces* sp. on cellulose, cellobiose, and glucose are given in Table VII. This table suggests that cellulose hydrolysis must occur at a rate greater than 0.02 to 0.04 g cellulose/g organism·hr for cell growth to occur. These data further illustrate just how important pretreatment of the cellulosic material is to good growth and yield of microorganisms on cellulose.

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Practical Cellulose Hydrolysis Processes

Both pilot-plant and plant-scale processes for cellulose hydrolysis or digestion by acids, enzymes, and microorganisms have been built (*1, 7, 15, 27*). Acid and enzyme processes usually have as their objective the production of a sugar syrup, while the microbial process usually results in microbial protein for animal feed. Figure 10 is illustrative of a microbial process (*29*) that has been developed to convert the unused cellulosic material in manure to recycle feed. Similar processes have been developed

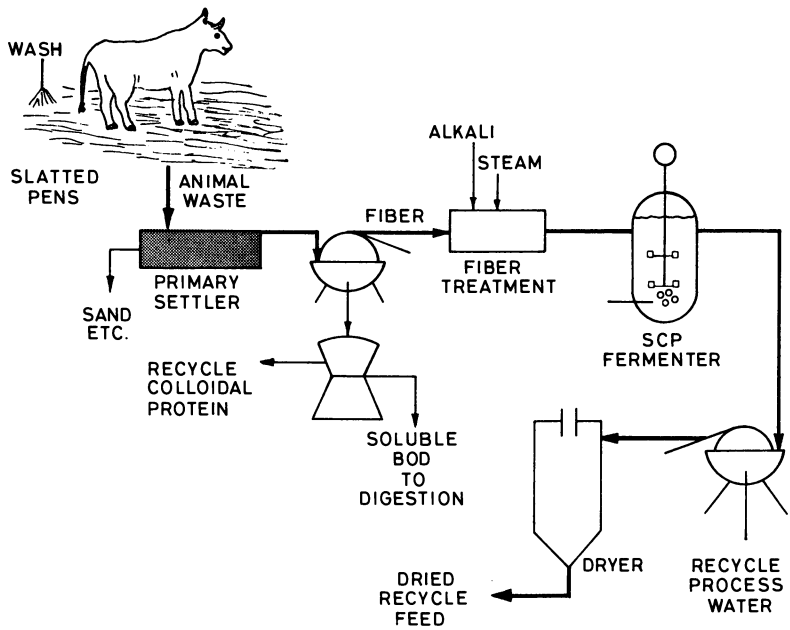

Figure 10. Animal recycle feedlot wastes system

for waste straw and for cane bagasse. As previously indicated, the problem is to achieve an adequate yet cheap cellulose pretreatment process. To date, simple grinding has been inadequate, drying and ball milling is too expensive, and combined alkali and heat treatment is marginally economical (*26*). Just recently, steam explosion of dried substrates and solvent delignification have been suggested as pretreatment procedures. It is the opinion of this author that the major bottlenecks to economical cellulose hydrolysis processes are the availability of large supplies of cheap biomass, the cost of the pretreatment step, and the conversion yield of the total biomass, i.e., hemicellulose as well as cellulose.

Figure 11 gives a scheme proposed by Wilke and Yang (*27, 37*) for the enzymatic hydrolysis of cellulose. The enzymatic process has a number of additional economic bottlenecks. These involve the cost of production of the cellulase enzyme and the recovery or reuse of enzyme. In order to achieve a high enzyme reuse, it is necessary to recover the enzyme from the glucose syrup product by adsorbing the enzyme on fresh cellulosic material. Similarly, it is necessary to recover the enzyme from spent cellulosic solids by washing. Both recovery systems are essential to economical enzymatic processes. Fortunately, the various cellulase components have similar adsorption characteristics (*see* Figure 5), thus their relative effectiveness is not significantly changed or reduced (*37*).

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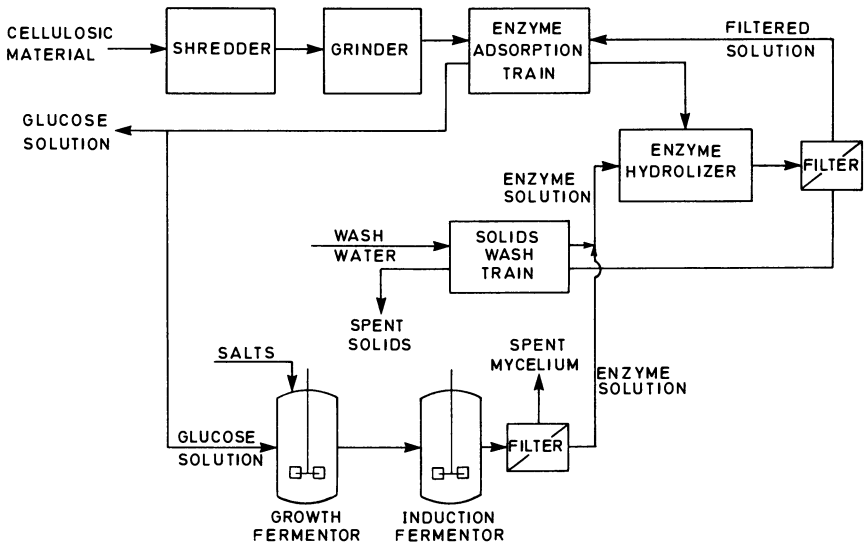

Figure 11. Cellulose hydrolysis process

The economic potential of enzyme hydrolysis is therefore dependent upon the evolution of (1) cheap, readily available, large biomass sources, (2) cheap and efficient pretreatment methods, and (3) cheap and potent sources of cellulolytic enzymes.

The data in Figure 12 highlight the effect of substrate costs on the cost of glucose for a 250,000-t/yr plant. Most people think that large sources of naturally occurring biomass (50–60% cellulose) will only be available at a delivered price of $30 per ton ODW (oven-dried weight) (*18*). If this is true, glucose syrups will only be produced by the enzymatic process at costs, depending upon the percentage of cellulose utilization, of 8 to 16¢ per pound of sugar. Such processes are marginally attractive at best in the present economic price climate for world sugar.

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Conversion of Sugar to Alcohol and Other Products

The conversion of sugar to alcohol has been highly touted as a way of solving the fuels and chemical feedstock problem. Clearly, ethyl alcohol is a technically feasible alternative to gasoline and ethylene for chemical feedstocks. The question is whether it is a practical or economic solution. Various economic exercises have been made (*7, 16, 22, 23, 34, 37, 38*). Some are favorable; others are highly unfavorable. The key is determining the basic assumptions; in particular, what is the cost of the raw material? The sensitivity of the process economics to raw material costs is illustrated in Figure 12. Another problem is by-products credits, i.e., the process cost reduction due to the by-products such as animal feed, spent solid fuel, recovered polymer grade lignin, etc. This is illustrated in Figure 13.

The other problem is the process energy demand due to "squeezing the water out" of the fermented alcohol product stream in order to produce high-purity alcohol. Unfortunately, most fermentations, such as alcohol fermentation, are carried out in dilute aqueous solution. Considerable energy is required to separate the alcohol from the water by steam distillation. These energy costs, in terms of tons of oil for energy per ton of alcohol product at various alcohol product concentrations, are illustrated in Figure 14. When oil costs were low, the energy costs of the separation process were relatively minor to the overall costs. Today, they are greater than 40% of the total process costs.

Examination of the data in Figure 14 indicates that the alcohol process should produce at least an 8% and preferably a 10–14% ethanol feed stream from the fermentation process in order to minimize separation costs. This means, assuming a 40% ethanol weight yield based on glucose in the syrup, that at least a 20–25% sugar syrup must be produced for fermentation. Without an intermediate sugar concentra-

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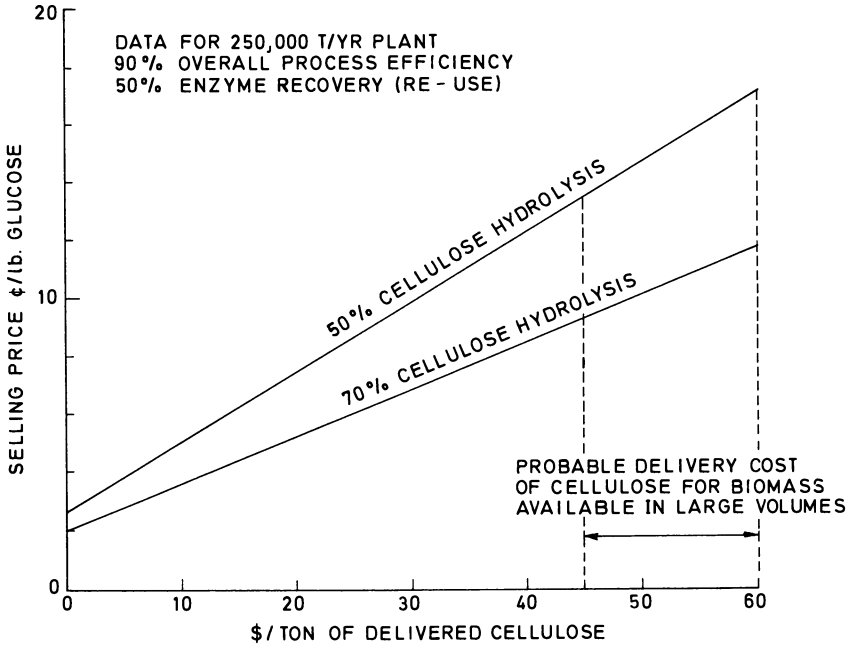

Figure 12. Relation of glucose selling price to delivered cellulose substrate costs for enzymatic hydrolysis process. Data for 250,000 t/yr plant: 90% overall process efficiency, 50% enzyme recovery (reuse).

tion step, the enzymatic hydrolysis process must be run on very high solids substrates. Indeed, solid bed reactors may be the only feasible reactor configuration for hydrolysis systems.

The ethanol selling price is very sensitive to the glucose substrate costs. This is illustrated in Figure 15. Present hydrolysis technology suggests that glucose substrates can be produced in the 10-to-14¢-per-pound range. Unfortunately, this results in costs of $1.60 to $2.00 per gallon for alcohol. In order to compete with (1978) U.S. gasoline prices, alcohol must sell for 60 to 80¢ per gallon. This requires a 2-to-4¢-per-pound glucose feed (Figure 14), which in turn means that cellulosic biomass must be available at a delivered price of less than $10/t of cellulose content. It would seem, therefore, that until the price of gasoline rises to the $1.60-per-gallon range, the use of ethanol produced from biomass to augment gasoline and other fuels is not economically attractive. However, there are places in the world where gasoline sells at this level and where large quantities of cheap biomass are readily available. An alcohol-based fuel economy may be feasible for these locations. Further, with decontrol of oil prices, gasoline prices of this level may soon be reached.

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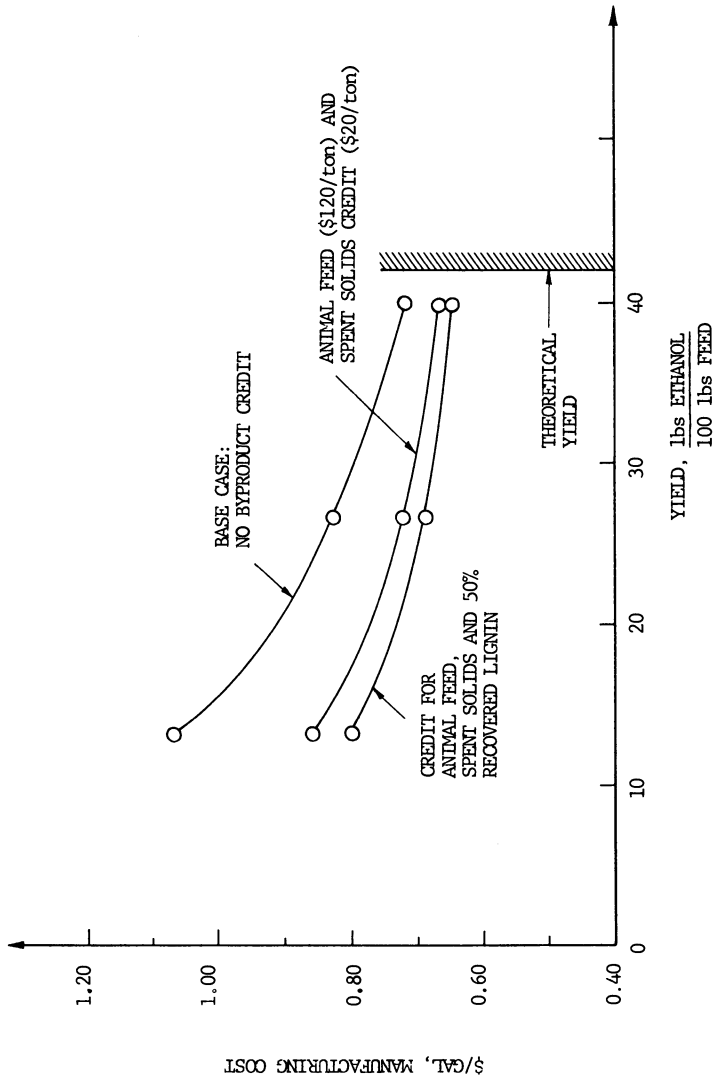

Figure 13. Manufacturing cost of ethanol by enzyme hydrolysis. Yearly production: 25 million gallons (95%). Includes: capital related costs, raw materials & chemicals, utilities, labor, maintenance. Feed: 56% cellulose, 28% hemicellulose, 16% lignin.

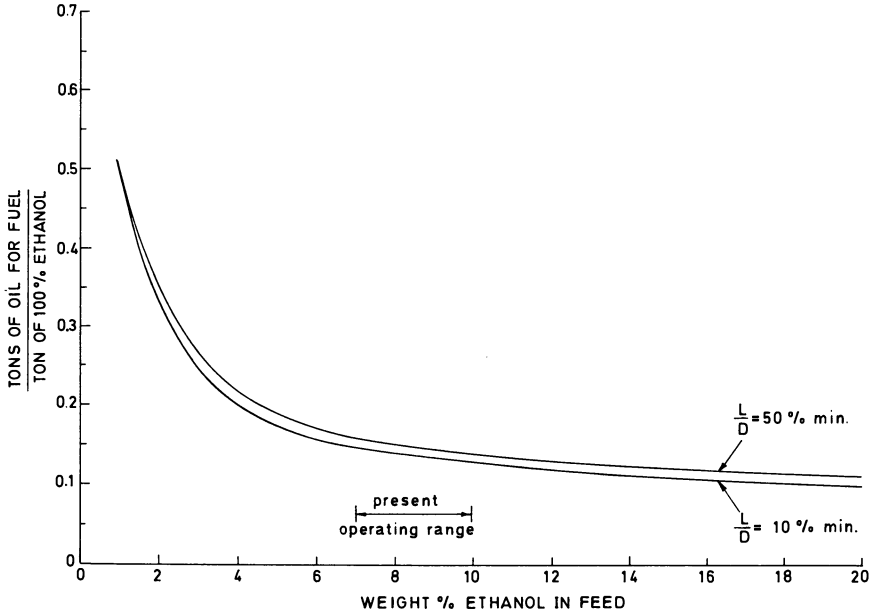

Figure 14. Heat required to distill ethanol from various concentration broths. Note: (L/D) *min* = *1.27.*

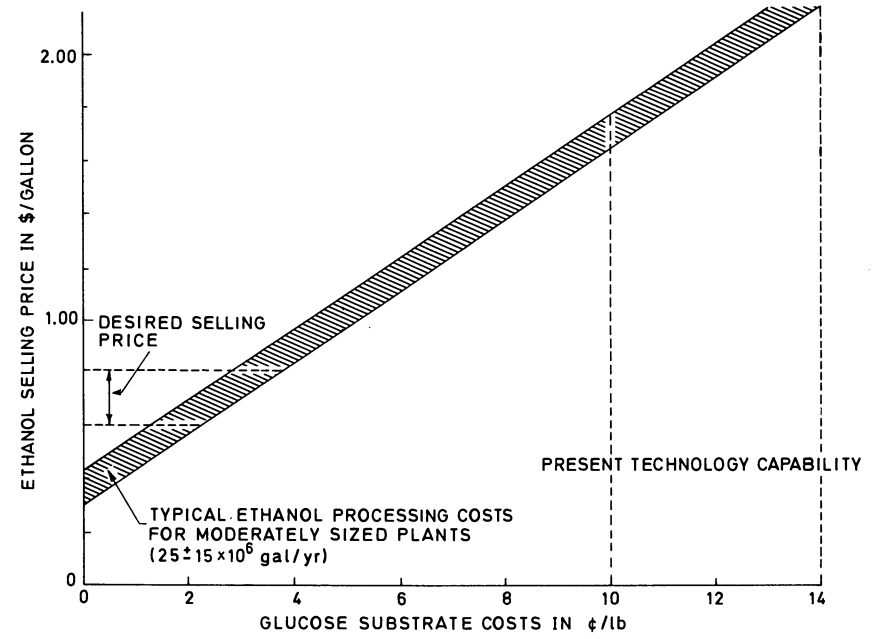

Figure 15. Relation of ethanol selling price to glucose substrate costs

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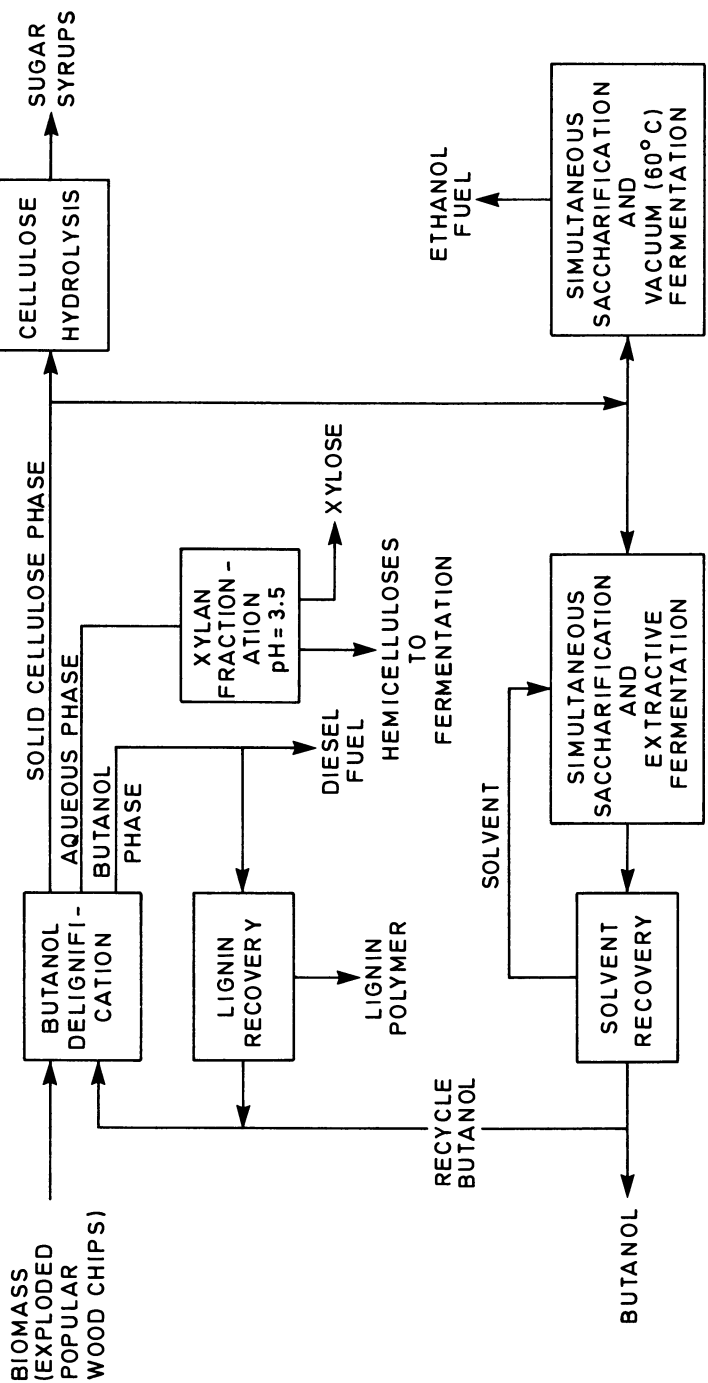

Figure 16. Total biomass utilization concept

Total Biomass Utilization Concept

In all the economic considerations, only the cellulosic portion of the biomass has been considered. Recently, several investigators have pointed out that by-product credits can change the process economics drastically. With simple economics, such as presented here, they have argued that

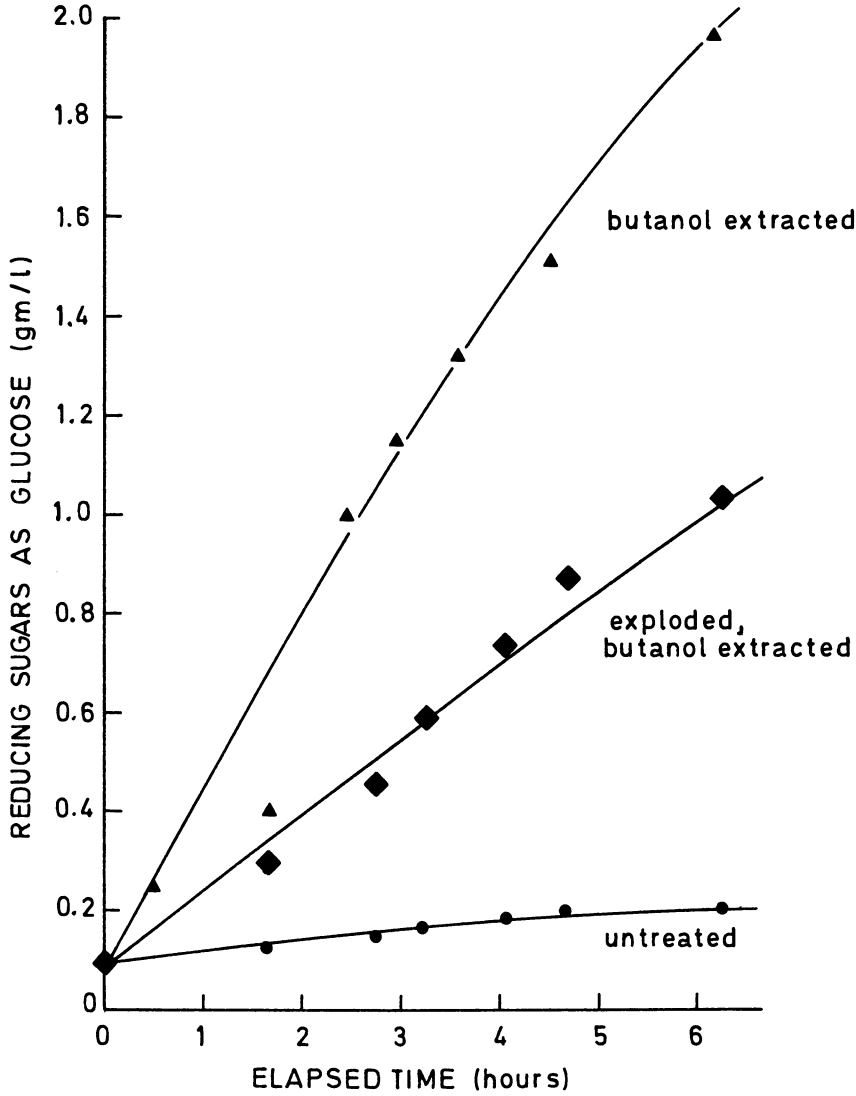

Figure 17. Hydrolysis of poplar chips by extracellular cellulase of Thermoactinomyces YX. *(25 dry g chips/L. Chips wet-milled in blender before hydrolysis.)*

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"total biomass utilization" and by-product credits must be taken into account in order to give cellulose hydrolysis processes any practical standing.

One such proposed "total biomass utilization" scheme is presented in Figure 16 (*31*). The basic concept is to solvent-delignify the biomass in order to recover a valuable lignin product, either for polymer or diesel fuel use, and to utilize the hemicelluloses for alcohol fermentation. Preliminary data suggest that solvent-delignified biomass can be very susceptible to enzymatic hydrolysis (*see* Figure 17 (*31*)). These preliminary results are most encouraging and give optimism to those searching for ways to develop economical hydrolysis processes.

Other developments have occurred that give additional optimism and hope in achieving an economical alcohol process from hydrolyzed cellulose. One such development is the combined saccharification and fermentation technique with saccharifying and alcohol-producing organisms (*35*), i.e., yeast or *Cl. thermocellums*. Another involves the suggestion that the water or dilute product problem might be overcome by high-temperature (60°C) vacuum or solvent-extractive fermentation (*9*). Here, the alcohol concentration relative to the water content of the product could be enhanced in a rather simple, one-step, low-energy-demand process.

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Summary

Based on present technology, cellulose hydrolysis processes do not appear to be economical for the production of sugar syrups or alcohol fuels, particularly if biomass costs are greater than $30/t. However, there is reason for optimism. Most important in contributing to this optimism is the emerging concept of total biomass utilization and the possibility of process by-product credits. Further, cheaper and more potent cellulases are being developed. Cheaper pretreatment methods that give greater cellulose utilization are emerging. Finally, new fermentation techniques are being evolved that minimize the problem of "squeezing the water" out of the fermentation product.

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3

Conformational Effects in the Hydrolysis of Cellulose

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It is proposed that the different conformations of the glycosidic linkages, indicated by Raman spectra of the various polymorphic forms of cellulose, result in different degrees of susceptibility to hydrolytic attack. Structural data for cellulose have been reexamined on the basis that anhydrocellobiose, rather than anhydroglucose, is the basic repeat unit. Comparisons with dimeric structures suggest that both right- and left-handed departures from the two-fold helix conformation are more stable than the two-fold helical structure. The Raman spectra indicate that the conformation in β-methylcellobioside is representative of Cellulose I, while that in cellobiose is representative of Cellulose II. The conformation of the anhydrocellobiose units in Cellulose I provides a basis for explaining the high resistance of native crystalline celluloses to hydrolytic agents.

Discussions of the hydrolysis of cellulose, in both acid media and enzyme preparations, have considered the influence of structure on the rates of hydrolytic degradation (*1–6*). In most instances the primary focus was on the variation in accessibility of the glycosidic linkage among the different types of cellulose. The differences between the responses of the various crystalline polymorphs were considered, but it was noted that the effect of crystalline polymorphic variation could not be separated from the effect of accessibility (*4*). In any event, the influence of polymorphic variation was assessed primarily in light of the prevailing notions concerning the structure of the different polymorphs—in terms of cellulosic chains possessing two-fold screw axis symmetry but packed differently in the crystalline lattice. This view of the structure of the different

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crystalline polymorphs has been based, in the main, on x-ray and electron diffractometric studies, with some elaboration based on infrared spectroscopic studies.

Recent studies of the Raman spectra of Celluloses I, II, and IV have indicated that the different polymorphic forms involve two basically different molecular conformations in addition to the differences in crystalline packing (*7, 8, 9*). The conformation variations suggested by the Raman spectra are such that they could play an important role in determining the susceptibility of glycosidic linkages to attack by hydrolytic agents. The questions raised by this possibility will be addressed in this chapter.

A fundamental conceptual question, which will be central in the following discussion, is should the structure of cellulose be interpreted on the basis that the anhydroglucose unit is the repeating unit in the structure, or is it more appropriate to adopt the view that the anhydrocellobiose unit is the basic repeat unit. It will be held that the latter view can provide a more consistent interpretation of structural information. It has been recognized for some time in studies of the chemistry of cellulose that the repeat unit must be taken as the anhydrocellobiose unit. Development of the view that it should be regarded as the basic repeat unit in structural studies, as well, is rooted in the realization that two anhydroglucose units are necessary to define the nature of the linkage between them, and that the steric constraints associated with the β-1,4-linkage are as important in determining the structure of cellulose as they are in determining its chemical reactivity. Within the framework set forth, the conformational variations possible for the β-1,4-linkage in cellulose will be considered, and the potential influence of such variations on the response to hydrolytic attack will be examined.

In order to place the discussion in perspective, the next section will be devoted to a brief review of current ideas concerning the structure of cellulose, as well as to consideration of the implications of two crystallographic studies of cellobiose and β-methylcellobioside. The response of different celluloses to acid and enzymatic hydrolysis will then be examined in light of the structural considerations.

Structures of Cellulose

The primary sources of information concerning the molecular structure of cellulose have been x-ray and electron diffractometric studies, conformational analyses, and vibrational spectroscopy. The work up to 1971 was very ably reviewed by Jones (*10*), and by Tønnesen and Ellefsen (*11, 12*). They generally concluded that although much evidence can be interpreted in terms of cellulose chains possessing a two-fold axis of symmetry, in both Celluloses I and II, none of the structures proposed

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for cellulose until that time could reconcile all of the known data derived from structural studies, primarily x-ray and electron diffractometric. Rees and Skerrett carried out a conformational-energy analysis on cellobiose and xylobiose (*13*), focusing primarily on the contribution of nonbonded interactions while excluding the influence of hydrogen bonding. For the isolated molecules they found that potential minima occurred on either side of the two-fold screw axis conformations. But the magnitudes of the differences in energy between the minima and conformations corresponding to two-fold screw axis symmetry were small enough for them to conclude that hydrogen-bonding energy in the lattice might compensate enough to stabilize a chain structure with two-fold screw axis symmetry. Until recently the more comprehensive studies in vibrational spectroscopy were those of Liang and Marchessault utilizing measurements of IR dichroism on oriented samples of ramie (*14*). On the basis of these studies they proposed a unit cell involving a particular pattern of hydrogen bonding between the chains, which has received popular acceptance as a representative structure of cellulose. Some remaining inconsistencies between the various structures proposed based on IR data, conformational analyses, and diffractometric studies were the basis for the conclusions by Jones and by Tønnesen and Ellefsen that some significant questions remained concerning the structure of cellulose.

Since the reviews cited were published, two groups of investigations of the structure of cellulose have been reported. The first has been based on refinements of the x-ray and electron diffractometric analyses, in one instance in conjunction with analyses of molecular packing in the crystalline lattice. The second group has been based on analyses of the Raman spectra of the four major polymorphs of cellulose. The results of the two groups of studies are not consistent. Those based on diffractometry reaffirm the view that the molecular chains possess two-fold axes of symmetry and continue to interpret the differences between the polymorphic forms in terms of different modes of packing of the chains in the crystalline lattice. The Raman spectral observations, on the other hand, can only be interpreted in terms of significant differences between the conformations of the molecular chains in the different polymorphs. The two groups of studies will be reviewed in somewhat greater detail in the following subsections. The studies of the structure of cellobiose and methylcellobioside will then be examined.

Diffractometric Studies

The primary difficulty in diffractometric studies of polymeric materials in general is that the number of reflections recorded in a fiber diagram is usually less than 100 (*15*). This is in contrast with observation of 1000–2000 reflections in a typical study of the structure of a small

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molecule, where single crystals of optimum size can be grown. In the case of cellulose it is generally difficult to obtain even as many as 50 reflections. Consequently, it is necessary to introduce assumptions concerning the structure of the monomeric entity and to utilize the limited scattering data to determine the orientation of the monomer groups with respect to each other (*15*). In all diffractometric studies of cellulose published so far, the monomeric entity has been chosen as the anhydroglucose unit; the parameters of the glycosidic linkage then are taken among those that are adjustable in search of an optimum fit to the crystallographic data. This is an assumption implicit in all of the analyses, but its validity needs to be questioned in light of the results on the dimeric species to be discussed later.

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In addition to the question concerning repeat unit selection in analyses of the diffractometric data, the studies of the structure of cellulose are complicated by the appearance of reflections that are disallowed by the symmetry generally ascribed to the cellulose chain. In one of the recent studies these reflections, which are rather weak relative to the other main reflections, have been assumed to be negligible (*16*). Thus, the refinement of the diffractometric data must be viewed as one constrained by the assumption of two-fold screw axis symmetry. In the other recent study on the structure of cellulose, the inadequate informational content of the limited number of reflections from the cellulosic samples was complemented by conduct of an analysis of lattice packing energy. Thus the final structures were those constrained to produce minima in the packing energy as well as optimum fits to the diffractometric data (*17*). One of the difficulties in this approach is that it is quite sensitive to statistical weighting in the energy computations. The calculation of packing energies used statistical weighting of hydrogen-bonding potentials relative to Van der Waals interactions that seem unrealistic; the weights were set at 20:1. These statistical weights had been initially derived in fitting the potential functions to crystallographic packing of monosaccharides, where the x-ray diffractometric data is adequate to establish the molecular structure and the relative position of the molecules in the unit cells. Serious questions must be raised, however, when these statistical weights are transferred to a polymeric species. The relative weights are particularly critical for the β-1,4-linkage, where structures possessing two-fold screw axes of symmetry of necessity have the two hydrogens on the 1- and 4-carbons significantly closer than the sum of their Van der Waals radii. Uncertainties concerning the distortions imposed by the statistical weighting, together with use of the anhydroglucose unit as the basic repeat unit in the cellulose molecule, raise serious doubts as to whether or not the remaining questions concerning the structure of the various polymorphs of cellulose have been resolved.

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Raman Spectral Studies

The Raman spectral evidence concerning the structure of cellulose, which has been discussed in detail elsewhere (*8, 9*), has suggested that the two most common polymorphs of cellulose, Celluloses I and II, have basically different molecular conformations. Furthermore, Celluloses III and IV may represent mixed lattices wherein the two basic conformations coexist; their spectra, in general, appear to be superimpositions of spectra of Celluloses I and II. The basic difference between I and II is suggested by the spectra of highly crystalline samples shown in Figure 1; the x-ray powder patterns of these samples are shown in Figure 2. A number of arguments based on theoretical considerations, on comparisons with model compounds, and on comparisons with spectra of solutions, were developed (*8*) to support the conclusion that only different conformations of the molecular chains could account for Raman spectral differences of the magnitude indicated in Figure 1. Analyses of the spectra of single fibers of native cellulose, before and after mercerization, suggested that the dominant conformation in native cellulose is a right-handed helical conformation, while that in mercerized cellulose is a left-handed helical conformation. Departures from the two-fold helix conformation need not be very large ones to account for the spectral differences. More recently, calculations have shown that rotations about the glycosidic linkage of the magnitude contemplated can indeed result in shifts in characteristic bands of the types observed in Figure 1 (*18, 19*).

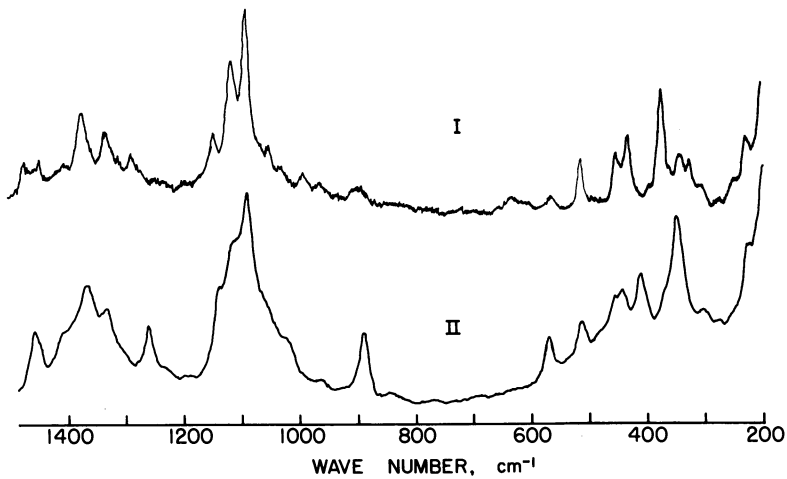

Figure 1. Raman spectra of highly crystalline samples of Celluloses I and II

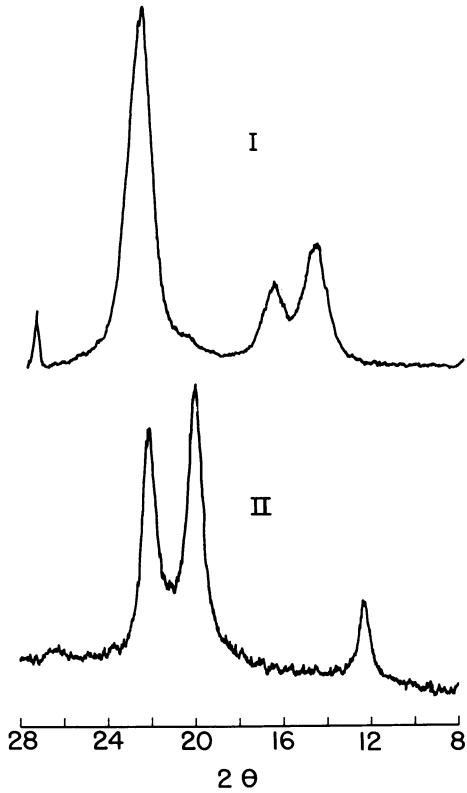

Figure 2. X-ray diffractograms for samples used for spectra in Figure 1. The reflection at 27.6° in diffractogram of Cellulose I is due to TiO_2 used for calibration purposes.

The findings summarized above, together with the information assembled by Rees and Skerrett on conformational analyses, led to a reassessment of the available information concerning the conformation of the glycosidic linkage, its variability, and the assumptions about it implicit in past investigations of the structure of cellulose.

Structures of Cellobiose and Methylcellobioside

A search of the literature on relevant disaccharides revealed two crystallographic studies that proved to be of considerable importance. The study by Chu and Jeffrey of the structure of cellobiose (*20*) had been referred to in the discussions by Rees and Skerrett. Soon thereafter the structure of β-methylcellobioside had been worked out by Ham and Williams (*21*). While the contrast between the two structures is very

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significant and will be considered in detail, it is important to note that in both structures the glycosidic linkage does not exist in a conformation consistent with two-fold screw axis symmetry.

Comparison of the two structures and their relationship to the proposed structures for cellulose is most conveniently discussed in terms of a diagram published by Rees and Skerrett (*13*). It defines conformations of the disaccharides as well as the structures of cellulose in terms of the dihedral angles about the bonds in the glycosidic linkage identified as ϕ and ψ. Though such maps were introduced by Ramachandran (*22*), we have found it convenient to adapt the map developed by Rees and Skerrett, Figure 3. In addition to indicating the regions of minimum potential energy, it includes the loci of structures possessing the same repeat distance for the anhydroglucose unit as well as the loci of structures possessing interoxygen distances of 2.5 and 2.8 Å for the intramolecular hydrogen bond between the C3 hydroxyl and the ring oxygen of the neighboring anhydroglucose unit. The loci of two-fold and three-fold helix structures are represented also. We have added the point corresponding to the structure of β-methylcellobioside, indicated by W.

The first point immediately obvious from the diagram is that the structure of β-methylcellobioside corresponds to a right-handed departure from the two-fold helix line while the structure for cellobiose corresponds to a left-handed departure from the line. The other important contrast between the two structures is that the intramolecular hydrogen bond in cellobiose is rather isolated in the lattice, whereas the corresponding bond in the β-methylcellobioside is bifurcated, with the C6 oxygen of the nonreducing pyranose ring participating significantly in it. The structures are compared in Figure 4 and the hydrogen bonding distances in Figure 5. The distance of 3.1 Å between the C6 oxygen and the C3 hydroxyl oxygen in the case of the cellobiose structure is clearly too large to imply any significant degree of hydrogen bonding. In the structure of β-methylcellobioside the distance is 2.91 Å, which is short enough to produce significant perturbation of the basic hydrogen bond between the C3 hydroxyl and the neighboring ring oxygen.

Observation of these differences suggested examination of the Raman spectra of the two compounds in the OH stretching region. The spectra are shown in Figure 6, where it is clear that isolation of the intramolecular hydrogen bond in cellobiose permits a narrow, rather intense band at 3440 cm^{-1}. In contrast, the spectrum of the β-methylcellobioside shows only broad bands in this region. The significance of this comparison is much enhanced when it is noted that it mimics comparisons of the spectra of Celluloses I and II in the OH stretching region. It was first observed by Marchessault and Liang in their studies of IR spectra of native and

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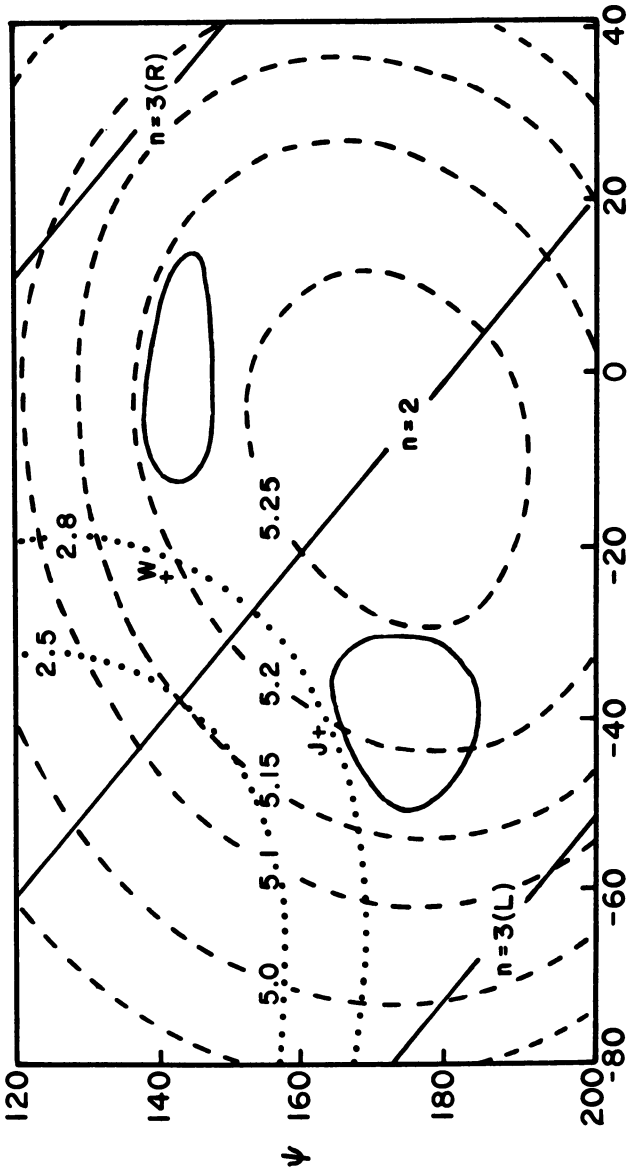

Figure 3. ψ/φ Map adapted from Ref. 13. (– – –) Loci of structures with constant anhydroglucose repeat periods, (· · ·) loci of structures of constant intramolecular hydrogen bond O–O distances, (——) contours of potential energy minima based on nonbonded interactions in cellobiose. J—cellobiose; W—β-methylcellobioside. n = 2, the two-fold helix line; n = 3 the three-fold helix lines, (R) right handed, (L) left handed. The Meyer–Misch structure is at ψ = 180, φ = 0.

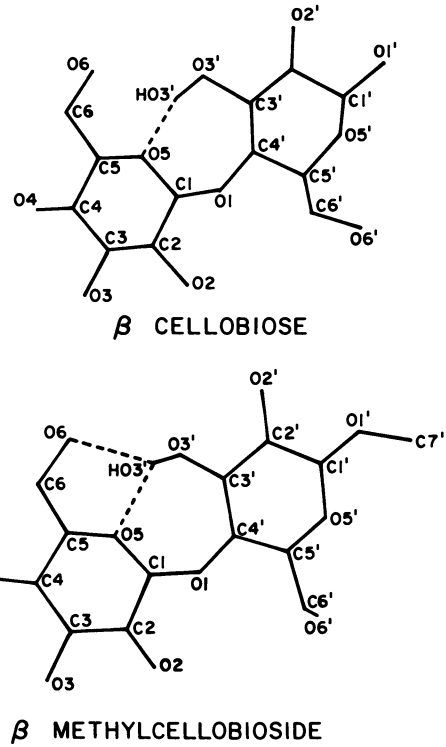

Figure 4. Structures of β-cellobiose (20) and β-methylcellobioside (21)

mercerized ramie (*14, 23*) that the latter possessed sharp absorption bands above 3400 cm^{-1} in the OH stretching region and that the dichroism of these bands was consistent with a transition moment parallel to the chain axis; absorption by native ramie fibers showed only broader bands at lower frequencies. Raman spectra of native and mercerized celluloses also reflect this effect (*24*).

The sharp narrow bands appearing in the OH region of cellobiose and mercerized celluloses imply isolated hydrogen bonds, unperturbed by the anharmonocities associated with participation in extended hydrogen-bonding networks. The appearance of only broad bands in the spectrum of β-methylcellobioside reflects the anharmonicity introduced into the intramolecular hydrogen bond by the additional participation of the C6 oxygen. It may be inferred from the similar spectra of the native celluloses that the C6 oxygens may indeed participate in bifurcated hydrogen bonds similar to the one observed in β-methylcellobioside.

The remaining questions in relation to the comparison made above is concerned with the appearance of two sharp bands in the IR and Raman spectra of mercerized cellulose, while only one such band is seen in the

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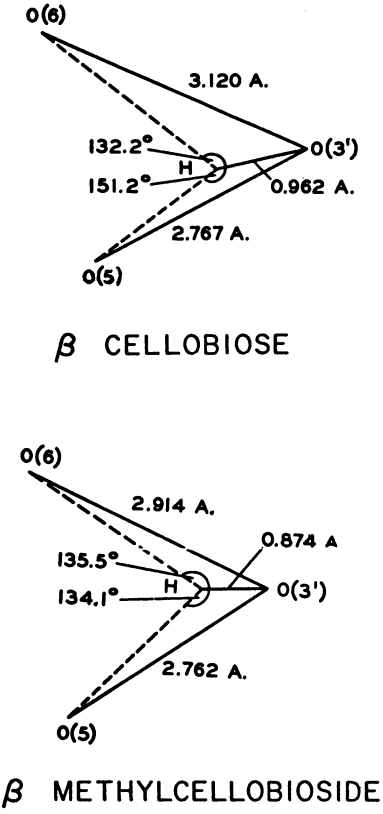

Figure 5. Interatomic distances at the site of the intramolecular hydrogen bond (21)

spectrum of cellobiose. The spectrum of cellotetraose, recorded in the same region, also possesses two sharp bands almost identical to those in the spectrum of mercerized cellulose (*18, 19*). There are indications that the broad band showing a high degree of dichroism in the IR spectra of native cellulose, as well as the band that might correspond to the same region in the Raman spectra of native celluloses, are both made up of two overlapping broad bands. The implication of all of these observations is that nonequivalent glycosidic linkages occur in both mercerized and native cellulose lattices.

The considerations involving comparisons of the structures of cellobiose and β-methylcellobioside with the structures of mercerized and native cellulose, respectively, when taken together with the additional observation that the basic repeat unit derived from the diffractometric studies is 10.3 Å rather than 5.15 Å, require that data relating to the structure of cellulose be reexamined with the constraint that the anhydrocellobiose unit, rather than the anhydroglucose unit, is the basic repeat unit. To the author's knowledge, no efforts have been made to interpret

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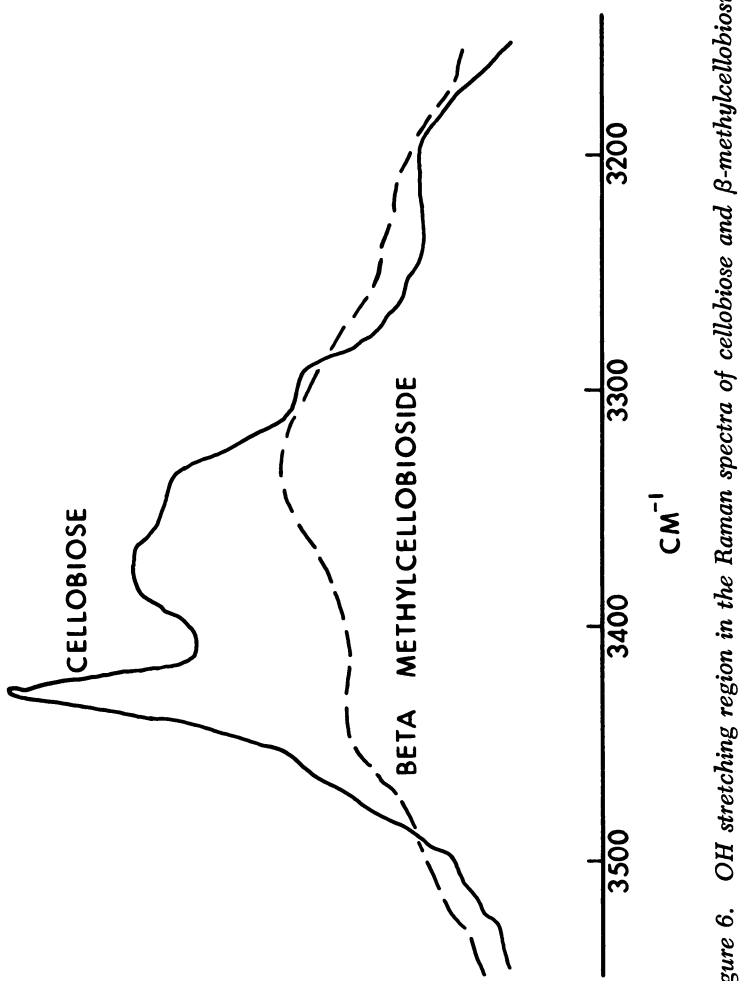

Figure 6. OH stretching region in the Raman spectra of cellobiose and β-methylcellobioside

the diffractometric data in terms of a repeat unit based on the structure of cellobiose or on the structure of β-methylcellobioside. Experience with other polymers suggests that this avenue should be fully explored before the variety of recently published structures are reaffirmed as the ones most closely approximating the various crystalline polymorphs of cellulose.

The relevance of the question discussed above to the problem of hydrolytic degradation of cellulose arises from both stereochemical and electronic factors associated with the difference in conformation of the glycosidic linkage as well as with the participation of the C6 oxygen in the bifurcated intramolecular hydrogen bond. The implications of these factors will now be considered.

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Conformational Effects in Hydrolytic Degradation

Among the patterns that emerge fairly early in any examination of the published literature on acid hydrolysis and on enzymatic degradation of cellulose are the many similarities in the response to the two classes of hydrolytic agents. In both instances a rapid initial conversion to glucose and cellodextrins is followed by a period of relatively slower conversion, the rate of conversion in this second period depending on the prior history of the cellulosic substrate. In general, the non-native polymorphic forms are degraded more rapidly during this second phase. In addition, it is found that the most crystalline of the native celluloses are particularly resistant to attack, with the most highly crystalline regions converted much more slowly than any of the other forms of cellulose.

The relationship of the patterns of hydrolytic susceptibility to the range of conformational variation discussed above can be interpreted in terms of the contrast between the states of the glycosidic linkage in cellobiose and β-methylcellobioside. The differences between the states that are likely to contribute to differences in observed reactivity are of two types. The first is differences in the steric environment of the glycosidic linkage, particularly with respect to activity of the C6 group as a steric hindrance to, or as a potential promoter of, proton transfer reactions, depending on its orientation relative to the adjacent glycosidic linkage. The second type of difference is electronic in nature and involves readjustment of the hybridization of the bonding orbitals at the oxygen in the linkage. The potential contribution of each of these effects will be considered.

Steric Effects. Careful examination of scale models of the cellodextrins reveals that when C6 is positioned in a manner approximating the structure in β-methylcellobioside, the methylene protons are so disposed that they contribute significantly to creation of a hydrophobic protective environment for the adjacent glycosidic linkage. If, however,

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rotation about the C5 to C6 bond is allowed, the primary hydroxyl group can come into proximity with the linkage and provide a potential path for more rapid proton transfer.

If, as suggested above on the basis of spectral data, the orientation of the C6 group in native cellulose is locked in by its participation in the bifurcated hydrogen bond to the C3 hydroxyl group, it may be responsible for the high degree of resistance to hydrolytic attack. Access to the linkage oxygen would be through a relatively narrow solid angle, barely large enough to permit entry of the hydronium ions that are the primary carriers of protons in acidic media (*25*). If, on the other hand, the C6 group has greater freedom to rotate, as is likely to be the case in Cellulose II, the hindrance due to the methylene protons can be reduced and, in some orientations, the oxygen of the primary hydroxyl group may provide a tunneling path, or a stepping stone, for transfer of protons from hydronium ions to the glycosidic linkages. This would result in greater susceptibility of non-native celluloses to hydrolytic attack.

The hypothesis put forth concerning steric effects in acid hydrolysis would have as its corollary the proposal that the role of the C_1 component in cellulase enzyme system complexes is to disrupt the engagement of the C6 oxygen in the bifurcated intramolecular hydrogen bond and thus permit rotation of the C6 group into positions more favorable to hydrolytic attack.

The key role of C6 in stabilizing the native cellulose lattice is supported by recent findings concerning the mechanism of action of the dimethyl–sulfoxide–paraformaldehyde solvent system, which is quite effective in solubilizing even the most crystalline of celluloses. The crucial step in the mechanism proposed for the action of this system is substitution of a methylol group on the primary hydroxyl at the C6 carbon (*26, 27*).

Electronic Effect. While the size of the system of bonds in the glycosidic linkage is clearly too large to permit computational investigation of the electronic structure by methods currently available, a number of qualitative observations related to the constraints of bond geometry are indicated. First it is clear that the hybrids of oxygen orbitals involved in the bonds to the carbons must be nonequivalent because the bond distances differ to a significant degree (*20, 21*). The angle of approximately 116 deg imposed on the linkage is likely to result in greater differences between the bonding orbitals and the lone pair orbitals than might be expected in a typical glycosidic linkage. Among themselves, the lone pair orbitals are likely to be nonequivalent because of their different disposition with respect to the ring oxygen adjacent to C1 in the linkage; the differences may be small and subtle, but they are no less real. Given these many influences on the nature of hybridization at the oxygen in the linkage, it seems most unlikely that they would remain

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unaltered by changes in the dihedral angles of the magnitude of the difference between cellobiose and β-methylcellobioside. Hence a difference in electronic character must be expected.

The present state of the art does not permit estimation of the magnitude of the effects discussed, nor any speculation concerning the direction of changes in the relative reactivity of the glycosidic oxygen in the two different conformations. Yet it is clear that differences can be anticipated and they may be viewed, within limits of course, as altering the chemical identity of the glycosidic linkage as its conformation changes. It remains for future studies to define the differences more specifically.

Concluding Proposal

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In light of the observations relating to the structures of the two primary polymorphs of cellulose, as well as the variations in reactivity that have been rationalized in terms of their differing conformations, it is useful to consider an alternative conceptual framework for describing the structures of cellulose. As noted in the discussion of diffractometric studies, it has been the general practice to regard cellulose as a polymer of anhydroglucose units connected by β-1,4-linkages. An alternative view, which is perhaps more provocative of new insight into the nature of cellulosic matter, is that the cellulose chain is a polymer of glycosidic linkages held together by anhydroglucose units. Such a view, when taken together with the possibility of different stable states of the linkage, corresponding to right-handed and left-handed conformations, elicits an interpretation of the phenomenology of cellulose in terms of a multistate model, not unlike the rotational isomeric model applied in the analysis of many other polymers with less constrained linkages. A detailed discussion of the implications of this proposal with respect to the physical and chemical properties of cellulose is beyond the scope of the present report. However, analyses of data on the hydrolysis of the glycosidic linkages need to be undertaken in light of the considerations which underly our proposal. In particular, variation of reactivity with conformation may require that the different polymorphs be viewed as differing in chemical character, as well as in physical structure.

Acknowledgments

The author wishes to express appreciation to K. P. Carlson for acquisition of the Raman spectra of the disaccharides and for much valuable discussion. The spectra of celluloses were acquired by R. Whitmore. Support of this work from institutional research funds of The Institute of Paper Chemistry is gratefully acknowledged.

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Influence of Fine Grinding on the Hydrolysis of Cellulosic Materials—Acid Vs. Enzymatic

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The relative influence of ball milling for various time periods on the course of enzymatic and dilute acid hydrolysis was investigated. The response of enzymatic hydrolysis to the extent of milling was quite dramatic. Cotton linters pulp was totally hydrolyzed in 10 days with 60 min of milling. The carbohydrates of red oak were 93% converted to sugar in the same time after 240 min of milling. It appears that, with sufficient milling, the carbohydrates of the three lignocelluloses investigated can be made almost totally accessible to enzymatic hydrolysis. Vibratory milling also yields substantial increases in the rates of dilute acid hydrolysis of all four substrates, nearly nine-fold for the cotton linters pulp and about five-fold for the three lignocelluloses. Thus vibratory milling represents an experimentally effective pretreatment.

Lignin and cellulose crystallinity are major deterrents to the chemical, enzymatic, and microbiological conversion of lignocellulosic residues to useful products. Lignin restricts enzymatic and microbiological access to the cellulose. Crystallinity restricts the rate of all three modes of attack on the cellulose. Thus if we are ever to make full use of the carbohydrate values contained in the many millions of tons of currently unused lignocellulosics generated in this country each year, some form of pretreatment must be employed to alter the fine structure of cellulose as well as disrupt or open up the lignin–carbohydrate complex.

[1] Maintained at Madison, WI, in cooperation with the University of Wisconsin.

Of the many physical and chemical procedures thus far applied to enhance lignocellulose reactivity (*1*), fine grinding appears to offer the most direct response to both lignin and crystallinity. When the grinding is done in a vibratory ball mill, particle size can be reduced to micron dimensions, with attendant expansion of external surface area, and crystallinity can be essentially eliminated (*2*). This combination of events markedly influences carbohydrate accessibility and, hence, the degree of response of a milled lignocellulosic material to chemical, enzymatic, and microbiological attack.

The enhancing influence of fine grinding on enzymatic and microbiological attack has been amply demonstrated (*3–11*). Thus far, however, the relationship between fine grinding and chemical reactivity appears to have been neglected. This investigation was undertaken to fill this void by evaluating the influence of fine grinding on the kinetics of the saccharification process, both enzymatic and dilute acid. It is based on the responses derived from four cellulosic materials: cotton linters, newsprint, Douglas fir, and red oak.

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Methods and Materials

Fine Grinding. Vibratory ball milling was done in the Forest Products Laboratory mill (*5*) using a sample charge of 10 g of dry material (48 hr at 40°C and 5 mm Hg) previously ground to pass a ½-mm screen. The milling jar and its charge of 5 kg of ½ in. (6.35 mm) polished and hardened steel balls was dried 4 hr under the same conditions and then cooled to room temperature in a desiccator. Milling times ranged from 0 to 240 min. Separation of the milled product from the balls was accomplished by mechanical agitation on a screened pan (Tyler Ro-Tap).

Cellulose Crystallinity. The cellulosic powders were pressed into pellets at 30,000 psi. X-ray diffraction traces were obtained on a Rigaku Denki diffractometer using nickel-filtered copper radiation over the angular range 10° to 30°. Crystallite size of the cellulose in the materials that were not ball-milled was determined by measuring the width of the 002 reflections. The crystallinity index (CI) proposed by Segal et al. (*12*) was used as a measure of the degree of lateral order. These indices are expressed as a percentage. Subject to the validity of the two-phase model of cellulose, this percentage is often used as a rough indication of the percentage of crystalline cellulose.

Enzyme Hydrolysis. Hydrolyses were conducted by the method of Moore et al. (*10*). This involves a digestion of 0.2-g samples with 6 mL of buffered enzyme solution at 40°C with constant rotation to effect continuous mixing. After the prescribed time period, a suitable aliquot is diluted, and Nelson's modification of the Somogyi procedure (*13*) is used to determine reducing sugar content. Maximum sugar yield for each level of vibratory milling was established on the basis of five hydrolysis periods ranging from 0 to 16 days. Residual potential sugar contents of hydrolyzed residues were determined by a quantitative acid saccharification as described in the following section.

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Dilute Acid Hydrolysis. Hydrolyses were carried out at 180°C in sealed glass ampoules charged with 25 mg of sample and 0.25 mL of 0.1*N* H_2SO_4 according to the procedures described by Millett and Goedken (*14*). Five hydrolysis times, ranging from 4 to 160 min, were used for each milled sample. The ampoules were then carefully opened and their contents quantitatively transferred to microgooch crucibles and washed with about 20 mL of water. The filtrates were collected in 100-mL volumetric flasks, diluted to volume, and analyzed for net sugar yield (*14*). Residual potential sugar content of the residues was determined by quantitative saccharification involving a primary hydrolysis with 0.5 mL of 72% H_2SO_4, a secondary hydrolysis with 4% H_2SO_4, neutralization with calcium carbonate, and colorimetric analysis for total reducing sugars (*14*). Initial potential sugar content of each of the milled samples prior to hydrolysis was determined in the same manner. The data thus obtained provided the basis for graphical estimation of rates of hydrolysis and maximum sugar yields for each of the various levels of milling given to the four cellulosic substrates.

Materials. The four cellulosic substrates used in this study were a high-purity, sheeted, cotton linters pulp obtained from the Buckeye Cellulose Corp., newsprint from one of our local daily papers, bark-free Douglas fir wood, and bark-free red oak wood. All materials were broken down in a Wiley mill by successive passages through a series of decreasing screen sizes, with the final test samples being that stock passing through a ½-mm mesh. As indicated above, 10-g portions of these ground samples were vacuum-oven-dried prior to vibratory ball milling.

Results and Discussion

Crystallinity. Diffraction traces of the cellulosic materials are presented in Figures 1–4. Table I lists the crystallinity indices obtained from the diffraction patterns. Also included in Table I are the crystallite sizes of the unmilled materials obtained from measurement of the half widths of the 002 reflections. The 002 reflection is noticeably sharper in the case of the cotton cellulose. Crystallite size measures 54 Å for the cotton cellulose compared to an average of 29 Å from the wood celluloses.

Table I. Crystallinity Indices of Ball-Milled Materials

Ball Milling Time (min)	*Cotton*		*Newsprint*		*Douglas Fir*		*Red Oak*	
	CI (%)	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*	*CI (%)*	*Crystallite Size (Å)*
0	79	54	55	31	48	29	38	27
10	43	—	37	—	26	—	24	—
30	14	—	10	—	~3	—	6	—
60	0	—	0	—	0	—	0	—
120	0	—	0	—	0	—	0	—
240	—	—	—	—	—	—	0	—

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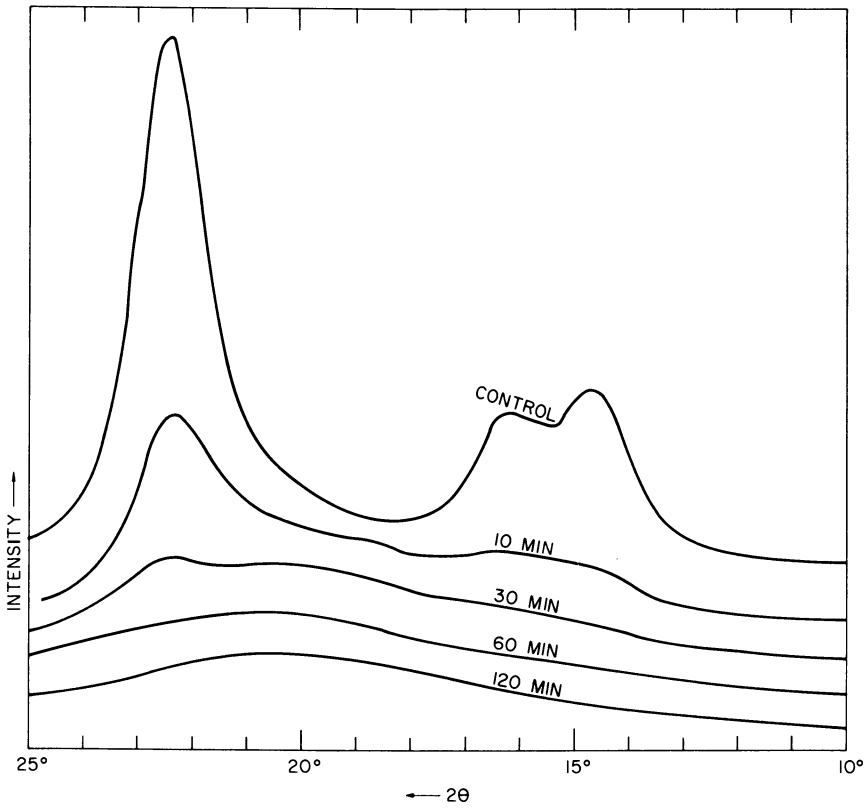

Figure 1. X-ray diffraction from cotton linters as affected by time of vibratory ball milling

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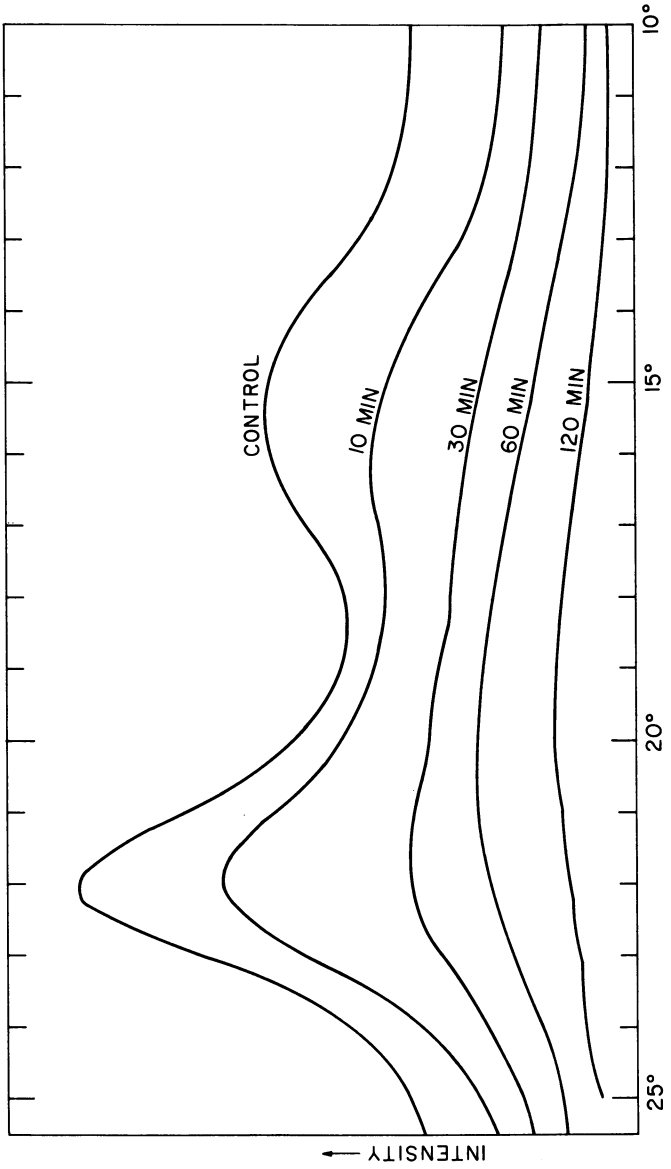

Figure 2. X-ray diffraction from newsprint as affected by time of vibratory ball milling

The effect of ball milling is similar in all cases. Within the first 10 min of milling, the 002 reflection is considerably broadened and appears slightly shifted to a smaller angle. This apparent shift is probably the result of the superposition of the broadened 002 peak upon the rising, broad, "amorphous" peak which centers about 18.5° (2Θ). As ball milling continues, the crystalline characteristics decrease and the amorphous characteristics increase. After about 1 hr of milling time, the crystalline characteristics of all the cellulosic materials have disappeared.

The use of CI to represent the percentage of the crystalline component is unjustified among such a diversity of cellulosic materials, whose lignin components vary from 0 to about 30%. CI, as used here, is not intended to represent the proportion of the crystalline component. Rather, CI provides a means of quantifying the characteristics of the x-ray diffraction patterns. When applied to a set of similar samples, CI is a convenient measure of the degree of lateral order.

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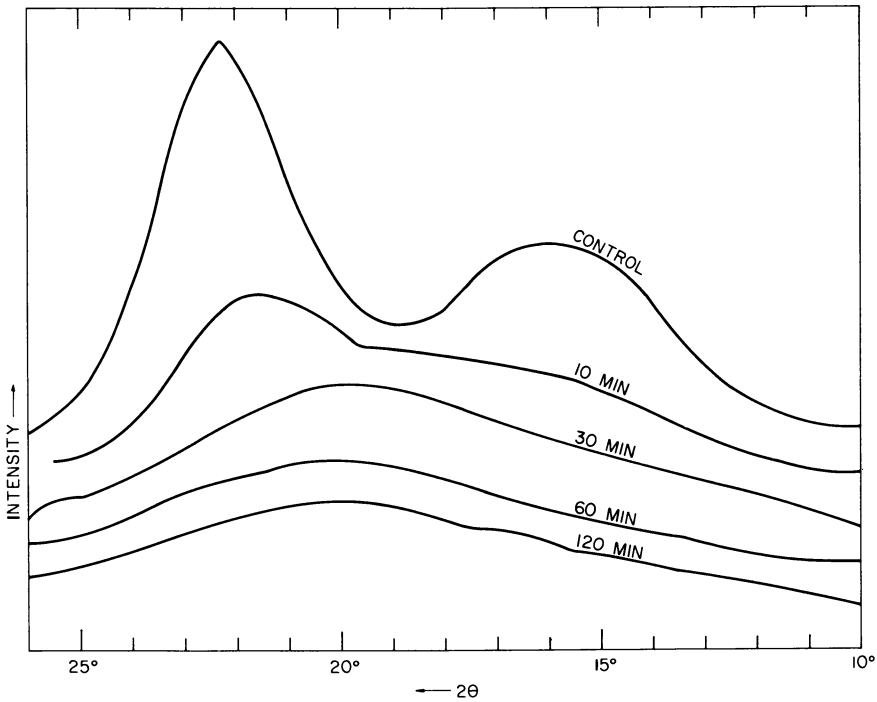

Figure 3. X-ray diffraction from Douglas fir as affected by time of vibratory ball milling

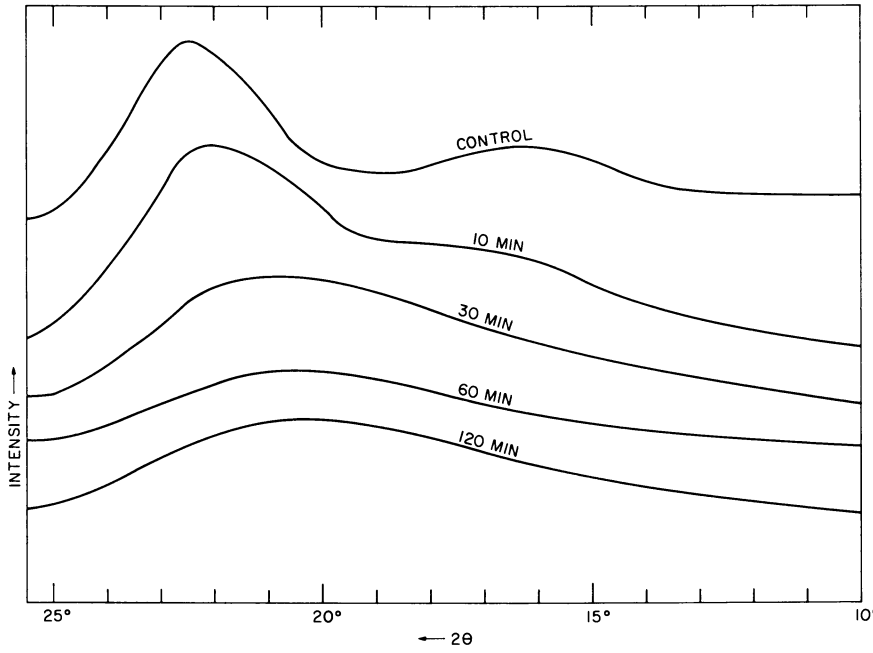

Figure 4. X-ray diffraction from red oak as affected by time of vibratory ball milling

Enzymatic Hydrolysis. All experimental data pertaining to the influence of fine grinding on the hydrolysis of the four cellulosic substrates are presented in Table II and plotted in the form of rate curves in Figures 5 and 6. The all-important roles played by cellulose accessibility and crystallinity in governing the course of enzymatic hydrolysis are readily apparent. With the unmilled starting materials, for example, approximately 10% of the total carbohydrates of Douglas fir and 6% of those for red oak are readily accessible and convertible to sugars in 2 days or less. Hydrolysis then ceases, probably due to complete shielding of the remaining carbohydrate by lignin. With cotton linters, hydrolysis proceeds at a moderate pace for the first 8 days of digestion and then slows down to an almost asymptotic approach to an 80% yield value (Figure 5). As suggested by Katz and Reese (*15*), this retardation in rate can well be the result of enzyme inactivation by hydrolysis products over the extended time periods used. Addition of fresh enzyme might have allowed hydrolysis to proceed to completion. In any event, total hydrolysis of a highly crystalline, lignin-free cellulose is a relatively slow process.

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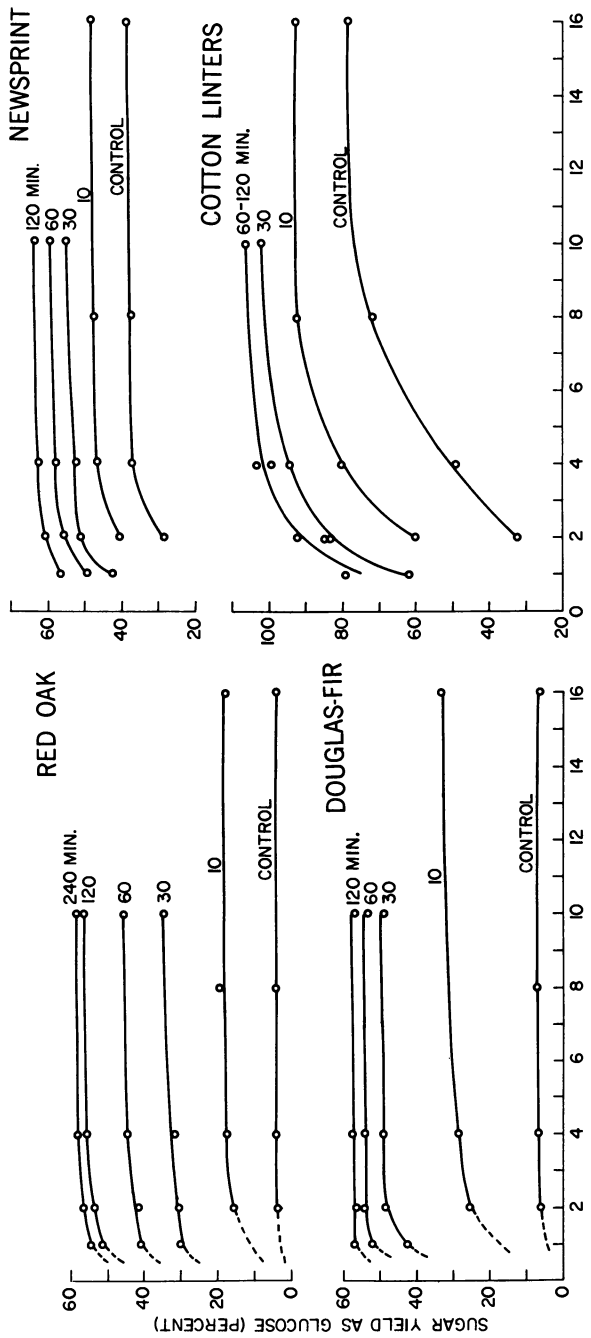

Figure 5. Effect of vibratory ball milling on the rate and extent of enzymatic hydrolysis

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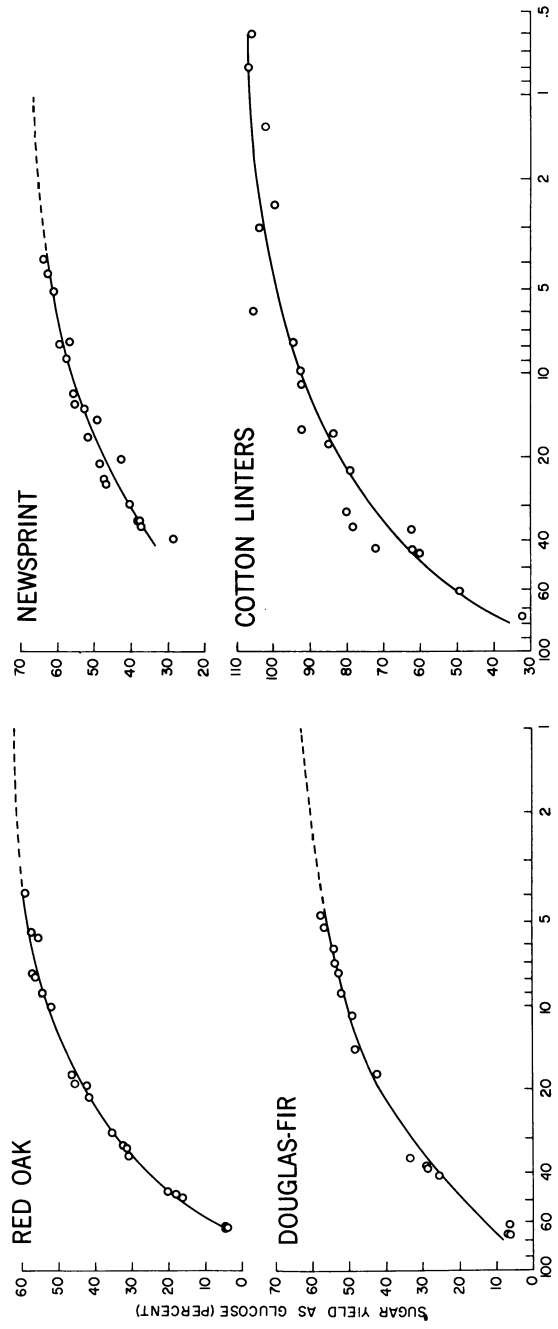

Figure 6. Effect of ball milling on sugar yield—enzymatic hydrolysis

Table II. Effect of Fine Grinding

Time of Milling (min)	*Time of Hydrolysis (days)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
0	0	—	—	—	—
	2	32.4	28.6	6.2	3.7
	4	49.3	37.1	6.7	4.0
	8	72.0	37.5	7.0	3.9
	16	78.5	38.7	6.3	4.0
10	0	—	—	—	—
	2	60.1	40.5	25.7	15.6
	4	80.1	46.8	28.8	17.3
	8	92.2	47.4	29.3	19.5
	16	92.4	48.4	33.6	17.3
30	0	—	—	—	—
	1	62.4	42.5	42.2	30.0
	2	85.2	51.2	48.5	30.4
	4	94.5	52.5	49.1	31.9
	10	102.0	55.1	48.9	34.9
60	0	—	—	—	—
	1	79.2	49.0	52.1	40.9
	2	92.4	55.4	53.9	41.5
	4	99.3	57.8	54.0	44.9
	10	106.7	59.4	52.9	45.6
120	0	—	—	—	—
	1	62.1	56.7	56.9	51.3
	2	83.5	60.9	56.7	53.7
	4	103.7	62.5	57.8	55.7
	10	105.4	63.9	56.9	56.6
240	0	—	—	—	—
	1	—	—	—	54.8
	2	—	—	—	56.5
	4	—	—	—	58.0
	10	—	—	—	58.3

[a] All listed values are averages of duplicate determinations.

Results with newsprint are roughly intermediate between those for wood and cotton linters. Hydrolysis of the delignified chemical pulp component (about 20% of the total newsprint furnish) and of the accessible carbohydrates of the groundwood component is accomplished with about 4 days of digestion. Further hydrolysis of the groundwood carbohydrate is then stymied by lignin as in the case of red oak and Douglas fir.

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on Enzymatic Hydrolysis[a]

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
108.0	69.6	69.4	62.8
75.9	39.2	63.5	62.1
61.4	35.4	68.2	62.1
42.4	33.8	62.9	61.0
35.8	33.5	61.3	60.4
104.4	70.3	69.4	62.8
44.6	29.8	40.9	48.5
36.5	25.0	38.2	47.6
15.9	23.8	37.4	46.3
10.8	21.1	35.1	45.7
103.2	69.0	69.2	59.6
36.2	20.2	17.7	34.5
18.0	16.9	14.3	32.2
7.8	13.2	10.9	31.4
1.3	12.9	10.7	28.7
109.7	68.9	71.8	60.8
22.4	14.7	9.0	21.2
9.7	11.8	7.1	19.2
2.5	8.8	6.2	19.0
0.8	7.9	7.6	17.8
105.6	67.9	69.0	58.1
43.0	6.7	5.2	10.0
16.4	5.1	4.9	9.0
3.0	4.4	4.7	7.9
0.6	3.9	4.8	7.8
—	—	—	57.0
—	—	—	5.7
—	—	—	5.4
—	—	—	3.9
—	—	—	4.1

Vibratory milling enhances both the rate and extent of enzymatic hydrolysis, as shown by the families of curves in Figure 5. With sufficient milling, nearly theoretical yields of reducing sugars appear to be obtainable on all four substrates. This is best illustrated by the curves in Figure 6, which represent a composite of all of the data listed for each substrate in Table II. The data in Table II show that, under the experimental

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conditions employed in this study, cotton linters pulp is totally hydrolyzable with about 60 min of milling and 10 days of enzyme digestion. The carbohydrates of red oak are 93% convertible to sugar with 240 min of milling over the same digestion period. Both Douglas fir and newsprint have a milling requirement in excess of 120 min to attain close to their theoretical conversion limits.

Dilute Acid Hydrolysis. Data relative to the acid saccharification of the four substrates at various levels of vibratory ball milling are given in Table III and plotted as rate and yield curves in Figures 7 and 8. Table IV summarizes the half-life and maximum sugar yield values derived from these curves.

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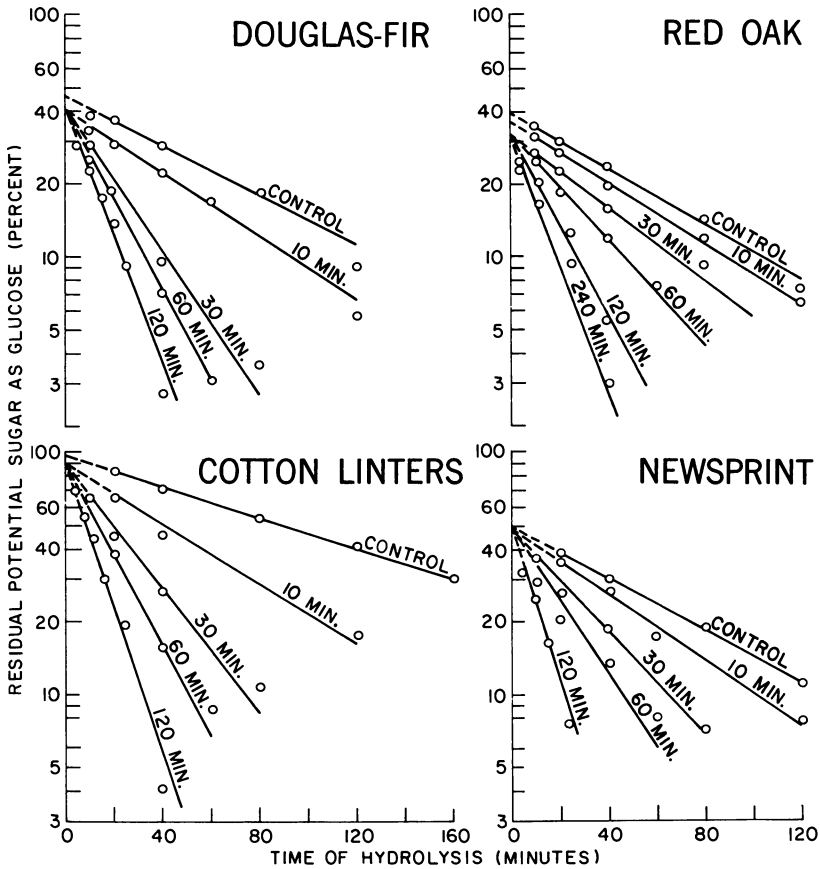

Figure 7. Effect of ball milling on rate of acid hydrolysis

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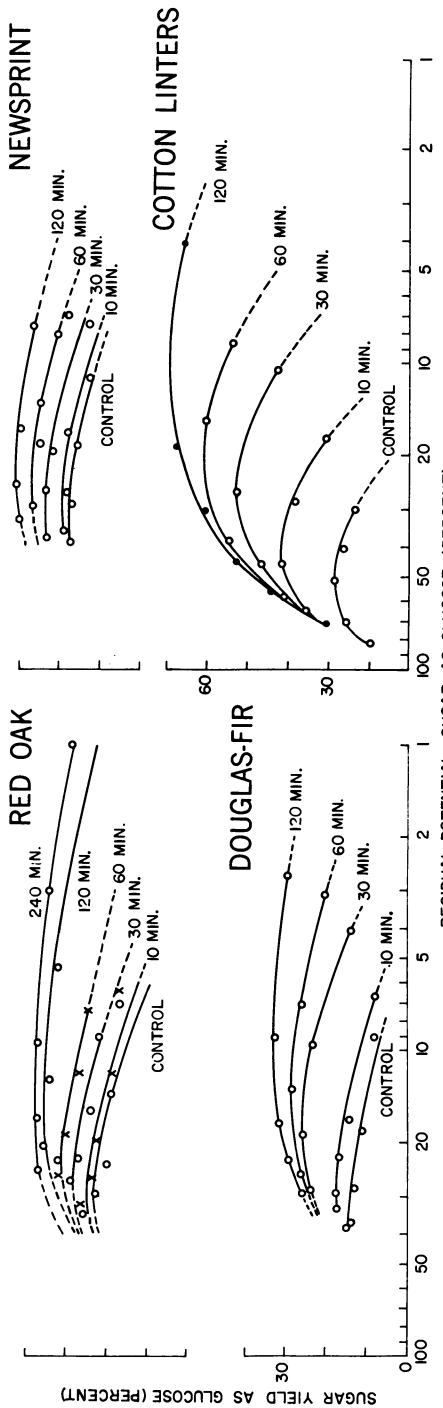

Figure 8. Effect of ball milling on sugar yield—acid hydrolysis

Table III. Effect of Fine Grinding on

Time of Milling (min)	*Time of Hydrolysis (min)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
0	0	—	—	—	0.6
	10	—	—	24.3	25.2
	20	19.7	17.4	28.4	22.2
	40	25.6	17.2	22.9	19.6
	80	28.5	15.4	21.0	18.4
	120	26.1	12.3	18.0	16.3
	160	23.4	—	—	—
10	0	—	—	—	1.0
	10	—	—	27.2	26.1
	20	35.4	18.6	27.2	23.7
	40	41.6	19.5	26.5	22.1
	60	—	18.0	23.9	—
	80	38.1	—	—	18.5
	120	30.5	12.4	18.1	16.3
30	0	—	—	—	1.2
	10	35.6	23.0	33.7	28.2
	20	46.3	23.2	35.6	26.2
	40	52.8	21.2	33.1	23.3
	80	42.5	18.6	24.2	21.1
60	0	—	—	—	1.1
	10	40.9	26.3	36.0	31.6
	20	54.5	24.8	38.3	29.7
	40	60.0	22.6	36.0	26.6
	60	53.2	20.4	30.8	24.4
120	0	—	—	—	1.1
	4	30.7	—	—	31.8
	5	—	29.6	35.7	—
	8	44.0	—	—	—
	10	—	30.7	39.0	—
	12	52.8	—	—	35.0
	15	—	29.5	41.2	—
	16	60.3	—	—	—
	25	67.5	26.4	42.5	33.4
	40	65.2	—	29.6	31.4
	60	—	—	—	25.2

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Acid Hydrolysis (0.1*N* H_2SO_4 at 180°C)[a]

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
108.0	69.6	69.4	62.8
—	—	38.5	34.6
82.6	38.4	36.7	29.8
70.3	29.6	28.7	23.6
52.8	18.5	18.3	14.0
40.9	11.1	9.1	7.1
30.0	—	—	—
104.3	70.3	69.4	62.8
—	—	33.0	81.6
65.0	35.9	29.5	26.8
45.2	26.8	22.2	19.7
—	16.9	16.9	—
28.4	—	—	11.9
17.4	7.5	5.7	6.4
103.2	69.0	69.8	59.6
64.8	37.2	28.8	26.7
45.3	26.1	19.0	22.7
26.4	19.6	9.6	15.6
10.5	7.0	3.6	9.0
109.7	68.9	71.8	60.8
58.6	29.3	25.3	25.1
38.3	20.3	13.4	18.7
15.3	13.6	7.1	11.7
8.6	8.1	3.1	7.4
105.5	67.9	69.0	58.1
71.3	—	—	22.9
—	32.4	29.1	—
55.3	—	—	—
—	25.0	22.8	—
44.7	—	—	20.5
—	16.4	17.3	—
30.1	—	—	—
19.1	7.6	9.1	12.5
4.1	—	2.7	5.4
—	—	—	0.9

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Table III.

Time of Milling (min)	*Time of Hydrolysis (min)*	*Sugar in Hydrolysate as Glucose (%)*			
		Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
240	0	—	—	—	1.0
	4	—	—	—	36.1
	5	—	—	—	—
	10	—	—	—	—
	12	—	—	—	36.5
	15	—	—	—	—
	25	—	—	—	36.2
	40	—	—	—	33.7
	60	—	—	—	27.1

[a] All listed values are averages of duplicate determinations.

Inspection of the data in Table IV shows that fine grinding causes a substantial increase in the rates of hydrolysis of all four substrates—nearly nine-fold for cotton linters and about five-fold for newsprint and Douglas fir after 120 min of milling. Red oak is somewhat less responsive, requiring 240 min of milling for a five-fold rate increase.

Maximum sugar yields exhibited a similar response to vibratory milling—a 2.4-fold increase in yield for cotton linters, a 1.8-fold increase for Douglas fir, a 1.7-fold increase for newsprint, and a 1.5-fold increase for red oak with 120 min of milling.

The batch hydrolysis conditions used in this work are not optimum for maximum production of sugar but were chosen many years ago at this laboratory for their sensitivity and convenience as a standard procedure for assessing the hydrolytic reactivity of a wide variety of cellulosic materials. Considerable improvement in maximum sugar yields is possible through the use of higher temperatures and acid concentrations in batch hydrolyses or through the use of percolation procedures whereby sugars are removed from the reaction zone as quickly as possible (*16, 17*). In no case, however, is it possible to conduct a dilute acid saccharification of a cellulosic material without some degree of sugar destruction. Thus maximum sugar yield obtained via dilute acid hydrolysis will always be somewhat lower than those attainable via enzymatic hydrolysis, even when both procedures are applied to highly reactive cellulose.

The effect of ball milling on the dilute acid hydrolysis of cellulose is similar in many ways to the effect electron irradiation has on enhancing saccharification (*18*). In the case of cotton linters, for example, maximum irradiation 10^8rd) resulted in a half life of hydrolysis of about 7 min

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Continued

Sugar in Residue as Glucose (%)			
Cotton Linters	*Newsprint*	*Douglas Fir*	*Red Oak*
—	—	—	57.0
—	—	—	24.6
—	—	—	—
—	—	—	—
—	—	—	16.6
—	—	—	—
—	—	—	9.4
—	—	—	3.0
—	—	—	0

Table IV. Effect of Vibratory Ball Milling on the Kinetics of Dilute Acid Hydrolysis[a]

Substrate	*Time of Milling (min)*	*Hydrolytic Half Life (min)*	*Maximum Sugar Yield (%)*
Cotton linters	0	95	29
	10	48	42
	30	23	53
	60	16	61
	120	11	69
Newsprint	0	56	18
	10	45	19
	30	29	24
	60	20	27
	120	10	31
Douglas fir	0	60	24
	10	47	27
	30	21	36
	60	16	39
	120	12	43
Red oak	0	53	23
	10	48	25
	30	40	28
	60	27	31
	120	16	35
	240	11	37

[a] 0.1*N* H_2SO_4 at 180°C.

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and a maximum sugar yield of about 70%. Ball milling for 120 min provides a maximum sugar yield of 69% and a hydrolytic half life of 11 min (Table IV). Electron irradiation also enhances markedly the yield and rate of acid hydrolysis of a lignocellulosic, extracted sprucewood (*18*).

It is not clear, however, whether electron radiation would have beneficial effects upon the enzymatic hydrolysis of cellulose. It has been shown that the particle size reduction and surface area increase upon ball milling are probably more important than the reduction of crystallinity (*19*). Electron irradiation may not disrupt the shielding layer provided by the lignin to expose fresh cellulose to the action of large enzyme molecules.

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Summary and Conclusions

The relative influence of vibratory milling on the course of enzymatic and dilute acid hydrolysis of four cellulosic substrates was investigated. The four substrates—cotton linters, newsprint, Douglas fir, and red oak—were vacuum-dried and then milled for various time periods ranging up to 240 min. Assays were then made of rate and extent of hydrolysis, maximum yield of reducing sugar, and cellulose crystallinity.

As anticipated, the response of enzymatic hydrolysis to extent of milling was quite dramatic. Under the assay conditions used, cotton linters pulp was totally hydrolyzed in 10 days with 60 min of milling. The carbohydrates of red oak were 93% convertible to sugar in the same period with 240 min of milling. It appears that, with sufficient milling, the carbohydrates of the three lignocelluloses are totally accessible to enzymatic attack. Thus when compared to the 6% maximum conversion of the original oak sawdust, vibratory milling does represent an experimentally feasible pretreatment.

Vibratory milling also yields substantial increases in the rates of dilute acid hydrolysis of all four substrates: nearly nine-fold for cotton linters and roughly five-fold for the three lignocelluloses. Increases in maximum sugar yields under simple batch conditions ranged from 60% to 140% over the yields for the unmilled materials.

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Absolute Viscosimetric Method for the Determination of Endocellulase (C_x) Activities Based upon Light-Scattering Interpretations of Gel Chromatographic Fractionation Data

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The action of cellulase on hydroxyethylcellulose was studied by agarose gel chromatography, and for molecular weights ranging from 1,200,000 to 100,000, the equation $\overline{M}_v = \overline{M}_n + 73{,}000$ *was valid. The activity was defined as the number of moles glycosidic bonds split per second (katal). Using the relationship between the first three virial coefficients, the decrease of* $\overline{M}_w$ *could be determined by the light-scattering technique. The changes in kinematic viscosity, determined by capillary viscosimetry, allowed the calculation of intrinsic viscosities* $[\eta]$*, extrapolated to zero shear rate, and of the* $\overline{M}_v$ *values. Equations for the endocellulase activity are given. The activity is proportional to the initial decrease of* $[\eta]$ *per second, and the proportionality constant is related to* $[\eta]$ *extrapolated to zero reaction time.*

Review

Assay of Endocellulase Activity. Cellulase is an enzyme complex; a synergistic action between the components is required for a complete hydrolysis of the insoluble cellulose. There is no consensus about the substrate to be used for the cellulase activity measurements.

A determination of the activities of the individual components after purification is very time-consuming and not suitable for practical work, since the number of components can be very high. There are at least 12

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different endo-β-glucanases (C_x) described in the literature, and in the fungus *Sporotrichum pulverulentum* (*1*), 20 cellulases are necessary for breakdown of the cellulose. After purifications, the components can be assayed individually as cellobiase, β-glucosidase, endo-β-1,4-glucanase (C_x), exo-β-1,4-glucanase, cellobiose oxidase, and so on. The assay of the whole complex can be performed by filter-paper or cotton hydrolysis methods by determining the loss in weight or the increase in reducing power, respectively (*2*).

Cellobiase can be measured by following the glucose production from cellobiose and cellodextrins, the saligenin from salicin, and the *p*-nitrophenol formation from its β-glucoside (*2*).

The glucose production from amorphous cellulose (by glucocellulase) and the cellobiose production from crystalline cellulose (by cellobiohydrolase, C_1) can be determined to assay the exo-β-1,4-glucanase.

Finally, the endo-β-1,4-glucanase (C_x) can be assayed using amorphous cellulose, cellodextrins, or water-soluble derivatives by determination of the reducing sugars released or by viscosimetry. It is generally accepted that the release of reducing sugars is not a typical measure for the random action of endocellulases.

Since these enzymes cut the cellulose chain randomly, the average chain length is lowered, decreasing the viscosity after a very short time, although the reducing power is only increased by a relatively minor amount.

The exocellulases, however, have the inverse effect; since small molecules such as glucose or cellobiose are then cut off, the reducing power rises very quickly, while the weight average chain length remains approximately the same.

It is clear that the measurement of the endocellulase activity in the cellulase complex (before purification) using reduction methods will suffer from lack of sensitivity and specificity. Since for low-dispersion cellulose derivatives the viscosimetric average molecular weight is almost equal to the weight average molecular weight, the measurement of the decrease of viscosity will not be influenced very much by cutting off glucose or cellobiose groups.

The viscosimetric method is sensitive due to the random action of endocellulases, since a few linkage breaks will give an appreciable lowering of the molecular weight and of the viscosity.

Different water-soluble cellulose derivatives have been used for the determination of endocellulase activities. Carboxymethylcellulose (CMC) has been proposed as substrate and recently Almin et al. (*3*) have improved their method for the calculation of endocellulase activities, using a medium-molecular-weight CMC ($\overline{M}_v$ = 299,000 and $\overline{M}_n$ = 142,000) with well-defined physicochemical properties. No corrections

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were made for shear stresses in the capillary viscosimeter, and it was assumed without experimental proof that the ratio of the viscosity average to the number average molecular weight was constant, at least during the initial stages of the enzymic degradation. They determined the molecular activities and the Michaëlis–Menten parameters of five different purified endocellulases of *Sporotrichum pulverulentum*.

A limitation of the use of ionic substrates is due to the fact that viscosity measurements depend then on ionic strength, pH, and polyvalent cations, making the comparisons of cellulase activities at different pH values difficult. It is generally necessary to dialyze each enzyme preparation or to use a heavily buffered substrate.

The K_m of the *Trichoderma viride* cellulase is strongly dependent on the degree of substrate substitution (DS), and an extrapolation to DS = 0 gave a K_m value of 3 mg/L for cellulose in a hypothetical solution.

Iwasaki et al. (*4*) proposed to use glycolcellulose as a water-soluble substrate to assay endocellulase. This substrate was obtained from swollen cotton fiber. The authors compare their results with those obtained with CMC but unfortunately give the activities in arbitrary units.

A third water-soluble substrate for endocellulase is hydroxyethylcellulose (HEC). As early as 1931, Ziese (*5*) used HEC and Sandegren et al. (*6*) defined the activity as being proportional to the change of the inverse of the specific viscosity per time unit. Child et al. (*7*) have also used HEC and based their calculation on a linearization of the viscosity measurements according to Eriksson et al. (*8*). They defined an enzymic activity unit as the amount of enzyme causing a viscosity change of 0.001 $\eta_{re}^{-1.26} \cdot \text{min}^{-1}$. The exponential factor was used in order to linearize the data. This unit is useful for comparison of cellulases of different origin, but it is based on an empirical relationship. The authors made an evaluation of their method in comparison with CMC hydrolysis.

Properties of the Substrate HEC in Relation with its Enzymic Hydrolysis. HEC is the reaction product of cellulose with ethylene oxide and has the special property that its hydroxyethyl groups can react again with ethylene oxide to form oligomeric side chains.

Commercial products with molecular substitution (MS) usually ranging from 1.8 to 2.5 are water soluble (*9*). Several studies have been done on the influence of substitution on the enzymolysis rate.

It has been shown (*10*) that if all three hydroxyl groups on every anhydroglucose unit are substituted, no enzymic attack is possible. A material with DS = 3 is very difficult to prepare because of steric hindrance and the effect of substitution on other physical properties. Wirick (*11*) has calculated the relationship between MS and DS, based

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on the relative reactivities of the different hydroxyl groups, and found a good agreement with the literature values, e.g., an MS of 2.5 corresponds to a DS of 1.3.

An increasing MS will decrease the reactivity of HEC. At the start of the hydrolysis, the chain scission is easier due to multiple unsubstituted anhydroglucose sequences. Klug et al. (*12*) have concluded that in the MS range 1.5–3, the differences in response to enzymic hydrolysis are low but that for MS 3–5.15, significant improvements in resistance to enzymic hydrolysis occurred. Many fungi grow on HEC, but if the initial viscosity is high, HEC is more resistant to fungal growth (*13*). In cellulose derivatives CMC and HEC the substituents have the same effectiveness in limitation of the hydrolytic cellulase action (*14*). Keilich et al. (*15*) showed, however, that HEC is more accessible to enzymic attack than CMC and that its sensitivity to degradation is inversely proportional to the DS.

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Analysis of Experimental Data and Current Results

Introduction. Our aim is to obtain the physicochemical background in order to be able to set up an absolute viscosimetric method for the assay of endocellulases in enzyme mixtures, using a buffered HEC substrate and giving the definition of enzymic activities in katals, according to the recommendations of the International Union of Biochemistry.

The first section will deal with the preparation of the HEC solutions, the determination of the refractive index increment, and the exact $\overline{M}_w$ determination by light scattering before and after endocellulase attack, using a simultaneous zero-angle and zero-concentration extrapolation of the reduced scattering values.

In the second section, a kinetic light-scattering technique will be developed that will be useful for reaction studies when high-cellulase activities are present. First, an isochronous interpolation method will be given, which enables one to correct for the fact that turbidity measurements at different angles are performed at different extents of the reaction. In this manner, the zero-angle extrapolations of the reduced scattering values are performed at well-defined reaction times. Further, we will set up the empirical relationship between the zero-angle extrapolated values and $\overline{M}_w$ at one concentration. An example of rapid cellulolysis is included. There are many possible applications of this method: the study of reaction kinetics, $\overline{M}_w$ determination at low-HEC concentrations, approximate $\overline{M}_w$ determinations in routine applications, etc.

In the third section, a gel chromatographic separation method will be set up in order to enable the evaluation of the changes of molecular weight distribution of the HEC substrate due to a slow cellulolysis. In

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the calculations, the above-mentioned light-scattering technique for the evaluation of $\overline{M}_w$ at one concentration will be applied. In the fourth section, an absolute, automatic, viscosimetric method will be developed based upon the knowledge of the molecular weight distribution during cellulolysis. An example of endocellulase activity determination will also be given.

Light-Scattering Properties of HEC. PREPARATION OF THE HEC SAMPLES. The substrate we used in all our experiments was a high-molecular-weight, commercial-grade HEC, Natrosol 250H Pharm. (Hercules, Wilmington, MA, USA). The amount of HEC to be weighed was corrected for its moisture content, since this varies with the surrounding humidity and temperature, with the restriction that a corrected amount of undried HEC was weighed.

The HEC powder was added to the desired amount of bidistilled dust-free water at 4°C, using a fast-stirring device. The rate of addition was very slow to avoid the formation of lumps. The substrate particles were further allowed to swell at 4°C during at least 12 hr. The swollen particles are then completely dissolved. One hour before use, the solution is swirled again and brought to the working temperature of 25.0°C. A supplementary correction can be needed for evaporation. This solution is stable for four days when kept at 4°C.

The cellulase preparation used in our studies was a generous gift of J. Storck of the Industrie Biologique Française (Gennevilliers, France) and was of fungal origin (*Aspergillus niger*). It was dissolved in water at 4°C 1 hr before use. When enzymic hydrolyses were studied, the HEC solution was buffered with 0.01*M* Na_2HPO_4 and 0.005*M* citric acid (Merck p.a.) at pH = 4.50. Between pH 4 and 5, a maximal and almost constant cellulase activity, using this *Aspergillus niger* cellulase (unpublished results), was found.

This buffer of low ionic strength (0.018) and of low proton concentration was also chosen in order to be able to neglect its influence on the light-scattering properties of the solution. Indeed, in the literature (*20*), a study was made of the influence of solvent composition on the light-scattering properties of HEC, and an ionic strength of 0.5 was found to have only a minor influence.

In a strong-acid medium (0.5*M* HCl), configuration changes were reported. We have not done an extensive study on this subject, but within the experimental error, no difference in light-scattering properties was found between aqueous and buffered HEC solutions.

All the solutions of HEC were made by gravimetric dilution due to the high pipetting errors occurring with viscous samples. Before the light-scattering measurement, the solutions were clarified, because dust is one of the most problematic limitations in light-scattering techniques.

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From the different methods existing in literature (*16*), we chose the use of pressure filtration at about 0.004 N/m², using the Millipore XX 4004740 filter system with Millipore MF screen filters (mixed cellulose acetate and nitrate) of different pore sizes. In order to obtain the best clarification of solutions of different viscosities, we used always the finest filter available that did not alter the solution properties. Indeed, when using very fine filters, the filter clogged after a few milliliters of filtrate, due to the formation of a viscous film on the filter. The finest filter, giving, within 0.2%, the same viscosity of the solution before and after filtration, was used, since a minor change of concentration gives serious changes in viscosity of the HEC solution (*17*).

DETERMINATION OF THE REFRACTIVE INDEX INCREMENT. In order to be able to calculate results obtained from light-scattering measurements, the refractive index increment (dn/dc) of HEC must be known. For this reason, an absolute differential refractometer (Brice-Phoenix Instrument Company, Philadelphia 40, PA; type 1120) was calibrated geometrically (*18*), using the next formula:

$$k = \frac{\Delta n}{\Delta d} = \frac{\cot i}{2m_0\,(a + b/2n_0 + t/n_w)} \tag{1}$$

All measurements were made according to Brice et al. (*18*), and the following results were found at 436 nm:

$\cot i = 0.366$ (cell partition angle)
$a = 119.34$ mm (slit-to-cell face)
$b = 15.00$ mm (cell length inside)
$t = 2.50$ mm (window thickness)
$n_0 = 1.340$ (refractive index, water)
$n_w = 1.52$ (refractive index, glass)
$m_0 = 1.502$ (magnification)

In this manner, a k value of 0.0009625 was found, which corresponded within 0.1% with a relative calibration using vacuum-dried sucrose solutions (*19*).

Using a concentration range between 0 and 0.003 g/mL, the following relationship was found, using linear regression analysis:

$$\Delta n = -0.000015\ (\pm\ 0.000004) + 0.152\ (\pm\ 0.002)\,c \tag{2}$$

The correlation coefficient was 0.9984.

The refractive index increment for HEC at 25°C and at 436 nm thus has a value of 0.152 mL/g. This value is not completely in agreement with the literature on HEC (*20*), where a value of 0.141 mL/g is

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reported. However, it is well known that differences of up to 30% and more between different authors for the same systems are frequently encountered (*19*).

DETERMINATION OF THE WEIGHT AVERAGE MOLECULAR WEIGHT. *Introduction.* The light-scattering measurements were performed at angle increments of 10° from 50° to 130° with an absolute light-scattering photometer (Brice-Phoenix, series 1870), using a symmetrical cylindrical cell with flat entrance and exit windows (Phoenix cell C-101). After gauging the apparatus with standard opal glass, it was possible to measure the scattered turbidities in absolute terms.

Correction for Reflection Effects in the Cylindrical Cell. Since HEC is an asymmetric molecule, corrections for reflections in the cylindrical cell are needed. In the literature, different corrections are proposed, adapted to the geometrical system of the apparatus and cells. Kratohvil (*21*) proposed a new formula for the reflections in cylindrical cells, taking into account four contributions to the scattering at a certain angle. He did not take into account the correction for the reverse reflected incident intensity due to reflection effects at the inner cell wall. On Figure 1 of his article (*21*) counts, e.g.: "3′ in solution equals 3′ multiplied by t_l." We propose the following correction formula:

$$I_\theta = \frac{(1 + A^2)}{t_a^2 t_l^2[(1 + A^2)^2 - 4A^2]} \cdot \left[I'_\theta - \frac{2AI'_{180-\theta}}{1 + A^2} \right] \tag{3}$$

$$A = f_l + t_l^2 \cdot f_a \tag{4}$$

I_θ is the true scattered intensity; I'_θ is the measured scattered intensity at angle θ, and $I'_{180-\theta}$ is at the supplementary angle. f_a and f_l are the Fresnel's coefficients for the fractions of light reflected at perpendicular incidence at the glass–air and glass–liquid interfaces, respectively; t_a and t_l are the corresponding transmission coefficients. They are defined by the following equations:

$$f_a = \left[\frac{(n_g - 1)}{(n_g + 1)} \right]^2 \tag{5}$$

$$f_l = \left[\frac{(n_g - n_l)}{(n_g + n_l)} \right]^2 \tag{6}$$

$$t_a = 1 - f_a \tag{7}$$

$$t_l = 1 - f_l \tag{8}$$

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n_g is the refractive index of glass with a value of 1.482 at 436 nm for the cylindrical cell used in our experiments (ref. nr. C101, Brice-Phoenix); n_l is the refractive index of the liquid and is approximately equal to 1.340 at 436 nm for aqueous solutions.

Putting the constant values in Equations 3 to 8, we obtain

$$I_\theta = 1.0906(I'_\theta - 0.0800 I_{180-\theta}) \quad (9)$$

We did not make corrections for the effects of the frost inside the cell since they are negligible (22).

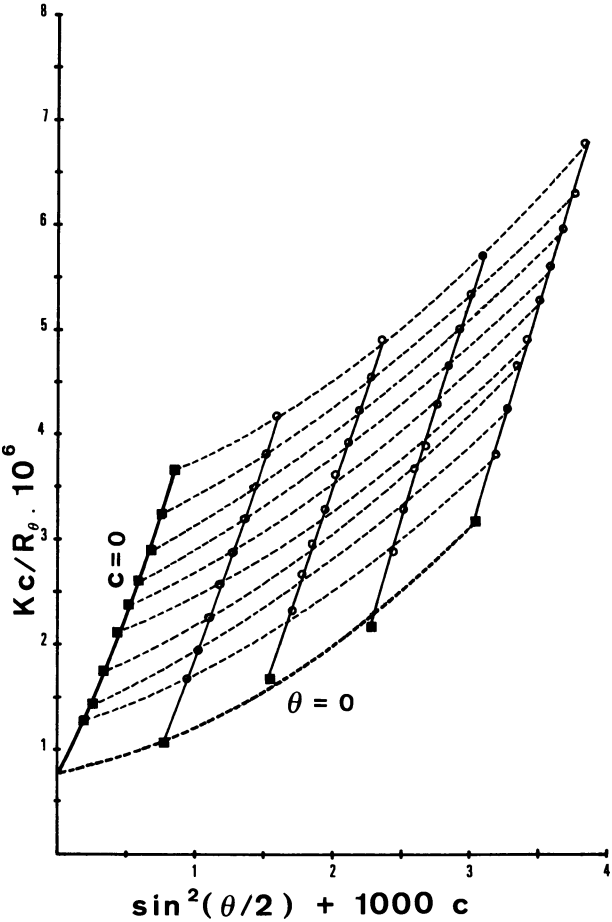

Figure 1. Computer plot of the light-scattering analysis of HEC before endocellulase hydrolysis. (○) Experimental Kc/R_θ *values, (■) extrapolated values (by quadratic least squares analysis). The inverse of the* Kc/R_θ *intercept,* $\overline{M}_w$*, equals 1,285,000.*

Light-scattering Equations. From the light-scattering data the $\overline{M}_w$ value can be found since it is related to the scattering in the following manner (*23*):

$$\lim_{\substack{\theta \to 0 \\ c \to 0}} \frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w} \tag{10}$$

K is the optical constant for the particular scattering system and is given by

$$K = 2\pi^2 n_0^2 \left(\frac{dn}{dc}\right)^2 \Big| \lambda_0^{-4} N^{-1} \tag{11}$$

where λ_0 is the wavelength of the light in centimeters and N is Avogadro's number; $K = 3.769 \times 10^{-7}$ for HEC at 436 nm. R_θ is the measured excess scattering intensity of solution over that of pure solvent and is defined by

$$R_\theta = \frac{\text{TD}\, a n_0^2\, R_w/R_c}{1.049\pi h} \cdot \frac{r}{r'} \cdot \frac{\sin\theta}{1 + \cos^2\theta} \cdot \frac{I_\theta}{I_w} \tag{12}$$

TD is the diffuse transmittance of the reference opal multiplied by the diffusor correction factor and is determined by the manufacturer as 0.249. a is the working standard constant (0.0367), and r/r' is its correction for narrow-beam geometry when using cylindrical cells; it must be determined experimentally before the measurement according to the instruction manual. R_w/R_c is a correction for incomplete compensation for refraction effects and is dependent upon the refractive index of the solvent and the size of cell used. For water at 436 nm, it is negligible and equal to 1.001. I_w is the measured intensity when viewing the working standard through the solution at 0°.

Experimental Method and Results. Using Equations 11 and 12, it is possible to calculate the Kc/R_θ values at different angles and concentrations from the I'_θ readings. They are calculated from the recorder readings after correction for the transmittance of a set of neutral filters in order to minimize the variations of the light intensities on the photomultiplier. Also, the scattered intensity of the solvent is subtracted. The scattered intensities were measured at four concentrations to determine the solute effects. For the simultaneous extrapolation to both zero concentration and zero angle, we used the method of Zimm (*24*).

For the calculation of the $\overline{M}_w$ values, a computer simulation of the Zimm plot has been written in reverse Polish notation. Also, a plotter program was provided. We used a Hewlett–Packard 9810A calculator

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connected to a 9862A plotter. A quadratic, least squares polynomial was used to fit the data, since higher polynomials would require presmoothing of data (25). The method of Zimm worked very well since the extrapolations to zero angle and zero concentration resulted in intersection points with the Kc/R_θ axis with differences of only 0.1%.

In Figure 1, the example is given of HEC before hydrolysis, where an $\overline{M}_w$ of 1,285,000 was found. Figure 2 gives the Zimm plot of HEC after endocellulase hydrolysis, with $\overline{M}_w$ of 413,000.

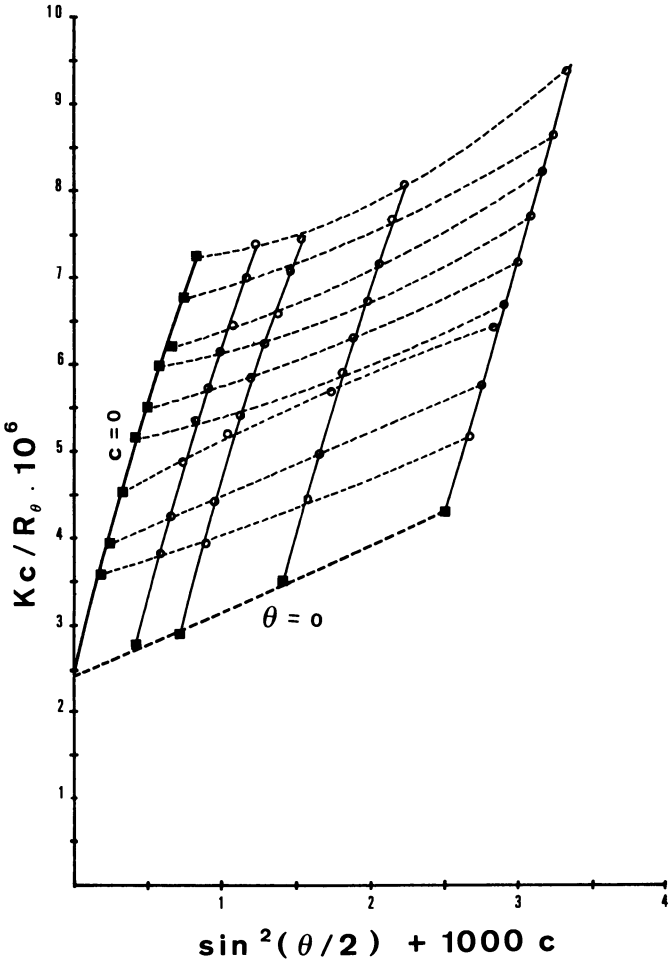

Figure 2. Computer plot of the light-scattering analysis of HEC after endocellulase attack. (○) Experimental Kc/R_θ *values, (■) extrapolated values.* $\overline{M}_w = 413{,}000$.

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From the $\lim_{c \to 0} Kc/R_\theta$, the radius of gyration can be calculated (*26*); for HEC before hydrolysis, a value of 81 nm was obtained. In all cases, the condition for the Zimm extrapolation was fulfilled—namely, that the ratio of the radius of gyration to the wavelength of the light lies between 0.05 and 0.5.

Kinetic Light-Scattering Method. ISOCHRONOUS INTERPOLATION. When high-activity samples of endocellulase are used, the reaction proceeds so quickly that, since the measurements of scattered intensity at different angles are not performed at the same extent of reaction, the extrapolations to zero angle and the subsequent calculations are erroneous. For this reason Kratochvil et al. (*27*) have proposed an "isochronous interpolation" method, whereby the Kc/R_θ values are plotted against $\sin^2|(\theta/2) + k't$. As in the double-extrapolation method of Zimm, the value of k' may be chosen arbitrarily in order to space the experimental data.

The interpolated values of Kc/R_θ at constant time are calculated or read from the graph, and then the extrapolation of the isochronous Kc/R_θ values to zero angle is made in the same manner as mentioned for the Zimm plot, by using quadratic least squares regression analysis.

For the interpolation of the data, we used a computer program as pictured in Figure 3. The interpolation is made as close as possible to the experimental values. The interpolation must be made without any presumption about the time function. To meet this assumption, the Lagrangian interpolation method for unequally spaced arguments was used (*28, 29*).

The interpolated curves are given as horizontal lines in Figure 3, and the Kc/R_θ values at constant time are given by Δ. The vertical parabolic curves result at their lowest end in the $\lim_{\theta \to 0} Kc/R_\theta$ values, given by ■.

CALCULATION OF THE WEIGHT AVERAGE MOLECULAR WEIGHT FROM LIGHT-SCATTERING MEASUREMENTS AT ONE CONCENTRATION. Since the light-scattering method will be used for kinetic measurement, we have to calculate the $\overline{M}_w$ value from measurements at one concentration, since any dilution will seriously influence the reaction kinetics. In our polymeric system, the light-scattering equation for larger particles (more then one-twentieth of the wavelength used) is valid:

$$\lim_{\theta \to 0} \frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w} + 2A_2c + 3A_3c^2 \qquad (13)$$

A_2 and A_3 are, respectively, the second and third virial coefficients. In polymer solutions, a relationship between the second and third virial coefficients was given by Flory et al. (*30*):

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$$A_3 = kA_2^2 \overline{M}_w \tag{14}$$

They calculated the value of k by using mathematical models and found a value of 5/8 for hard spheric particles. This value was obtained in an equivalent manner many years ago for a gas composed of hard spheric molecules (*31*). They derived this equation from osmotic pressure measurements and showed the parallelism between these virial coefficients and those obtained from light scattering. Stockmayer et al. (*32*) made an

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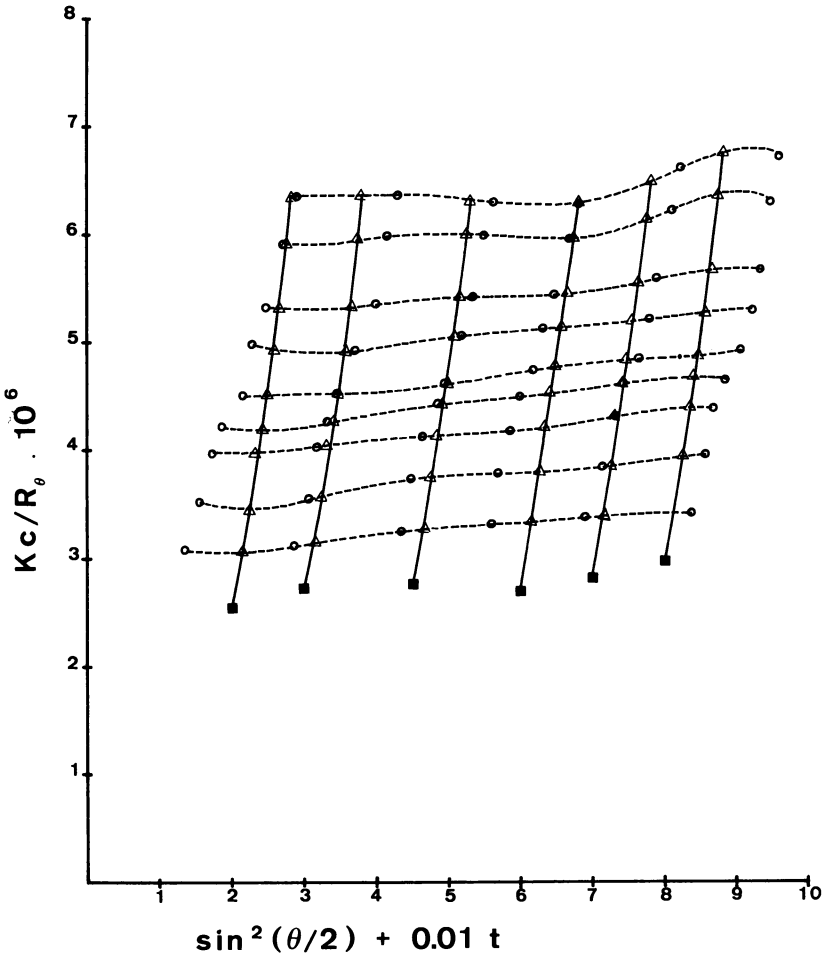

Figure 3. Computer plot obtained by isochronous interpolation of the experimental light-scattering data (○) of HEC during endocellulase attack. The Langrangian interpolation functions are given by the horizontal curves and the isochronous interpolated Kc/R$_\theta$ *values by △. The quadratic least squares extrapolations to zero angle (■) are given by the vertical parabolas.*

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extensive study of the third virial coefficient in real polymer solutions and showed that, for "soft" molecules, the k value must be much lower than 5/8. It has been shown (33) that, in practice, the k value lies in the vicinity of 0.25.

A least squares technique was used to optimize the virial coefficient and the k values (34), using the light-scattering measurements at $\overline{M}_w$ = 1,285,000 and 413,000. The k value could not be resolved statistically since variations in k gave only slight variations in the least squares deviations from the experimental values. For this and the above-mentioned reason, k was considered to be equal to 0.25. With this value, the virial coefficients were optimized.

The following equation was obtained:

$$A_2 = 0.0053\overline{M}_w^{-0.212} \tag{15}$$

Combining equations 13, 14, and 15, we obtain

$$\lim_{\theta \to 0} \frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w} + 0.01056\overline{M}_w^{-0.212}\, c + (2.091 \times 10^{-5})\overline{M}_w^{0.575}\, c^2 \tag{16}$$

From this equation, $\overline{M}_w$ is known implicitly and can be calculated using the Newton–Raphson iteration technique (35):

$$\overline{M}_{w',i+1} = \overline{M}_{w,i} + \frac{1/\overline{M}_{w,i} + 0.01056\overline{M}_{w,i}^{-0.212}\, c + (2.091 \times 10^{-5})\overline{M}_{w,i}^{0.575}\, c^2 - \lim_{\theta \to 0} Kc/R_\theta}{\overline{M}_{w,i}^{-2} + 0.002242\overline{M}_{w,i}^{-1.212}\, c - (1.203 \times 10^{-5})\overline{M}_{w,i}^{-0.425}\, c^2} \tag{17}$$

$\overline{M}_{w,i}$ is the last approximation of $\overline{M}_w$ and $\overline{M}_{w,i+1}$ is the next-to-last approximation. The function converges very quickly, and after a few approximations performed by computer, the $\overline{M}_w$ value differs only 0.001% from the exact solution of the equation.

Now for the calculation of $\overline{M}_w$ from Equation 17, we need only the $\lim_{\theta \to 0} Kc/R_\theta$ value at one concentration c (in g/mL), so that kinetic studies can now be performed at concentrations where there is a serious contribution of second and third virial coefficients. In this case, $\overline{M}_w$ will not differ more than 10% from the exact $\overline{M}_w$.

An indication of the validity of the extrapolation method could be given by light-scattering analysis of an intermediate-molecular-weight hydrolysate due to a very low cellulase activity. Indeed, at a relatively high HEC concentration of 3.152×10^{-3} g/mL, a $\lim_{\theta \to 0} Kc/R_\theta$ value of

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3.642×10^{-6} and an $\overline{M}_w$ of 790,000 was calculated; the contribution of second and third virial effects was 65%. The same hydrolysate gave, after dilution to 5.679×10^{-4} g/mL, a $\lim_{\theta \to 0} Kc/R_\theta$ value of 1.6052×10^{-6} and an $\overline{M}_w$ of 798,000; the contribution of second and third virial effects was 22%. The $\overline{M}_w$ values were in agreement, since only a difference of 1% was observed. In Figure 4 an example is given of the result of the kinetic study of the changes of $\overline{M}_w$ due to endocellulase attack. The first points correspond to Figure 3 and are subject to some scatter, due to the limited accuracy of light-scattering. Indeed, results with 10% error (23) are generally considered to be good. Another reason for the scatter is the operation of the photomultiplier power supply of the Brice–Phoenix light-scattering photometer, resulting in a noise of 1–2%, probably due to the slightly obsolete lamp electronics.

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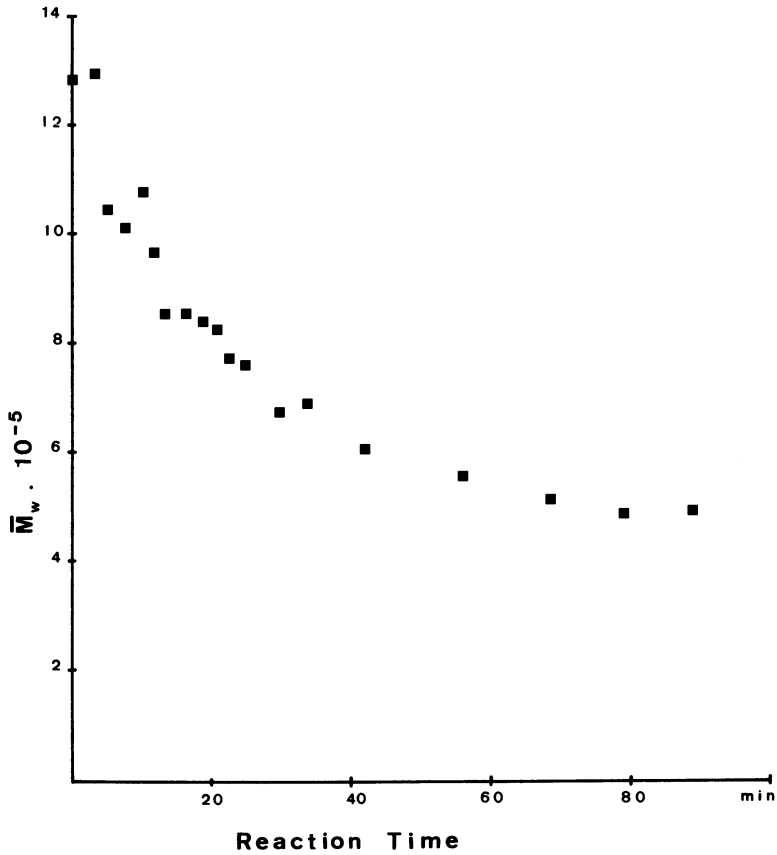

Figure 4. Changes in $\overline{M}_w$ as a function of the enzymic hydrolysis times, calculated from the light-scattering data by isochronous interpolation and by subsequent extrapolation to zero concentration using Equation 17.

Gel Chromatographic Separation of HEC and Determination of the Molecular Weight Distributions. INTRODUCTION. In order to evaluate the dispersion of the HEC, it is necessary to determine some distribution parameters.

One of the most frequently used parameters for the characterization of a distribution is the ratio $\overline{M}_w/\overline{M}_n$. For very narrow distributions, this value equals one, and for highly dispersed polymers, this value can be 30 and more. The best method for the direct determination of $\overline{M}_n$ is the measurement of the colligative properties of HEC by osmometry. In comparison to the reducing end group determinations, the osmometric method has the advantage of not measuring the cellobiose and glucose molecules produced by the exocellulase attack. Indeed, if a membrane with a good permeability for oligomers (e.g., Sartorius membrane n° 11539 with a molecular weight cutoff of 20,000) is chosen, mono- and disaccharides diffuse freely, and their apparent osmotic pressure equals zero. The increase of the osmotic pressure will thus only be affected by the endocellulase attack.

For this purpose we used an electronic membrane osmometer (Knauer & Co., Berlin, Germany) connected to a Wheatstone bridge. However, the osmotic pressures were too low and not reproducible. The most important reasons for this were the high molecular weights of the samples and the viscosity of the solutions, making the equilibrium times too long. Furthermore, an uncontrolled drift of the base line at high sensitivities made it difficult to interpret the recorder readings.

EXPERIMENTAL SETUP OF THE GEL CHROMATOGRAPHIC SEPARATIONS. The HEC was separated by gel chromatography in order to determine the complete distribution before and after enzymic hydrolysis. The separation was carried out on cross-linked agarose (Sepharose C14B, Pharmacia Fine Chemicals AB ,Uppsala, Sweden) using a 4% concentration. In order to obtain the large fractions needed for the determination of molecular weight, a semipreparative column of 1-m length and 50-mm diameter was used (column K50/100, Pharmacia Fine Chemicals AB, Uppsala, Sweden). The optimal flow rate was determined by gravimetric filtration, using a constant hydrostatic pressure of 10^4 Pa. We used a peristaltic pump to keep the flow rate constant during the separations.

The temperature was 25°C, and the fractions of 8 mL were collected at 4°C. It was important to reverse the direction of flow through the column after each separation, using a four-way valve, in order to prevent compression of the gel beads. The sample volume was never higher than 1% of the bed volume in order to obtain an optimum separation (*36*). The sample concentration was about 2 g/L; this is the maximal concentration that can be accepted, since higher concentrations will give

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viscosity errors (*36*). The reason for using this high concentration will be discussed later.

The gel chromatographic separation can be described by the following formula:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} = b \log M + a \tag{18}$$

K_{av} is the availability constant of a substance with molecular weight M, V_e is the elution volume, V_t is the total volume of the column, V_0 is the void volume or the interstitial space between the beads in the bed. The void volume could not be determined by the elution volume of Blue Dextran 2000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden, $\overline{M}_w$ about two million) since it would have separated in the column. We used a 0.5% lipopolysaccharide solution (Bacto Lipopolysaccharide W, *Salmonella typhosa* 0901 ref. nr. 3124-25, Difco Laboratories, Detroit, MI, USA) with a molecular weight of above 150,000,000, which is excluded by the gel.

A UV detector was not used to determine continuously the HEC since the UV absorption maximum changed with the HEC concentration. The following linear relationship was found between the maximal wavelength (in nm) and the concentration (between 0 and 0.003 g/mL):

$$\lambda_{max} = 202 + 6500c \tag{19}$$

The correlation coefficient was 0.98.

Other anomalies were observed, since Beer's law was fulfilled at 220 nm and between 0 and 2 g/L and not at other wavelengths. Considering the possibility of unattended anomalies of the HEC after fractionation, we had to abandon this detection method. For this reason, for the continuous concentration determination, we used a flow-differential refractometer (type R403, Waters Associates, Milford, MA, USA) connected to an integrating recorder. Since there is no monochromator on the apparatus, it was not possible to use the refractive index increment value used for the light-scattering measurements.

The proportion between the recorder signal and the concentration of a dilution series was constant and accorded to the ratio of the integrated recorder signal after a separation run to the initial amount of HEC sample brought upon the column. In order to keep the base line constant, the differential refractometer was thermostatted at 25°C($\pm$ 0.01) and shielded from external, heat-radiating sources.

One of the most important advantages of gel chromatographic separations could not be used—namely, the calibration of the column using narrow-molecular-weight standard substances to determine the *a*

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and b parameters of Equation 18. Indeed, standard proteins and dextrans approximating the molecular weight of HEC are not available or do not have the appropriate availability constant. (The highest-calibrated dextran available from Pharmacia, Dextran T 500, has an $\overline{M}_w$ of 500,000 and $\overline{M}_n$ of 200,000). The $\overline{M}_w$ of the individual fractions was thus determined by using the time-consuming light-scattering technique as mentioned above. Our experimental data were in agreement with Equation 18, and interpolations of $\overline{M}_w$ values were possible.

The error due to peak broadening in the gel column was minimal since the individual fractions were evaluated on their molecular weight and since we used a low flow rate. Granath et al. (*37*) showed by rechromatographing the fractions of a first gel chromatographic run of dextrans and after distribution analysis that no corrections are needed for peak broadening. The broadening in the elution profiles of subfractions is compensated for in the elution of the heterogeneous sample. For the distribution analysis after enzymic attack, the above-mentioned cellulase solution was mixed with the HEC solution, and the pH was brought to between 4 and 5 by using HCl.

A buffer was omitted to avoid peak formation in the chromatogram; a differential refractometer is indeed not specific and the signal is influenced by the solvent composition. Twice distilled water was used as eluent. Since the molecular weights of three cellulases isolated from *Aspergillus niger* were characterized as being 49,000, 23,800, and 13,000 (*38*), the high-molecular-weight HEC substrate and the cellulases are supposed to be separated very quickly when entering the column. The time between the mixing of the HEC and the enzyme and the application of the sample on the column was thus considered to approximate the reaction time.

FRACTIONATION DATA AND DISTRIBUTION ANALYSIS OF THE HEC BEFORE HYDROLYSIS. The results of the fractionation of HEC not subjected to cellulase attack are given in Table I. It appeared that the distribution of these fractionation data could be described by the Lansing–Kraemer distribution (*39*), also known as the logarithmic normal distribution, i.e.:

$$W(M) = \frac{1}{\beta \sqrt{\pi}} \cdot \frac{1}{M} \cdot \exp\left(-\frac{1}{\beta^2} \ln^2 \frac{M}{M_0}\right) \tag{20}$$

The two adjustable parameters are β and M_0; β increases with the increase of the breadth of the distribution. The cumulative fraction $I(M)$ can be described by the following formula:

$$I(M) = 0.5\left(1 + \frac{1}{\sqrt{2\pi}} \int_{-z}^{z} e^{-z^2/2}\, dz\right) \tag{21}$$

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The z values can be calculated from the $I(M)$ values or by the use of statistical tables (*40*) and are given in the last column of Table I.

Table I. Fractionation Data of HEC After Gel Chromatographic Separation on Sepharose C14B (Pharmacia Fine Chemicals AB, Uppsala, Sweden)

Fraction Tube No.	*Weight Fraction Differential Refracto-metric*	*Cumulative Weight Fraction*	*Molecular Weight Light Scattering* $\overline{M}_w \times 10^{-6}$	*Cumulative Lansing–Kraemer Distribution Value (z)*
30–32	0.0535	1	2.178	—
33–35	0.1598	0.9467	1.905	1.614
36–38	0.1917	0.7869	1.671	0.796
39–41	0.1639	0.5952	1.466	0.241
42–44	0.1485	0.4313	1.285	−0.173
45–47	0.1298	0.2828	1.125	−0.575
48–50	0.0758	0.1530	0.986	−1.024
51–53	0.0503	0.0772	0.865	−1.424
54–56	0.0203	0.0269	0.7586	−1.928
57–59	0.0066	0.0066	0.6637	−2.480

The unhydrolyzed HEC obeys the Lansing–Kraemer distribution: a straight line was obtained, putting the z values against the logarithm of the molecular weights. The following experimental equation was obtained:

$$z = 8.37 \log M - 51.17 \tag{22}$$

The correlation coefficient was 0.996, and at $z = 0$, the value of $M = M_0 = 1{,}310{,}000$ is obtained. It can be shown that at $z = 1$, β can be calculated from the M value in the following manner:

$$\beta = \sqrt{2} \ln \frac{M}{M_0} \tag{23}$$

For Equations 22 and 23, the β value was calculated to be 0.389.

Figure 5 shows the weight distribution curve according to Equation 20.

From the next equations, the different average molecular weights were calculated:

$$\overline{M}_n = M_0 e^{-\beta^2/4} \tag{24}$$

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$$\overline{M}_w = M_0\, e^{\beta^2/4} \tag{25}$$

$$\overline{M}_z = M_0\, e^{3\beta^2/4} \tag{26}$$

For unhydrolyzed HEC, we obtained the following values: $\overline{M}_n$ = 1,261,000; $\overline{M}_w$ = 1,360,000; $\overline{M}_z$ = 1,467,000. Since $\overline{M}_w/\overline{M}_n$ equals 1.08, the initial molecular weight distribution is narrow. Comparing this result with the $\overline{M}_w$ found in our light-scattering experiments, we found only a difference of 5%. This difference could be caused by inhomogeneities in the bulk HEC powder.

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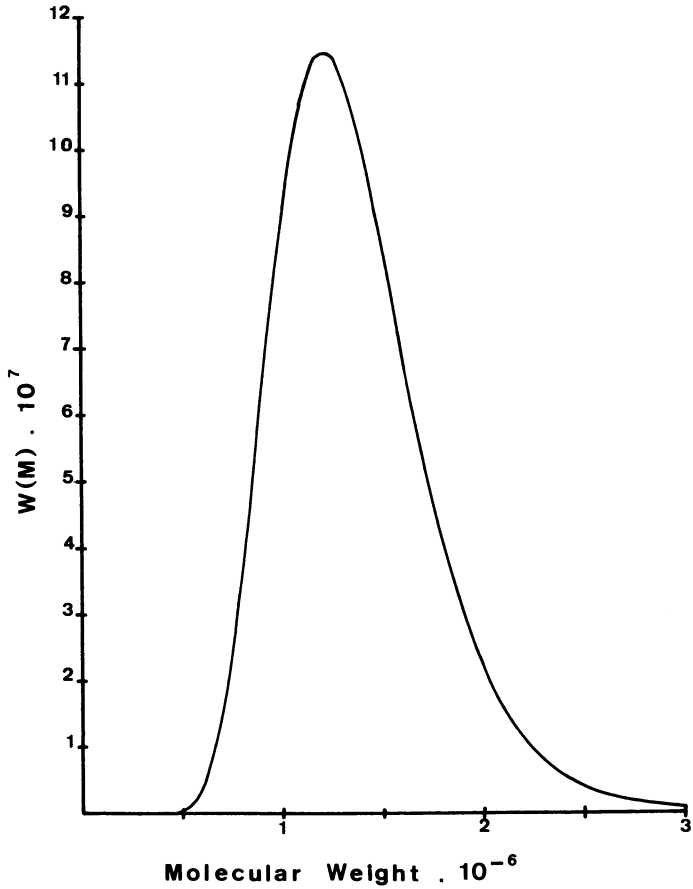

Figure 5. Lansing–Kraemer weight distribution function of HEC before enzymolysis, calculated from the fractionation data given in Table I and according to Equation 20.

Table II. Fractionation Data of HEC According to Table I After One Hour of Enzymic Hydrolysis

Fraction Tube No.	*Weight Fraction*	*Cumulative Weight Fraction*	*Molecular Weight* $\overline{M}_w \times 10^{-5}$
79–81	0.0134	1	10.08
82–84	0.0356	0.9866	8.959
85–87	0.0487	0.9510	7.960
88–90	0.0608	0.9023	7.072
91–93	0.0675	0.8415	6.283
94–96	0.0667	0.7740	5.583
97–99	0.0673	0.7073	4.960
100–102	0.0760	0.6400	4.407
103–105	0.0802	0.5640	3.916
106–108	0.0794	0.4838	3.479
109–111	0.0729	0.4044	3.091
112–114	0.0706	0.3315	2.746
115–117	0.0609	0.2609	2.440
118–120	0.0498	0.2000	2.168
121–123	0.0477	0.1502	1.926
124–126	0.0425	0.1025	1.711
127–129	0.0322	0.0600	1.521
130–132	0.0258	0.0278	1.351
133–135	0.0020	0.0020	1.200

FRACTIONATION DATA AND DISTRIBUTION ANALYSIS OF HEC AFTER ONE HOUR OF CELLULASE ATTACK. The results of the gel chromatographic separation of HEC after one hour of enzymic hydrolysis are given in Table II. These fractionation data did not correspond to any of the distribution functions mentioned by Peebles (*41*) and by Tung (*42*). In the middle of the distribution it corresponded to the Lansing–Kraemer distribution functions, but deviations occurred at the low- and high-molecular-weight ends.

The average molecular weights were calculated according to Tung (*42*):

$$\overline{M}_n = \sum_1^\lambda w_i M_i \tag{27}$$

$$\overline{M}_w = \frac{1}{\sum_1^\lambda w_i/M_i} \tag{28}$$

$$\overline{M}_z = \sum_1^\lambda w_i M_i^2 \Big/ \sum_1^\lambda w_i M_i \tag{29}$$

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The following values were obtained: $\overline{M}_n = 771{,}000$; $\overline{M}_w = 839{,}000$; $\overline{M}_z = 909{,}000$. It should be noted that Equation 29 was not correct in (*42*) and that due to the polydispersity of the fractions, $\overline{M}_n$ is a little too high and $\overline{M}_z$ is a little too low.

FRACTIONATION DATA AND DISTRIBUTION ANALYSIS OF HEC AFTER ONE DAY OF CELLULASE ATTACK. In Table III, the fractionation data of HEC are given after one day of enzymic hydrolysis. As after one hour of enzymic hydrolysis, no theoretical distribution function accorded well with the fractionation data, but we evaluated the parameters by numerical analysis, using the Gauss–Laguerre method (*43*, *44*). This method has one advantage over other numerical methods, e.g., (*45*)—all the calculations involved can be done manually without the need of high-speed computers.

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Table III. Fractionation Data of HEC According to Table I After 24 Hr of Enzymic Hydrolysis

Fraction Tube No.	*Weight Fraction*	*Cumulative Weight Fraction*	*Molecular Weight* $\overline{M}_w \times 10^{-5}$
93–95	0.0098	1	5.832
96–98	0.0154	0.9902	5.178
99–101	0.0238	0.9748	4.597
102–104	0.0324	0.9510	4.082
105–107	0.0358	0.9186	3.624
108–110	0.0418	0.8828	3.218
111–113	0.0473	0.8410	2.857
114–116	0.0499	0.9737	2.536
117–118	0.0551	0.7437	2.252
120–122	0.0583	0.6887	1.999
123–125	0.0594	0.6303	1.775
126–128	0.0594	0.5709	1.576
129–131	0.0646	0.5115	1.399
132–134	0.0651	0.4469	1.242
135–137	0.0588	0.3818	1.103
138–140	0.0552	0.3230	0.9792
141–143	0.0508	0.2678	0.8694
144–146	0.0505	0.2170	0.7719
147–149	0.0477	0.1666	0.6853
150–152	0.0478	0.1189	0.6085
153–155	0.0336	0.0711	0.5402
156–158	0.0229	0.0375	0.4796
159–161	0.0122	0.0146	0.4258
162–164	0.0025	0.0025	0.3781

The method starts with an expansion in normalized, associated, Laguerre polynomials $p_n^s(z)$:

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$$W(M) = a(aM)^s \exp(-aM) \sum_0^\infty c_n p_n^s(aM) \tag{30}$$

where

$$c_n = \int_0^\infty Mf(M)p_n^s(aM)\, dM \tag{31}$$

and where $f(M)$ is the number–density function. These expansions are also described as the Poisson–Charlier series (*46*).

The approximation consists in the truncation of the series of Equation 30 after the fifth term. This presumes that the distribution is determined within the experimental accuracy by the first six moments $\mu_0, \ldots, \mu_5$, where the moments are defined by the following formula:

$$\mu_r = \int_0^\infty M^r f(M)\, dM \tag{32}$$

The moments of the distribution function are of practical value, since the average molecular weights can be calculated from their ratios:

$$\overline{M}_n = \frac{\mu_1}{\mu_0} \tag{33}$$

$$\overline{M}_w = \frac{\mu_2}{\mu_1} \tag{34}$$

$$\overline{M}_z = \frac{\mu_3}{\mu_2} \tag{35}$$

In Equation 30 the variables a and s are the scaling parameters and must be optimized by a method that converges as rapidly as possible, since the method must be applicable to manual calculations.

A very rapidly converging method is given by Goodrich (*44*). Using this method, approximations were made for s and a, applying matrix multiplication of the column matrix $I(M)$—read from the graph $I(M)$ as a function of M at five interpolated points according to (*44*)—and a rectangular matrix (Q) at the appropriate s value. The different approximations are given in Table IV. Since the s values are given at 0.5 increments between 0 and 3, it can be seen that the s value of 1.5 gives a rapidly converging, corresponding a value. The following moments were obtained: $\mu_0 = 8.991 \times 10^{-6}$; $\mu_1 = 0.9988$; $\mu_2 = 172{,}300$; $\mu_3 = 4.062 \times 10^{10}$; $\mu_4 = 1.070 \times 10^{16}$. From Equations 33–35, the average molecular weights are calculated as $\overline{M}_n = 111{,}000$, $\overline{M}_w = 172{,}500$, and $\overline{M}_z = 235{,}800$. The calculated cumulative distribution function corresponds

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with the experimental cumulative distribution, although at the low-molecular-weight end some deviation is observed. If the weight distribution $W(M)$ contains only a single maximum, the Gauss–Laguerre method gives good estimates of the exact average molecular weights. Since the matrix tables are only given at 0.5 s intervals, the error will be maximal when the properly scaled s value falls midway between the tabulated values. In this case, μ_4 may be off by 10%, while in the case when the scaled s value corresponds with a tabulated s value, the maximal error in μ_5 will be 10%. As can be seen from Table IV, the distribution data are in the most unfavorable conditions, and only the moments μ_0 to μ_3 are known accurately. The $\overline{M}_{z+1}$ value is in error by about 10%.

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Table IV. Successive Approximations of the Scaling Parameters s and a for the Distribution Data of Table III According to Equation 30

$s_{calculated}$	$s_{tabulated}$	$a \times 10^5$
	1	2.6
1	1	1.168
1.78	2	3.16
1.99	2	1.742
1.55	1.5	1.47
1.77	1.5	1.448
1.72	1.5	1.4496

From the associated Laguerre functions according to $s = 1.5$, discrete values of the distribution function $W(M)$ as a function of M are calculated by using the four first terms of Equation 30; they are given in Table V. The continuous distribution function is calculated by Lagrangian interpolation (*34, 35*) of the discrete values given in Table V. The interpolation errors were less than 0.01%. The distribution function is given in Figure 6.

We have used the same Gauss–Laguerre numerical method to calculate the moments of the data given in Tables I and II. However, scaled s values higher than five were obtained, for which no mathematical tables exist. For narrow distributions with an expected $\overline{M}_w/\overline{M}_n$ below 1.3, the method could thus not be used.

ANALYSIS OF THE MOLECULAR WEIGHT DISTRIBUTION CHANGES CAUSED BY THE CELLULASE ACTION. The most important fractionation data are summarized in Figure 7. From the straight lines obtained, it is clear that over a broad molecular weight range (100,000–1,200,000), the $\overline{M}_w$ changes are parallel with $\overline{M}_n$, within the experimental error. The following relationship is then valid:

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$$\overline{M}_w = \overline{M}_n + 78{,}000 \tag{36}$$

The correlation coefficient r equals 0.9994.

When a polymeric chain of infinite length is cut by random fracture, an exponential distribution is obtained (*47*), for which the following relationships between the molecular weights are valid:

$$\overline{M}_v = \overline{M}_n[\Gamma(2 + \alpha)]^{1/\alpha} \tag{37}$$

$$\overline{M}_w = 2\overline{M}_n \tag{38}$$

$$\overline{M}_z = 3\overline{M}_n \tag{39}$$

where Γ is the gamma function and α is the exponent in the exponential relationship between $\overline{M}_v$ and $[\eta]$.

Table V. Weight Distribution Function of HEC After 24 Hr of Enzymic Hydrolysis Calculated from the Gauss–Laguerre Numerical Computation Method Using the Four First Terms of Equation 30 with the Scaling Factors $a = 1.4479 \times 10^{-5}$ and $s = 1.5$

Scaled Molecular Weight aM	*Weight Distribution Function* W(M) $\times 10^6$	*Molecular Weight* M $\times 10^{-5}$
0	0	0
0.2	0.387	0.138
0.4	1.278	0.276
0.6	2.371	0.414
0.8	3.415	0.552
1	4.254	0.691
1.2	4.821	0.829
1.4	5.149	0.967
1.6	5.144	1.105
1.8	4.983	1.243
2.0	4.678	1.381
2.5	3.610	1.727
3.0	2.536	2.072
3.5	1.714	2.417
4.0	1.178	2.763
5.0	0.682	3.453
6.0	0.487	4.144
7.0	0.336	4.834
8.0	0.196	5.525
9.0	0.090	6.216
10.0	0.025	6.906

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From Equations 36 and 38, it follows that an exponential distribution should be observed at $\overline{M}_n = 78{,}000$. At this molecular weight, the extrapolated $\overline{M}_z$ value equals 191,000, and $\overline{M}_z \cong 2.5\overline{M}_n$; the distribution is narrower than a true exponential distribution. Since α equals 0.87 (*20, 48*), then from Equation 37, for an exponential distribution of HEC of $\overline{M}_v/\overline{M}_n = 1.94$, the expected $\overline{M}_v$ value equals 151,000.

During the enzymic hydrolysis, $\overline{M}_z$ and $\overline{M}_w$ decrease in parallel with $\overline{M}_n$, and thus also $\overline{M}_v$, so that

$$\overline{M}_v = \overline{M}_n + 73{,}000 \tag{40}$$

which is valid for molecular weights ranging from 100,000 to 1,200,000. This result is important for the derivation of an absolute viscosimetric endocellulase activity method.

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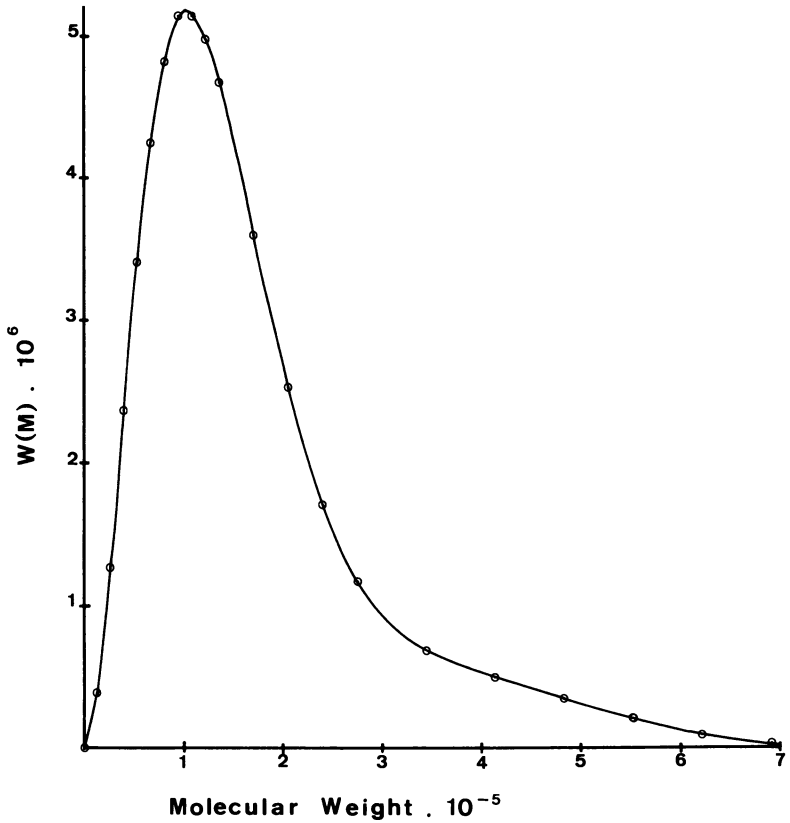

Figure 6. Gauss–Laguerre weight distribution function of HEC after one day of enzymolysis, calculated by Lagrangian interpolation of the W(M) *values (⊙) of Table V.*

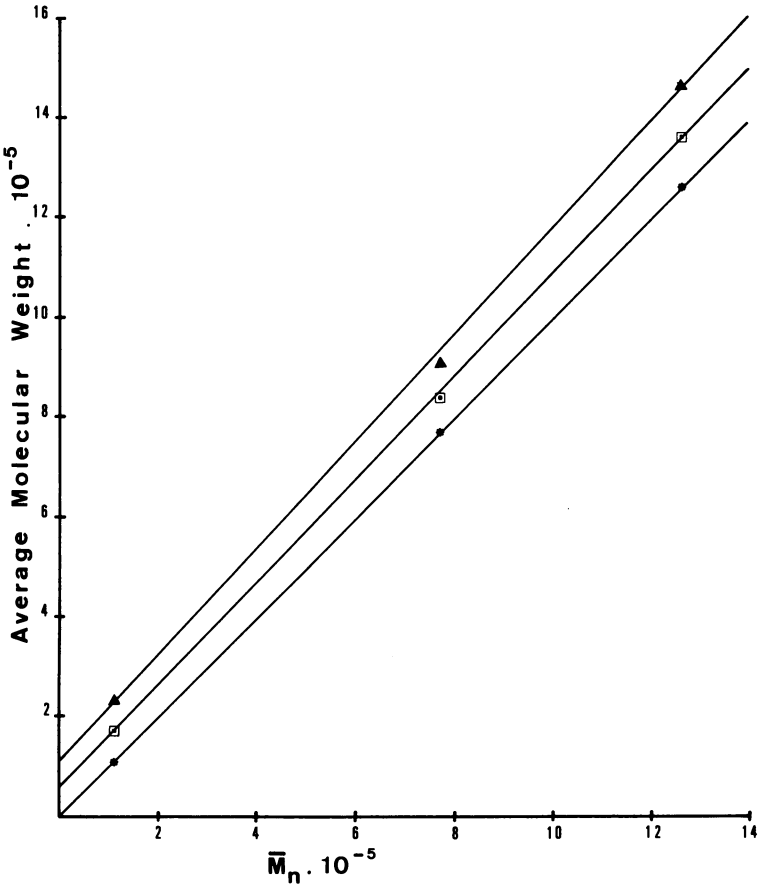

Figure 7. Parallelism between (▣) $\overline{M}_w$, (▲) $\overline{M}_z$, and (*) $\overline{M}_n$ values, obtained from the distribution analysis of the gel fractionation of HEC before and during endocellulase attack. The experimental $\overline{M}_n$ values are given by the abscissa and they are repeated in the ordinate to show the parallelism between the different average molecular weights.

Absolute Viscosimetric Method for the Determination of Endocellulase Activities. EXPERIMENTAL SETUP OF THE VISCOSIMETRIC MEASUREMENTS. The substrate, buffer, and enzyme solutions are prepared in the same manner as described in the section on light scattering. However, no clarification of the substrate solution by pressure filtration is needed.

The final concentration of the buffered HEC was about 2.5×10^{-3} g/mL and the enzyme concentration about 3×10^{-4} FIP units/mL. The FIP (Fédération Internationale Pharmaceutique) unit is a unit of endocellulase activity (*49*) calculated from capillary viscosimetric measurements.

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The experimental part of the viscosimetric method used in this study is based upon an automatization of the FIP method (*49*). A calibrated capillary viscosimeter of the Ubbelohde type (nr. 2453723 Jenaer Glaswerk, Schott & Gen., Mainz) is used. The length of the capillary is 13 cm and its diameter is 0.096 cm, determined experimentally by filling the capillary with Hg. The volume of the bulb is 0.71 cm^3.

The automatic apparatus consists of a viscosimeter and phototransistorized sensing devices mounted in a precision thermostat ($\pm$ 0.005°C) connected to a cooled prethermostat ($\pm$ 0.1°C). The base apparatus is commercially available (Schott Viscotimer, Jenaer Glaswerk, Schott & Gen., Mainz), but the viscosimeter control functions and the time measurements are performed by using an electronic computer-controlled interface. This modification enables one to follow slow reactions and to reduce standard errors on the outflow times to 2 msec. The final results are evaluated numerically by an on-line computer–plotter system.

CALCULATION OF THE INTRINSIC VISCOSITY DURING THE CELLULOLYSIS. The kinematic viscosity (ν) is related to the outflow time (t) by

$$\nu = K_\nu \left(t - \frac{K'_\nu}{t^2} \right) \tag{41}$$

K_ν is the viscosimetric constant and equals about 3×10^{-7} $m^2 \cdot s^{-2}$ for the viscosimeter used. $K'\nu$ is a correction factor for kinetic energy effects during the outflow. Its value is about 250 sec^3 for viscosimeters as used in routine assays (*49*), but it can be neglected for our viscosimeter at outflow times above 20 sec.

From the kinematic viscosity of HEC, the viscosity ratio excess, η_{re}, can be calculated:

$$\eta_{re} = \left(\frac{\eta - \eta_0}{\eta_0} \right) \approx \frac{\nu}{\nu_0} - 1 \tag{42}$$

This approximation of the kinematic viscosity ratio to the dynamic viscosity ratio is valid since the densities of HEC and the solvent, water, are almost the same.

The intrinsic viscosity $[\eta]$ of the unhydrolyzed HEC has been calculated by using the polynomial formula by regression analysis:

$$\frac{\eta_{re}}{c} = [\eta] + \sum_1^n k_i [\eta]^{i+1} c^i \tag{43}$$

The k_i values are interaction constants. With $n = 5$, a correlation coefficient of 0.9998 is obtained. This equation is not useful in practice since five constants are involved.

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Different empirical equations relating the viscosity ratio excess to the intrinsic viscosity were tested, and the Martin equation (*50*) correlated the data with an error of 1% with respect to the results obtained using Equation 43:

$$\log\left(\frac{\eta_{re}}{c}\right) = \log[\eta] + k[\eta]c \tag{44}$$

A k value of 0.1910 was found and is in perfect accordance with an extensive study made by Wirick et al. (*51*) on 26 different HEC samples with intrinsic viscosities ranging from 1700 mL/g to 170 mL/g and with MS values between 1.6 and 2.7.

In Equation 44, the intrinsic viscosity is known implicitly and can be approximated by the Newton–Raphson iteration technique (*35*). The iteration formula is

$$[\eta]_{i+1} = [\eta]_i - \frac{\log[\eta]_i + 0.191[\eta]_i c - \log(\eta_{re}/c)}{(1/([\eta]_i \ln 10)) + 0.191c} \tag{45}$$

$[\eta]_i$ is the last approximation of $[\eta]$ and $[\eta]_{i+1}$ is the next to last approximation. After four approximations, $[\eta]$ deviates only 0.001% from the exact solution of the Martin equation.

Since HEC solutions have a pseudoplastic flow behavior, the apparent viscosity will change with the shear rates applied, and the intrinsic viscosity found experimentally will generally differ up to about 10% from the true intrinsic viscosity extrapolated to a shear rate of $q = 0$. The following empirical correction formula for this effect was proposed by Vink (*48*):

$$[\eta]_{q=0} = [\eta](1 + \beta[\eta]^2 q) \tag{46}$$

The β value is an empirical constant and equals 1.79×10^{-10} g^2 · sec · mL^{-2} for HEC. The shear rate (q) is, by definition, the radial velocity gradient in the capillary, and its value can be calculated by the following formula (*52*):

$$q = -\left(\frac{dv}{dr}\right)_{wall} = \frac{4V}{\pi r^3 t} \tag{47}$$

where V is the volume of the bulb of the capillary viscosimeter and t is the outflow time.

Using Equations 41, 42, 45, 46, and 47, it is possible to calculate the intrinsic viscosity extrapolated to zero shear from the outflow time measurements during an enzymic hydrolysis.

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CALCULATION OF THE ENDOCELLULASE ACTIVITY FROM THE INTRINSIC VISCOSITY VALUES. The enzymic degradation of polymeric substrates can occur at different bonds in the same substrate molecule, and the enzymic activity has to be defined here as the initial number of moles of glycosidic bonds split per second (*53*). This definition corresponds to the definition of the katal, symbolyzed "kat." This unit is defined as "the catalytic amount of any catalyst (including any enzyme) that catalyzes a reaction rate of one mole per second in an assay system" (*54*), and it is recommended by the International Union of Pure and Applied Chemistry (*55*) for the quantitative evaluation of catalytic activities.

The amount (A) of katals per milliliter of the reaction mixture is given by

$$A = \left[\frac{d}{dt}\left(\frac{c}{\overline{M}_n}\right)\right]_{t=0} \tag{48}$$

where c is the initial substrate concentration (g/mL). For HEC the modified Staudinger equation is valid (*20, 48*):

$$[\eta]_{q=0} = 1.1\ (\bar{x}_v)^{0.87} \tag{49}$$

where $[\eta]_{q=0}$ is given in mL/g and $\bar{x}_v$ is the viscosimetric average degree of polymerization.

The HEC used in our experiments has an MS value of 2.5. The molecular weight of one substituted anhydroglucose group equals 274.7, so that Equation 49 can be written in the form

$$[\eta]_{q=0} = 0.0083\ (\overline{M}_v)^{0.87} \tag{50}$$

The relationship between $\overline{M}_n$ and $\overline{M}_v$ is known from the Equation 40; the absolute activity can be calculated from Equations 48 and 50 by differential calculus. The following formula is obtained:

$$A = -k_A \cdot c\left[\frac{d[\eta]_{q=0}}{dt}\right]_{t=0} \tag{51}$$

where k_A is a constant depending on the initial intrinsic viscosity:

$$k_A = 4.65 \times 10^{-3}\ \frac{[\eta]^{0.15}_{t=0,\,q=0}}{([\eta]^{1.15}_{t=0,\,q=0} - 296)^2} \tag{52}$$

Equations 51 and 52 are valid when the concentration (c) is expressed in g/mL and $[\eta]$ in mL/g.

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The enzymic activity can be calculated from the plot of $[\eta]_{q=0}$ as a function of the reaction time. The true reaction time is the time t_1 when the outflow time measurement begins plus half of the outflow time t_2. In Equation 51, the value $d[\eta]_{q=0}/dt$ is calculated from the slope of the curve. Since the initial viscosity of the HEC solution can vary due to the limited stability of the substrate, it is necessary to calculate the k_A value by using Equation 52, where $[\eta]_{t=0,\ q=0}$ is determined from the $[\eta]_{q=0}$ axis intercept. According to the kinetics of random degradation, $1/\overline{M}_n$ should be a linear function of time during initial reaction stages. This condition was met up to $\overline{M}_n \cong 500{,}000$. The enzymic activity is calculated from Equation 48 using linear regression analysis of $1/M_n$ as a function of the reaction time.

EXAMPLE OF AN ENDOCELLULASE ASSAY. *Experimental Data.*
Capillary viscosimeter:

viscosimetric constant $K_\nu = 3.204 \times 10^{-7}\ m^2 \cdot sec^{-2}$
kinetic energy correction factor $K'_\nu \approx 0$
capillary radius $r = 0.048$ cm
bulb volume $V = 0.715\ cm^3$. .
shear rate according to Equation 47 $q = \dfrac{8300}{t}\ sec^{-1}$

Solvent: aqueous buffer at 25°C:

kinematic viscosity at 25°C $\nu_0 = 8.93 \times 10^{-7}\ m^2 \cdot sec^{-1}$

Substrate solution:

buffered HEC concentration $c = 2.511 \times 10^{-3}\ g \cdot mL^{-1}$

Enzyme solution: *Aspergillus niger* cellulase obtained from the Industrie Biologique Française. The concentration in the reaction mixture equals 3.25×10^{-3} mg/mL.

The experimental values are given in Table VI and the $1/M_n$ values were calculated from $[\eta]_{q=0}$ using Equations 40 and 50. The following equation was obtained:

$$1/\overline{M}_n = 1/1{,}069{,}000 + 1.444 \times 10^{-9}t$$

with a correlation coefficient of 0.997. According to Equation 48, the activity A equals 3.627×10^{12} kat/mL and the specific activity 1.12×10^{-9} kat/mg; the value of $[\eta]_{q=0}$ is 1546; $[d[\eta]/dt]_{t=0}$ equals −1.960 and k_a, 7.37×10^{-10}.

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Table VI. Example of an Endocellulase Assay

Reaction Time t_1 *(sec)*	*Outflow Time* t_2 *(sec)*	*Corrected Reaction Time* $t_1 + t_2/2$ *(sec)*	η_{re}/c [a] *(mL/g)*	$[\eta]$ [b] *(mL/g)*	$[\eta]_{q=0}$ [c] *(mL/g)*
97.5	38.745	116.9	5137.9	1267	1346
152.7	34.517	169.9	4533.8	1202	1277
201.9	31.938	217.9	4165.2	1159	1231
248.9	29.111	263.4	3761.3	1107	1177
294.8	27.525	308.5	3534.7	1077	1144
337.7	24.882	350.1	3157.1	1022	1085
377.3	23.730	389.2	2992.5	996	1058

[a] According to Equations 40 and 41.
[b] According to Equation 44.
[c] According to Equations 45 and 46.

Discussion and Summary

This study describes an absolute method for the evaluation of endo-β-glucanase (C_x) or endocellulase activities in the cellulase complex without the need of a time-consuming isolation of the endocellulase fractions. In the method proposed by Almin et al. (*3*), an assumption was needed in the theoretical derivation of endocellulase activities, namely, that $\overline{M}_v/\overline{M}_n$ is constant in the initial stages of the enzymic reactions. We found, however, a linear relationship between $\overline{M}_v$ and $\overline{M}_n$. They used CMC as substrate, and since the mode of action of endocellulases on CMC can be regarded as similar to that on HEC, it is possible that their assumption is not fulfilled.

We worked with an HEC substrate, which has been used for a long time in routine applications by many pharmaceutical laboratories. It has a molecular weight above one million, and the decrease in viscosity and $\overline{M}_w$ is affected mostly by endocellulases. A disadvantage of the high molecular weight of HEC is that it is difficult to analyze by simple chemical methods such as reducing end group determinations. The interesting measurement of the colligative properties of HEC by osmometry is also not possible.

The physical properties of HEC were analyzed using absolute differential refractometry, after geometric calibration of the apparatus, and light-scattering analysis. The light scattering of HEC is dissymmetric, and the appropriate corrections for reflection effects in the light-scattering cell were applied. An improvement of the formula of Kratohvil (*21*) was proposed in order to take into account the reflections at the inner wall of the cylindrical cell. Since water is not a theta solvent for HEC, the

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scattering intensities were made as a function of the concentration and the scattering angles. For the determination of $\overline{M}_w$, the method of Zimm (*24*) was modified to a computer method; the extrapolations made to zero angle and zero concentration differed only at 0.1%, and $\overline{M}_w$ was calculated accurately.

When rapid enzymic reactions are studied by light scattering, the different angle readings will not correspond with the same extent of the reaction. We used an isochronous interpolation method to correct for this effect. The method consists of plotting Kc/R_θ values as functions of $\sin^2(\theta/2) + k't$ and the interpolation at constant time by the Lagrangian method (*28, 29*). When performing quadratic least squares extrapolation of the interpolated Kc/R_θ values, the $\lim_{\theta \to 0} Kc/R_\theta$ is found at a well-defined reaction time.

Using least squares statistical techniques, the relationship between the second and third virial coefficients with molecular weight was obtained. This was necessary to make kinetic light-scattering measurements at a constant concentration. An iteration formula was given to calculate $\overline{M}_w$ from the $\lim_{\theta \to 0} Kc/R_\theta$ value at one concentration.

More information on the action of endocellulases on the molecular weight distribution of HEC was obtained by a gel chromatographic study using a 4% agarose gel. UV detection was not possible due to the wavelength shift of the UV absorption maximum as a function of the HEC concentration. A continuous integrating differential refractometer was used, and the molecular weight of the fractions was determined by light scattering. A linear relationship between the availability constant and the logarithm of the molecular weight was observed. From a distribution analysis of HEC before endocellulase attack, it was concluded that the distribution function corresponds to the Lansing–Kraemer distribution. The molecular weight distribution was narrow, since $\overline{M}_w/\overline{M}_n$ equals 1.08. The HEC was also fractionated after endocellulase attack. The fractionation data could not be described by any theoretical distribution function. The data after one day of enzymic hydrolysis could be analyzed numerically by the Gauss–Laguerre method (*43, 44*) and the moments μ_0 up to μ_4 were calculated. This method was only applicable for distributions with $\overline{M}_w/\overline{M}_n$ above 1.3. From the gel chromatographic fractionation and the subsequent distribution analyses, the following important conclusion was made on the relationship between the different average molecular weights: in a broad molecular weight range (100,000 to 1,200,000), the $\overline{M}_w$ value changes in parallel with $\overline{M}_n$ and $\overline{M}_z$, within the experimental error.

From an extrapolation of the experimental data to a higher extent of hydrolysis, an exponential distribution can be expected at $\overline{M}_n =$ 78,000. Since for this distribution the relationship between $\overline{M}_v$ and $\overline{M}_n$

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is known, and since $\overline{M}_v$ will decrease during enzymic hydrolysis in parallel with $\overline{M}_w$, the relationship between $\overline{M}_v$ and $\overline{M}_n$ was easily calculated. This result is needed to calculate the absolute endocellulase activities using viscosity methods. For the viscosimetric determination of endocellulase activities, a high-precision, computer-controlled, capillary viscosimetric system was set up. The errors in outflow time measurements were as low as 2 msec. The effect of the kinetic energy on the viscosity measurements was taken into account. The intrinsic viscosity was calculated with only 1% error with respect to the exact polynomial equation. Due to the pseudoplastic flow behavior of water-soluble cellulose derivatives, especially if the molecular weight is high, an extrapolation of the intrinsic viscosity to zero shear rate was necessary. For this calculation, the following parameters are involved: the volume of the viscosimeter bulb, the radius of the capillary and the viscosimetric outflow time. Using the modified Staudinger equation and taking into account the molecular substitution degree of HEC, the $\overline{M}_v$ value during the enzymic hydrolysis was calculated. The endocellulase activity was expressed in katal units according to the recent international recommendations.

The enzymic activity was proportional to the change of the inverse of the numerical average molecular weight per time unit. The linear relationship between $\overline{M}_v$ and $\overline{M}_n$ allowed a simple calculation of the activity from the knowledge of the intrinsic viscosity at zero shear rate as a function of the reaction time. A practical example is given.

The HEC used has useful properties for an absolute endocellulase assay: high molecular weight, a low dispersion, and a viscosity that is practically not influenced at all by such factors as pH, ionic strength, and polyvalent cations. An HEC substrate with similar properties was accepted by the Fédération Internationale Pharmaceutique ("Centre for Standards" of the International Commission on Pharmaceutical Enzymes, Wolterslaan 12, 9000 Gent, Belgium). The characterization of the physicochemical properties of the polymeric substrate allowed its use as substrate for enzyme assays. Studies to elucidate the mechanism of the endocellulolysis and kinetic and inhibition parameters are in progress.

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6

Influence of Cellulose Physical Structure on Thermohydrolytic, Hydrolytic, and Enzymatic Degradation of Cellulose

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After a brief survey of the mechanism of cellulose acid hydrolysis and the influence of the structure of cellulose on the reaction rate under heterogeneous conditions, thermohydrolysis, hydrolysis by dilute acid, and enzymatic degradation are compared with regard to the influence of the structure of cellulose. Cotton linters, dissolving pulp from beech and spruce, and cellulose powders have been used as starting materials with and without pretreatment by dry and wet disintegration, mercerization, or NH_3 decrystallization. In thermohydrolysis, chain degradation is counteracted by thermal cross-linking, probably through transglycosylation. Comparing acid and enzymatic hydrolysis in an aqueous medium, most of the differences relating to various structural parameters can be explained by the difference in molecular size and in the selectivity of the hydrolyzing agent.

The molecular mechanism of acid hydrolysis of glycosides is rather well understood today, much confusion being resolved now by the achievements of conformational analysis of carbohydrates (*1, 2*). With regard to the three consecutive steps of reaction (cf. Figure 1)—i.e., the formation of a conjugate acid by protonation of either one of the acetalic

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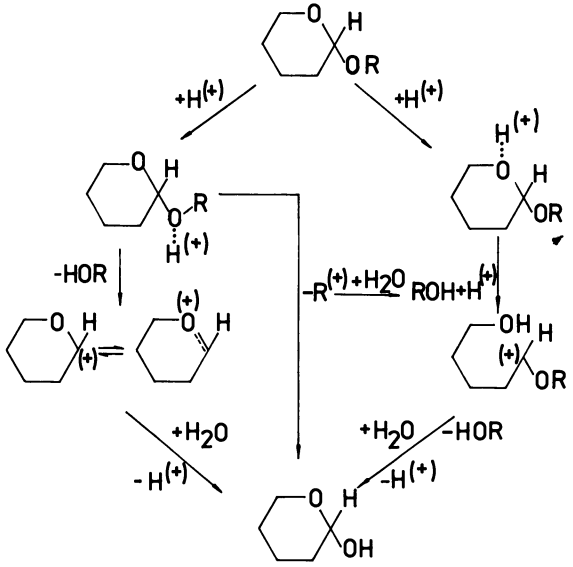

Figure 1. Mechanism of acid hydrolysis of glycosidic bonds (compare with 2)

O atoms, the formation of a carbonium cation due to cleavage of the C–O bond, and the stabilization of the product by heterolysis of a water molecule—there is still some discussion about the structure of the carbonium cation. This may be either cyclic or acyclic, depending on the primary site of protonation. Possibly, this discussion can be settled by assuming a partial protonation of both oxygen atoms caused by the structured nature of water (2), as shown in Figure 2.

The overall rate law of homogeneous acid hydrolysis of glycosides is given by

$$S + H_3O^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SH^+ \xrightarrow{k_2} R \qquad \text{and} \qquad k_{total} = K_{SH^+} \cdot k_2$$

with S being the substrate, R the reaction product, and K_{SH^+} the equilibrium constant of the protonation step. In accordance with this scheme, the overall rate of homogeneous hydrolysis increases with the Hammett acidity function of the medium.

With the heterogeneous hydrolysis of polysaccharides like cellulose, these general considerations are valid, too, of course, but the rate of cleavage is slowed down by one or two orders of magnitude by the limited accessibility of the acetalic O atoms. The rate of reaction depends largely on the physical structure of the original samples and on the state

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of swelling achieved by the hydrolyzing medium (*3*). Much knowledge is available today concerning correlations between the rate of weight loss and the decrease in chain length in heterogeneous hydrolysis of cellulose by dilute aqueous mineral acid and the state of the lateral order of the sample. The well-known LODP concept of Battista (*4*), i.e., the approaching of a limiting DP remaining constant at prolonged hydrolysis and depending largely on the lateral order of the cellulose sample, as well as the work of Sharkov (*5*) on measuring cellulose accessibility by ethanolysis may be cited here as examples.

Much less information is available with regard to the action of hydrolyzing reagents in the gaseous state in relation to the physical structure of cellulose. With respect to enzymatic cleavage, the possibility of enhancing the reaction rate by increasing the accessibility is well known (*6*), but there are still open questions as to the relevance of the different ways to do this.

In this context, some experimental results relevant to these open questions of enzymatic degradation will be presented and will be discussed from the viewpoint of cellulose chemistry, together with a summary of our recent work on thermohydrolysis and acid hydrolysis of cellulose, performed in connection with research on cellulose powder manufacture (*7*). After a short survey of the experimental techniques applied, this contribution will be centered on three problems: (1) the interaction of chain degradation and cross-linking in thermal and thermohydrolytic treatments of cellulose, (2) the influence of mechanical strain

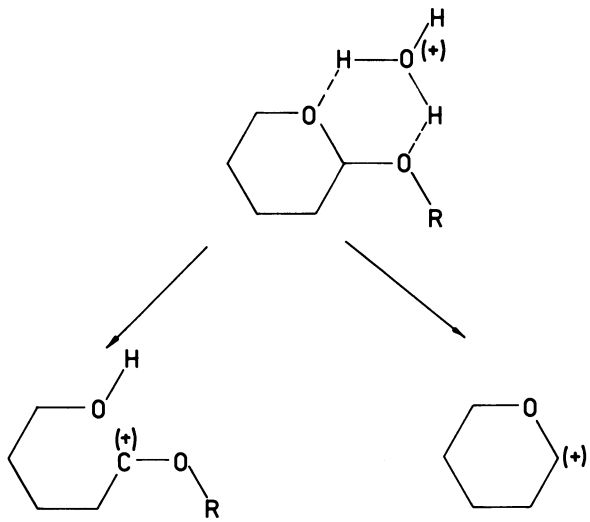

Figure 2. Pattern of simultaneous protonation of acetalic O-atoms (compare with 1)

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and structural stress on cellulose hydrolysis, especially by dilute aqueous acid, and (3) the effect of changes of cellulose morphology and lateral order on accessibility to hydrolytic degradation, comparing enzymatic and acid hydrolysis.

Experimental

Our experimental work started from the following original cellulose samples: (a) acetate-grade, bleached cotton linters, DP ~ 1800; (b) hot, refined, spruce, sulphite-dissolving pulp, machine dried, ca. 93% α-cellulose, DP = 750; (c) never-dried, normal, rayon-grade, beech sulphite pulp, ca. 90% α-cellulose, DP = 825; (d) commercial cellulose powders obtained by hydrolytic degradation and/or mechanical disintegration of cotton linters or spruce sulphite pulp.

For further variation of the physical structure, some of the original samples were given different pretreatments, i.e., (a) different modes of drying, (b) dry or wet disintegration by milling or beating, respectively, (c) decrystallization by liquid ammonia at −50°C, with subsequent evaporation of NH_3 at room temperature, (d) mercerization with 18%-by-weight aqueous NaOH at 20°C, or (e) heterogeneous hydrolysis with 5%-by-weight aqueous HCl at 100°C.

The thermal, thermooxydative, and thermohydrolytic degradations were performed in the range between 100 and 200°C with 5-g samples contained in a glass tube heated to the approximate temperature, within ±2°C, and using a streaming gaseous reaction medium of either N_2, or $N_2 + O_2$, or $N_2 + H_2O + HCl$, respectively (cf. Ref. 8). If not stated otherwise, acid heterogeneous hydrolysis was done with 3-g samples in 90 ml of 5%-by-weight aqueous HCl at 100°C.

For enzymatic degradation, culture filtrates of selected strains of *Trichoderma viride* ("Cellulase T") and *Gliocladium spec.* ("Cellulase G") were used, strains being selected by screening with respect to C_1 activity. Generally, 1-g samples of substrate were incubated at 40°C with a mixture of 80 ml of culture filtrate and 20 ml of acetate buffer (pH = 5.0) for 12 to 170 hours.

Analytical data relevant to all degradation processes covered here are reported in percentage by weight of cellulosic residue and cuprammonium DP. These data have been supplemented in enzymatic degradation by determination of the "reducing substance" (RS) and the glucose content. For an additional characterization of sample morphology, scanning electron microscopy, mercury porosimetry, and particle size classification were applied, using for the latter a fully automatized, computer-coupled evaluation of light-scattering data of a streaming fiber suspension, as developed by Unger (cf. Ref. 9).

Results

Interaction of Chain Destruction and Cross-linking in Thermal and Thermohydrolytic Degradation of Cellulose. Starting points of these investigations were the statement of a "thermal level-off-DP" by Golova and Krylova (*10*) and observations of our own (*8*) of an increase

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Table I. "Minimum DP Value" in Thermohydrolytic Degradation of Linters

Composition of Gaseous Phase	*Temperature of Degradation (°C)*	*Minimum DP Value*
~80% N_2, ~20% H_2O, and 0.5% HCl (by volume)	200	270
"	170	210
"	140	200
"	105	180
86.7% N_2, 13% H_2O, and 0.3% HCl (by volume)	140	260
"	105	168

of DP in thermal treatment of hydrolytically degraded cellulose powders (DP ~ 150). Results with high-DP cotton linters already published (*11*) confirmed the leveling off of a decrease in DP in thermal (N_2), thermooxidative ($N_2 + O_2$), and thermohydrolytic ($N_2 + H_2O + HCl$) treatments. Especially during thermohydrolytic treatment, a definite minimal DP value is reached; it depends on temperature and somewhat on concentration of acid (Table I) and is followed by an increase in DP and the formation of cuprammonium insoluble parts of the specimen on prolonged treatment.

The initial rate of chain-length degradation $\Delta S/\Delta t$, with $S = 1/DP$ (Table II), increased, of course, with degradation temperature, both in the thermal and the thermohydrolytic treatments of the linters, but the Arrhenius energy of activation was much lower in thermohydrolysis. The difference in the rate between both kinds of treatment decreased significantly after a decrystallizing pretreatment of the linters with liquid

Table II. Initial Rate of Thermal and Thermohydrolytic Degradation of Linters

Sample	*Medium of Degradation*	*Initial Rate of Degradation ($DP^{-1} \cdot hr^{-1} \cdot 10^5$)*				E_A *(kcal/mol)*
		105°C	*140°C*	*170°C*	*200°C*	
Linters without pretreatment	N_2	0.34	0.9	1.4	7.2	14
"	$N_2 + H_2O + HCl$	40	63	102	272	7
Linters, pretreated with NH_3	N_2	—	—	22	—	—
"	$N_2 + H_2O + HCl$	—	—	162	—	—

In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

ammonia. For interpretation, either a strong influence of HCl diffusion on the rate of thermohydrolysis and/or an interaction of chain-length degradation and a counteracting cross-linking reaction may be assumed, the rate of the latter increasing with temperature and with state of order of the specimen.

We turn now to some results with hydrolytically degraded linters powders with a DP of about 150. Figure 3 demonstrates the increase in DP on long-time thermal treatment. In these experiments, as well as in analogous ones on thermooxidative and thermohydrolytic treatments, reproducibility of change of DP with time is rather poor. Pretreatment of the specimen by liquid ammonia without and with subsequent acid posthydrolysis did not cause a principal change in the effect of thermal treatment on DP (Figure 4). The initial decrease of DP with the NH_3 decrystallized sample is in accordance with a lower LODP due to the lowered state of order.

For further elucidation of the presumed cross-linking after prolonged thermal treatment, degradation of appropriate samples by aqueous HCl at 100°C and by our cellulase culture filtrates was investigated (Tables III and IV). The increase in DP induced by thermal treatment was reversed completely by an acid treatment, arriving at the same LODP

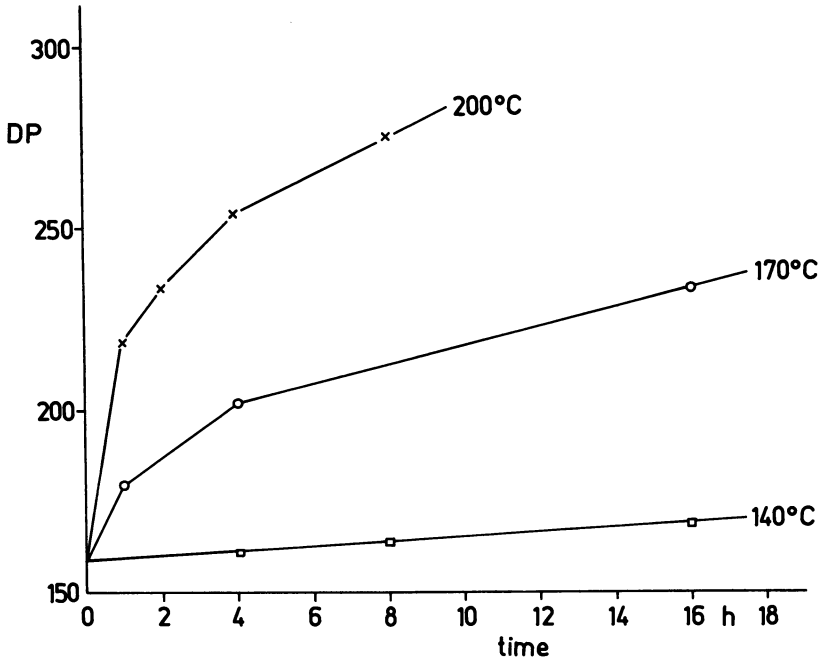

Figure 3. Increase in DP of linters cellulose powder during thermal treatment in nitrogen

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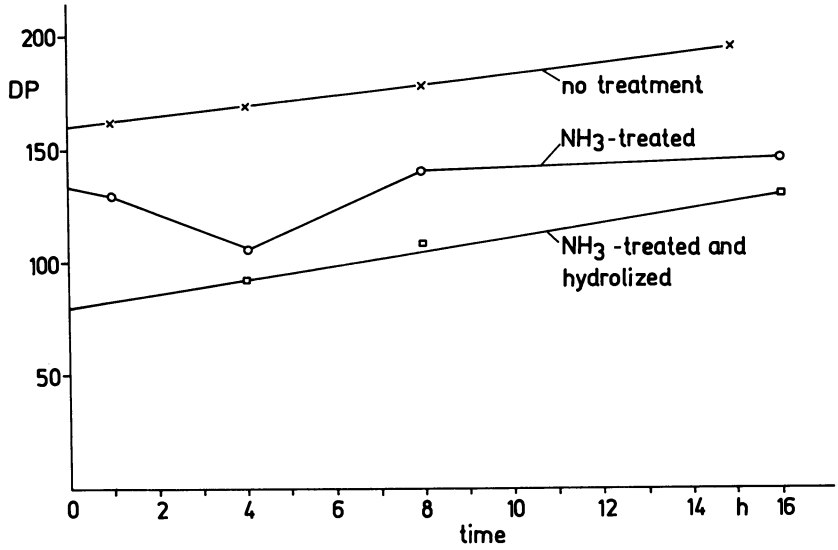

Figure 4. Effect of thermal treatment (N_2, 170°C) of linters cellulose powder after different pretreatment on DP

as with an acid posthydrolysis of the original cellulose powder. Comparison of zero-order rate constants calculated according to $1/(DP_t - LODP) - 1/(DP_o - LODP) = k_o \cdot t$ indicated a somewhat more rapid decrease in DP with the thermally treated sample than with high-DP cotton linters. On the other hand, treatment by water alone or by aqueous alkali did not result in a significant decrease in DP of the thermally treated cellulose powder. In contrast to the rather fast cleavage of bonds

Table III. Chain-length Degradation of Different Cellulose Samples at 100°C in Aqueous Media

Sample	*Medium of Degradation and Time of Treatment*	*Initial DP*	*Final DP*	*Zero-Order Rate Constant* ($DP^{-1} \cdot hr^{-1} \cdot 10^2$)
Linters	0.1*N* HCl; 8 hr	1530	148	·0.5, . . . , 1.3
	1*N* HCl; 1.5 hr	1530	141	22
Linters powder (hydrolytically degraded) after treatment 24 hr/170°C/ $N_2 + O_2$	0.1*N* HCl; 4 hr	160	140	—
	1*N* HCl; 1 hr	160	139	—
	0.1*N* HCl; 6 hr	200	139	4.5
	1*N* HCl; 1 hr	200	140	38
	H_2O, 16 hr	200	185	—
	0.1*N* NaOH; 8 hr	200	198	—

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Table IV. Enzymatic Attack (68 hr/40°C) of Linters Cellulose Powder before and after Thermal Treatment

Sample	*Culture Filtrate*	*Residue (%)*
DP = 160	Cellulase G	65; 67
Without treatment	Cellulase T	73; 77
After treatment	Cellulase G	95.5
3 days/170°C/N_2 + O_2	Cellulase T	95.2
DP = 190	H_2O for comparison	96.5

by aqueous acid, weight loss of the thermally treated sample by enzymatic attack was nearly nil, while the untreated sample showed a weight loss of about 30% after the same enzymatic treatment.

With regard to the type of thermally induced cross-links, the following conclusions may be drawn from our results: Formation of C—C bonds can be definitely excluded due to the cleavage observed by aqueous acid. Intramolecular ether bonds within the glucose unit might explain the change in cuprammonium viscosity, but they should be cleaved also by aqueous alkali. The most probable conclusion is, according to our opinion, a formation of intercatenary acetal bonds (*12*).

Influence of Mechanical Strain and Structural Stress on Hydrolytic Degradation of Cellulose. The general question of the effect of combined chemical and mechanical action on polymer degradation is relevant to cellulose hydrolysis, too. As detailed by us earlier (*13*) and summarized in Table V, the zero-order rate constant of chain-length degradation and the DP of residue obtained after prolonged treatment in acid hydrolysis of cotton linters definitely depends on the reaction medium. This influence may be interpreted, at least partially, by the assumption that relaxation of structural stress in the sample by application of liquids of high swelling power retards hydrolytic chain cleavage, while preservation of this stress by use of nonswelling media enhances the rate of chain cleavage and decreases the LODP.

Table V. Rate of Degradation of Linters in 1*N* H_2SO_4 at 80°C in Different Media

Medium	*DP of Residue after 1 hr of Hydrolysis*	*Zero-Order Rate Constant* ($DP^{-1} \cdot hr^{-1} \cdot 10^3$)
DMSO	227	1.8
Water	166	2.8
n-Propanol-water 90:10 by volume	143	15.5
Methylethylketone	141	37.4

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In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

Table VI. Influence of Drying on Acid and Enzymatic Hydrolysis of Beech Pulp

Sample	*Zero-Order Rate Constant ($DP^{-1} \cdot hr^{-1} \cdot 10^3$) 7.5% HCl/100°C*	*% Residue after 68 hr/40°C Treatment with Cellulase T*
Never dried	0.25	< 1
Air dry	15	19
Air dry + wet beating	8	8.6
Air dry + 2 hour/140°C	22	40
Freeze-dried	22	—

In agreement with this hypothesis is the remarkable low rate constant with a never-dried beech sulphite dissolving pulp compared with the dried sample at identical conditions of aqueous acid hydrolysis (Table VI).

Wet beating of the air-dry pulp lowers the rate constant again but by no means down to the level of the never-dried pulp. Starting from a machine-dried spruce sulphite pulp, heterogeneous hydrolysis by aqueous acid is accelerated by mechanical strain, i.e., stirring, during hydrolysis. A disintegration of the original sample, too, enhances the rate of chain-length degradation and lowers the LODP finally reached.

Table VII. Influence of Disintegration and of Mechanical Strain on Acid and Enzymatic Hydrolysis of a Machine-Dried Spruce Sulphite Dissolving Pulp

	Hydrolysis with 5% HCl at 100°C				*Hydrolysis with Cellulase T at 40°C for 68 hr*	
Sample	*Stirring During Hydrolysis*	*DP after 1 hr*	*LODP*	*% Residue after 2 hr*	*DP*	*% Residue*
Original, DP 750	none	181	130	89	770	35; 32
	+	170	—	—	—	—
Dry disintegrated DP 660	none	97	90	84.5	705	50; 50
	+	94	—	—	—	—
Wet disintegrated	none	145	116	92	—	7.6
For comparison:						
Acid hydrolyzed, DP 200	none	—	—	—	225	56

In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

Table VIII. Enzymatic Degradation of Cellulose Powders of Different Origin and Particle Size (Cellulose T, 1% Substrate; 68 hr; 40°C)

Sample	*Origin and Preparation*	l_L[a] *(μm)*	% *Residue*
FNA (Filtrak)	linters, degraded by hydrolysis subsequently disintegrated to different particle sizes	57	76
FNS "		41	73
FND "		28	66
01 L (HEWETEN)	linters, mechanically disintegrated	133	62
10 HL "	linters, degraded by hydrolysis	43	69
20 HL "	linters, degraded by hydrolysis, additionally disintegrated	27	65
40 HL "	linters, degraded by hydrolysis, wet-beaten, spray-dried	31	56
01 Z (HEWETEN)	wood pulp, mechanically disintegrated	144	50; 48
10 HZ "	wood pulp, degraded by hydrolysis	28	53

[a] l_L = length average of particle length.

A dry-milling procedure is much more efficient in this respect as compared to wet beating (Table VIII). These results may also be understood by the concept already mentioned, i.e., an acceleration of hydrolysis by structural stress imposed either by stirring during or by dry disintegration prior to the action of the acid. In wet beating, this internal stress cannot be "stored" due to swelling and fibrillation. As can be seen from the data in Tables VI and VII, this reasoning obviously cannot be transformed to interpret the results of enzymatic hydrolysis obtained with the appropriate samples: Never-dried pulp was attacked much more quickly than a dry sample by cellulase T, and an increase in drying temperature correlated with a further decrease in weight loss by enzymatic degradation. With the spruce pulp (Table VII), the wet beaten sample was degraded fastest, followed by the machine-dry original sample, followed by the dry disintegrated one. Obviously, in enzymatic attack, the influence of internal stress discussed in this context is overcompensated by an enhanced accessibility of the fibrillar structure of the cellulose sample in the never-dried or wet-beaten state.

Enhancement of Heterogeneous Hydrolytic Degradation of Cellulose by Changing Its Morphological and Supermolecular Structure. It is well known from the recent work of several authors (*6, 14, 15*) that enzymatic digestibility of cellulose in the pure as well as in the lignified state may be increased by mechanical disintegration, by lowering the state of lateral order—for example, by treatment with liquid ammonia—

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or by mercerization. But from the results reported so far, obviously no definite conclusion can be drawn on the effectiveness of the different structural changes with regard to enzymatic attack.

Starting from a series of cellulose powders of rather well-known physical structure, and high-DP cotton linters given different pretreatments, we compared enzymatic and acid hydrolysis to gain more insight into correlations between cellulose structure and accessibility to hydrolytic agents. In discussing our results, two limitations imposed by the experimental technique used are to be kept in mind: (1) all our experiments so far have been performed with cellulase culture filtrates, i.e., with multienzyme systems, and not with isolated enzyme components; (2) our kinetic measurements on heterogeneous enzymatic degradation are still at the very beginning, and most statements are based on results obtained after just one definite time interval of incubation (usually 68 hr at 40°C).

With a series of air-dry cellulose powders differing in origin, mode of preparation, and particle size, no general correlation can be stated between residue remaining after enzymatic attack and average particle size. Our results indicate again a significant influence of submicroscopic morphology and state of lateral order on enzymatic degradation (Table VIII). Only with samples of the same origin and mode of preparation was a significant influence of particle size on enzymatic degradation found.

Thus, further experiments were performed with two hydrolytically degraded linters powders subsequently disintegrated to different particle sizes (Filtrak, FNA and FND, VEB Spezialpapierfabrik Niederschlag, GDR), the lateral order of these samples being varied by liquid ammonia treatment or by mercerization with 18% aqueous NaOH. A posthydrolysis of these samples with aqueous acid (5% HCl, 2 hr, 100°C) resulted in changes in DP and in residue as indicated in Table IX and as to be expected from previous publications (*11, 16*).

Between the NH_3-treated samples and the mercerized ones, no significant difference in DP has been found after posthydrolysis, while the residue value of the NH_3-treated samples was definitely lower.

Table IX. Acid Hydrolysis of Cellulose Powders after Different Pretreatment (5% HCl, 2 hr, 100°C)

Sample	*% Residue*	*DP*
FNA (DP = 180)	94	152
FND (DP = 160)	94	141
FNA, NH_3 treated	78	67
FND, NH_3 treated	78	64
FNA, mercerized	86	58
FND, mercerized	86	67

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Residue values after an enzymatic degradation of these samples by different cellulase culture filtrates are summarized in Table X. These data also give an impression of the influences of type of enzyme, storage time of culture filtrate, substrate concentration, and incubation time on residue value obtained with identical substrate samples. Obviously, differences in substrate structure have a more pronounced effect on residue value at low substrate concentration. We can conclude from these experiments that (1) a smaller average particle size favors enzymatic attack, even after pretreatments lowering the state of lateral order and/or changing the lattice type of the samples, and (2) a mercerization pretreatment proves to be much more efficient in increasing accessibility to enzymatic attack than a decrystallizing pretreatment with liquid NH_3, while the reverse holds true for heterogeneous acid hydrolysis.

The DP of all the cellulose powder samples investigated here was not changed significantly by this enzymatic treatment. Differences between the original and the enzyme-treated samples did not exceed ±10 DP units at residue values between 30 and 70%. Also, in gross morphology (light microscope, magnification about 150 ×), no significant differences between original and enzyme-treated samples were observed, but—according to some preliminary results—these differences obviously do exist at the level of submicroscopic morphology—for example, in pore-size distribution (Table XI). Though our results are not yet free of some contradictions, we may conclude that, especially in the larger size range, the pore volume of the sample is enhanced by enzymatic treatment.

Table X. Influence of NH_3 Pretreatment and of Mercerization on Enzymatic Degradation of Cellulose Powders

Conditions of Enzymatic Treatment	*% Residue*					
	Original		*NH_3-treated*		*Mercerized*	
	FNA	*FND*	*FNA*	*FND*	*FNA*	*FND*
Cellulase G, 10% substrate, 68 hr/40°C	87	88	—	—	82	82
Cellulase G, 1% substrate, 68 hr/40°C	65	50	47	38	33	36
Cellulase G, 1% substrate, 7 days/40°C	—	50; 54	—	—	—	14
Cellulase G, 1% substrate, 68 hr/40°C	67	50	56	45	39	19
Cellulase T, 1% substrate, 68 hr/40°C	73	61	57	49	37	19
	76[a]	63[a]	63[a]	54[a]	47[a]	30[a]

[a] Same culture filtrate of cellulase T applied under identical conditions 14 days later.

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Table XI. Change in Pore Volume of Linters Powder Samples by Enzymatic Treatment (Cellulase G, 68 hr/40°C, 1%)

Sample	*Pore Volume (cm^3/g)* *Pore Size Range 0.015, . . . , 0.77 μm Enzyme Treatment*		*Pore Size Range 0.77, . . . , 13.6 μm Enzyme Treatment*	
	Before	*After*	*Before*	*After*
FNA	0.071	0.078	0.342	0.515
FND	0.071	0.079	0.820	0.932
FNA, NH_3 treatment	0.059	0.048	0.190	0.426
FND, NH_3 treatment	0.061	0.047	0.955	1.318
FNA, mercerized	0.061	0.049	0.217	0.137
FND, mercerized	0.046	0.058	0.209	0.684

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It seemed reasonable to supplement our results obtained with low-DP linters cellulose powders by some experiments with high-DP cotton linters, given analogous pretreatments in the original state and after mechanical disintegration.

Acid hydrolysis of these samples led to the results to be expected, i.e., a lowering of LODP and residue value by NH_3 pretreatment and mercerization as well as by mechanical disintegration prior to the hydrolytic treatment (Table XII). With regard to residue value, an NH_3 pretreatment again proved to be more efficient in enhancing accessibility than a mercerization, while the rate constant of chain-length degradation was increased somewhat more by mercerization.

In enzymatic degradation, only the pretreatment with NaOH proved to be efficient in lowering the residue value obtained. After incubation times of 12 and 68 hr a remarkable difference was observed between the mercerized and the NH_3-treated sample, the former showing much lower residue values during the whole course of enzymatic treatment (Figure 5) and following also another overall rate law of weight loss. After a short time of incubation, degradation of the NH_3-treated sample seemed to be somewhat retarded as compared to the original linters. The outstanding effect of mercerization is also reflected by the change in DP during enzymatic treatment: With the original and the NH_3-treated samples, only a small decrease in DP was observed, if the drop in DP from about 1800 to about 1660 by a 68-hr treatment with the slightly acid buffer solution is taken into account. With the mercerized sample, however, a remarkable decrease in DP by action of the enzyme system was seen after only 12 hr of incubation, followed by a further slow decrease and then by a significant increase in residue DP.

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Table XII. Acid and Enzymatic Hydrolysis of

Sample	*Acid Hydrolysis with HCl at 100°C*	
	LODP (5% HCl)	*% Residue After 2 hr (5% HCl)*
No treatment, DP = 1800	155	93
NH_3 treated, DP = 1620	71	85
Mercerized, DP = 1580	68	89
Dry disintegrated, DP = 1425	110	89
Dry disintegrated, NH_3 treated, DP = 1120	~ 50	66
Dry disintegrated, mercerized, DP = 1000	51	70

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With the samples at first mechanically disintegrated, all the statements regarding the influence of an NH_3 and an NaOH pretreatment were confirmed (Table XII). In any case, the previous mechanical disintegration decreased the rate of enzymatic attack as compared with the appropriate nondisintegrated samples. Probably some hornification and/or change in pore structure occurs during mechanical disintegration in the dry state.

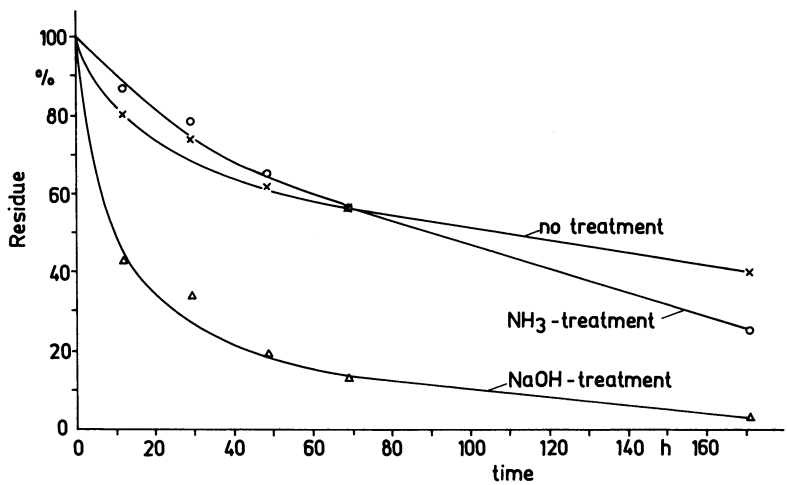

Figure 5. Enzymatic degradation of acetate linters after different pretreatment (Cellulase T, 1% substrate, 40°C)

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Acetate Linters after Different Pretreatments

Acid Hydrolysis with HCl at 100°C	*Enzymatic Hydrolysis with Cellulase T (1% Substrate) 40°C*		
Zero-Order Rate Constant ($DP^{-1} \cdot hr^{-1} \cdot 10^3$) *(1% HCl)*	*% Residue After*		*DP After*
	12 hr	*68 hr*	*68 hr*
41	79	55	1490
53	82	55	1550
72	43	13	920
51	81	59	1336
—	83	63	1369
—	71	23	463

Discussion and Conclusions

A common feature of vapor-phase thermohydrolysis, liquid-phase acid hydrolysis, and enzymatic hydrolysis of cellulose is a significant influence of lateral order on the rate of chain cleavage, the DP finally reached, and weight loss. But with regard to this influence of the physical structure of cellulose, there also exist remarkable differences between these three modes of hydrolytic degradation.

In thermohydrolysis, chain cleavage is counteracted by thermal cross-linking, probably due to acetal bonds formed by transglycosylation, as proposed by Back (*12*) and demonstrated in a simplified way in Figure 6. Cross-links are formed here by reactions of cellulosic OH groups with carbonyl groups originating from oxidation processes in thermal treatment. These cross-linking reactions obviously are favored by a high state of lateral order.

The influence of mechanical strain and structural stress on the rate of weight loss and the decrease in DP in acid hydrolysis is in agreement with some considerations of Elema (*17*), who discussed the role of structural stresses in connection with "weak links" in the cellulose chain. It may be mentioned here, however, that our results on heterogeneous acid hydrolysis did not yield further evidence of these "weak links."

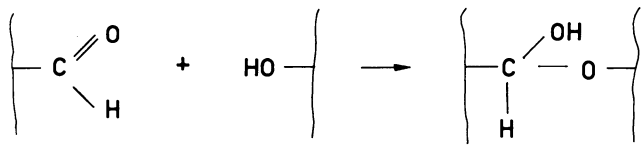

Figure 6. Simplified pattern of transglycosylation according to Back (12)

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The thermal cross-linking as well as the influence of internal structural stress may give rise to some limitation of the LODP concept if hydrolytic media of different swelling powers are compared or if the range of temperatures considered exceeds 100°C. In those cases, as well as after mechanical strain on the sample, the LODP obviously is not unambiguously determined by the physical structure of the sample but depends to some extent on the conditions of hydrolysis.

In comparing the two modes of heterogeneous cellulose hydrolysis in aqueous medium—i.e., acid and enzymatic degradation—it is obviously not justified to transfer statements regarding the influence of cellulose structure from acid to enzymatic hydrolysis and vice versa. Two decisive points of difference have to be kept in mind here: (1) the different size of the active agent, i.e., the rather small H_3O^+ ion on the one hand, the large protein molecules on the other, thus leading to a strong influence of pore structure and fibrillar morphology on enzymatic but not on acid hydrolysis; and (2) the difference in selectivity of the active agents, with the H_3O^+ ion cleaving acetal and hemiacetal linkages of any kind but the enzyme molecules acting rather specifically.

These rather simple considerations explain most of our experimental results—for example, the behavior of thermally treated samples, the different influences of dry and wet disintegration on acid and enzymatic hydrolysis, or the pronounced increase in accessibility to acid hydrolysis as compared to enzymatic degradation by treatment with liquid NH_3. No definite interpretation can be given so far of the unexpected strong increase in weight loss during enzymatic degradation after a previous mercerization.

Further progress in understanding this effect, as well as others, of the physical structure of cellulose on enzymatic degradation may be expected from combining physicochemical and morphological techniques and from kinetic measurements in heterogeneous enzymatic hydrolysis, applying substrates of well-defined physical structure and isolated components of the enzyme systems.

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Hydrolysis of Polysaccharides with Trifluoroacetic Acid and its Application to Rapid Wood and Pulp Analysis

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For the total hydrolysis of polysaccharides, trifluoroacetic acid (TFA) has important advantages over sulfuric acid. The reaction time is short and there is no need for conventional neutralization, as TFA is volatile and can be removed by evaporation. Several methods have been developed, depending on the substance to be hydrolyzed. Soluble saccharides (e.g., polyoses) can be hydrolyzed with diluted TFA, while cellulose, pulp, and wood need treatments with concentrated TFA in homogeneous solution. The presence of lignin impedes the hydrolysis of polysaccharides; thus, especially for wood samples, an intensive treatment with TFA is necessary, and correction values have to be considered. Several application examples show that the hydrolysis with TFA enables a rapid quantitative determination of the composition of polysaccharides, pulps, and woods.

Hydrolysis plays an important role in wood and pulp chemistry and means a defined degradation of polysaccharides at the glycosidic bonds for analytical and preparative purposes. In analysis the application of acids, particularly of sulfuric acid, is usual (*1, 2*). No problems would arise if the reaction between acid and polysaccharides would stop after the splitting of glycosidic bonds. But the monosaccharides also react with acid, decreasing their yields. The splitting rate varies for different glycosidic bonds and for different monosaccharide units. The attack of the acid may also be hindered by supramolecular structures. Thus, many factors influence acid hydrolysis and preclude a good yield of sugars.

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For several years, trifluoroacetic acid (TFA) has been used for the hydrolysis of proteins, glycoproteins, and glycosides (*3, 4, 5, 6*), where the acid treatment is carried out under pressure at temperatures over 100°C.

We applied TFA to hydrolysis of polysaccharides under various conditions (*7, 8, 9*). Many tests have shown that some troubles known from sulfuric acid treatment can be eliminated using TFA. In addition, the tests resulted in a wide applicability and flexibility of the method, and it can be easily adapted to many polysaccharides.

TFA is a strong acid with a pungent smell. As it is volatile, no neutralization is necessary: the acid can be removed by evaporation.

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Results and Discussion

For the study of soluble polysaccharides, a treatment with diluted TFA is sufficient and the reaction time can be kept short (*7*). Soluble polysaccharides of wood are separated from holocellulose by extraction with alkali. Wise et al. (*10*) term the extract with 5% KOH polyoses (hemicelluloses) A. Polyoses A can be hydrolyzed completely with 2*N* TFA within 1 hr. The chromatograms of the hydrolysates of polyoses A from spruce and beech holocelluloses recorded with a sugar analyzer (Biotronik ZA 5100) are shown in Figure 1.

In the chromatogram of spruce polyoses, mannose, galactose, and glucose from galactoglucomannan, and arabinose, xylose, and 4-0-methylglucuronic acid from arabino-4-0-methylglucuronoxylan are present. The main components in the hydrolysates of beech polyoses are xylose and 4-0-methylglucuronic acid deriving from 4-0-methylglucuronoxylan.

A comparison of a TFA hydrolysis with a sulfuric acid hydrolysis (Table I) demonstrates that all sugars—but not glucose—are obtained in higher yield with TFA than with sulfuric acid; this is evidence for the controlled action of TFA.

Table I. Hydrolysis of Polyoses A from Spruce Holocellulose with 72(%) Sulfuric Acid[a] and 2*N* TFA[b]

Sugar	*H_2SO_4 (%)*	*TFA (%)*
Mannose	6.2	7.5
Arabinose	2.3	3.5
Galactose	3.6	4.4
Xylose	16.0	25.1
4-O-Methylglucuronic acid	4.6	8.9
Glucose	2.8	2.7

[a] According to Jayme and Knolle (*2*).
[b] According to Method 1.

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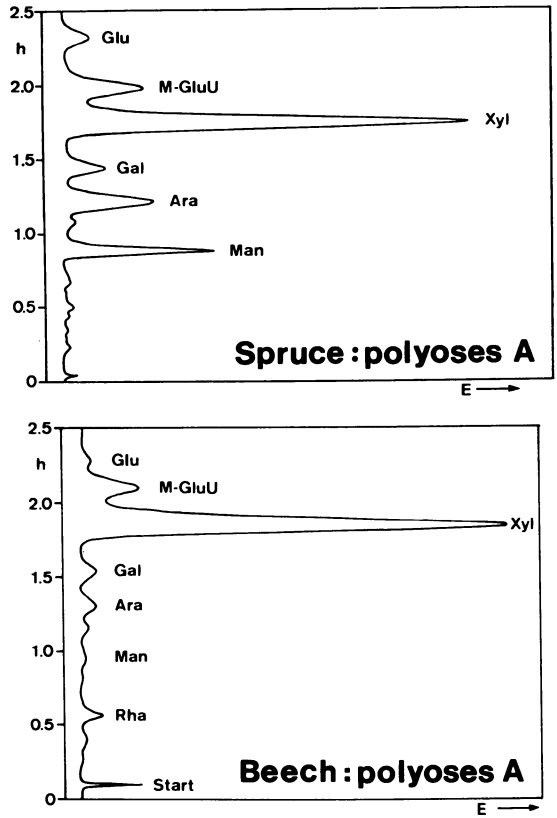

Figure 1. Chromatograms of the hydrolysates of polyoses A from spruce-wood and beechwood. Rha = rhamnose, Man = mannose, Ara = arabinose, Gal = galactose, Xyl = xylose, M-GluU = 4-O-methylglucuronic acid, Glu = glucose.

In Figure 2 the chromatograms of the hydrolysates of wheaten bran, draff, and pease-meal are presented; these substances can be hydrolyzed easily with 2*N* TFA in 1 hr.

If 2*N* TFA is applied to cellulosic material, e.g., α-cellulose of wood, only a small portion (about 20%) is completely hydrolyzed. Higher TFA concentrations and prolonged reaction time do not improve the hydrolysis. Figures 3 and 4 show electron micrographs of cellulose treated with TFA at different concentrations. The treatment with very highly concentrated TFA (95%) results in relatively long needlelike fragments (Figure 3); after treatment with 2*N* and 5*N* TFA, apart from shorter fragments, a formation of oblong laminae of recrystallized cellulose can be observed (Figure 4). In these cases, the insoluble residues amount to 60–70% of

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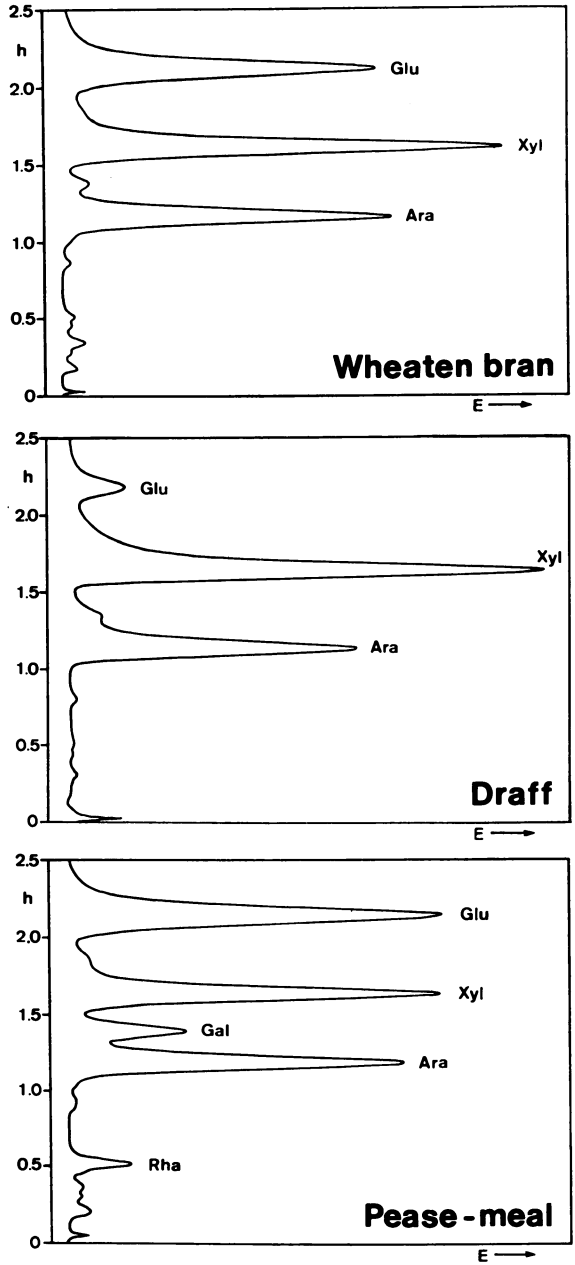

Figure 2. Chromatograms of hydrolysates of wheaten bran, draff, and pease-meal

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the charge. At highest concentration, i.e., 100% TFA, cellulose is dissolved, a phenomenon observed previously by Valtsaar and Dunlap (*11*) and Geddes (*12*).

But also a treatment with 100% TFA at high temperature results in a poor hydrolysis of cellulose. After a reaction time of 4 hr at 120°C in a closed tube, about 80% of the charge was precipitated when diluting the TFA. This residue could not be completely hydrolyzed with diluted TFA, though the average degree of polymerization was only about 30. In the electron microscope, samples of cellulose precipitated from TFA solution and treated with diluted TFA show broad crystallike platelets (Figure 5).

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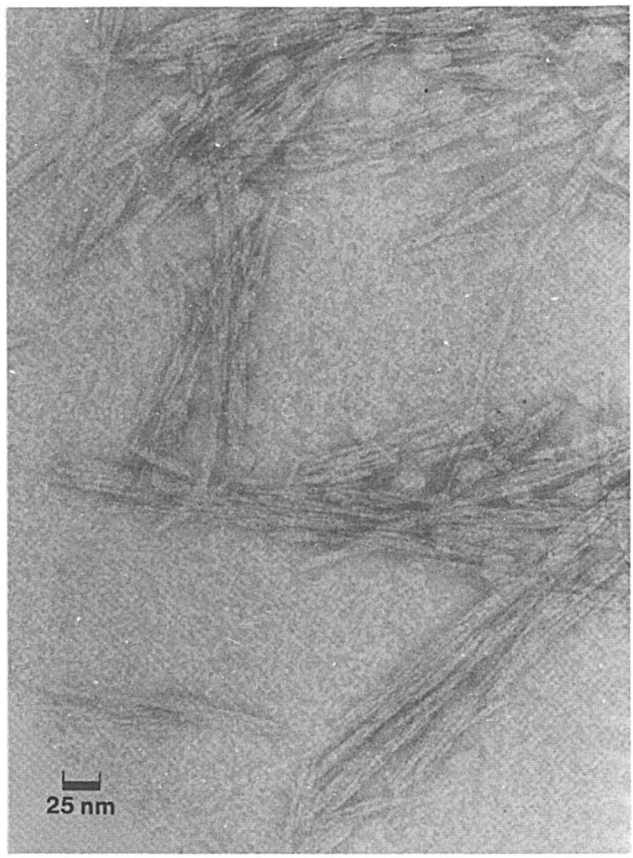

Figure 3. Electron micrograph of cellulose from beechwood, boiled with 95% TFA for 8 hr. Negative staining with uranyl acetate.

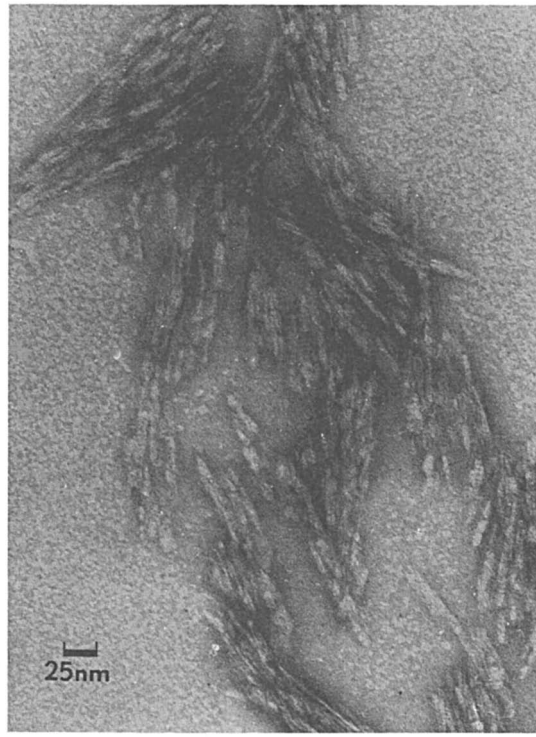

Figure 4. Electron micrograph of cellulose from beechwood, boiled with 2N TFA for 2 hr and subsequently with 5N TFA for 5 hr. Negative staining with uranyl acetate.

A series of hydrolysis tests led to the idea to keep the cellulose in solution until the total hydrolysis is performed. In doing so, we observed that short cellulose chains also remain in solution in diluted TFA. Thus a method was developed in which cellulose is dissolved in 100% TFA at boiling temperature (73°C). Thereupon the solution is diluted with water (without precipitating the cellulose), boiled, and diluted again.

The whole procedure normally takes about 1 hr. The acid is then evaporated, and the dry matter can be analyzed. This method can be applied to cellulose from wood, as α-cellulose or pulp, or to other celluloses (e.g., cotton) as well as to cellulosic materials with higher amounts of other polysaccharides (e.g., holocellulose). The chromatograms of the hydrolysates of α-cellulose from beechwood and of holocellulose from sprucewood (Figure 6) are examples of the application of this method. Compared with sulfuric acid hydrolysis, the total sugar yield from the spruce holocellulose is higher after the hydrolysis with concentrated TFA (Table II). Regarding the individual sugars, it can be seen that the

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Table II. Hydrolysis of Holocellulose from Spruce With 72(%) Sulfuric Acid[a] and 100(%) TFA[b]

Sugar	*H_2SO_4 (%)*	*TFA (%)*
Mannose	10.5	11.9
Arabinose	1.0	1.1
Galactose	1.1	1.3
Xylose	4.6	4.5
4-O-Methylglucuronic acid	0.0	1.3
Glucose	50.2	59.3

[a] According to Jayme and Knolle (*2*).
[b] According to Method 2.

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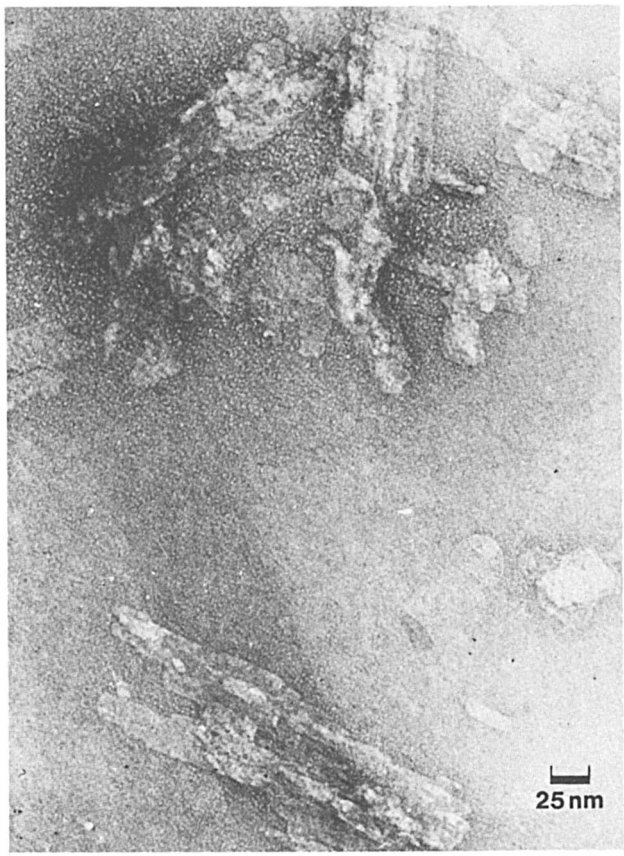

Figure 5. Electron micrograph of cellulose from beechwood, dissolved in 100% TFA at 120°C, precipitated, and boiled with 2N TFA for 6 hr. Negative staining with uranyl acetate.

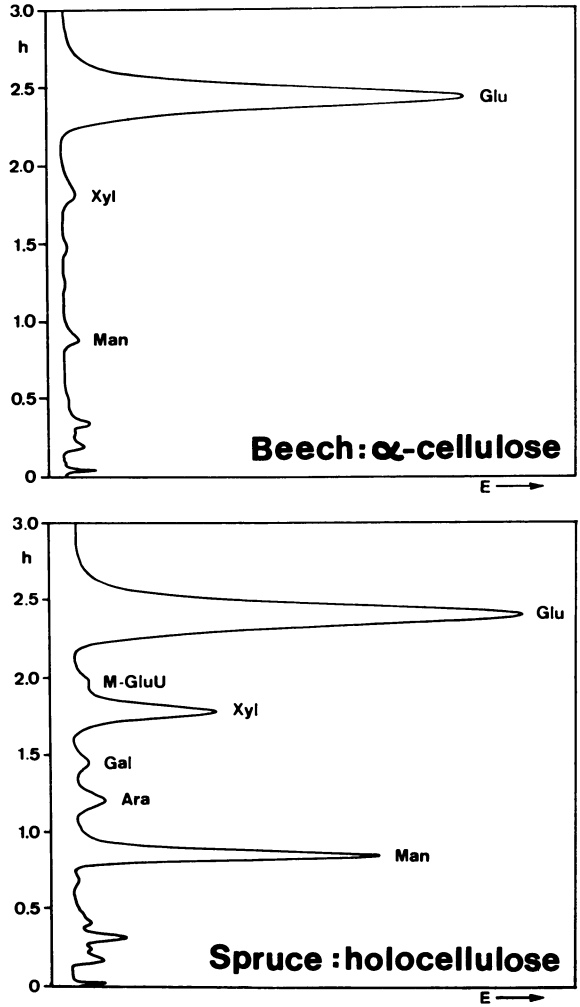

Figure 6. Chromatograms of the hydrolysates of α-cellulose from beechwood and holocellulose from sprucewood

Table III. Yield and Fluorine Content of TFA Reaction Products

			After H_2O Treatment	
Reaction Product	*Yield (%)*	*F (%)*	*Yield (%)*	*F (%)*
TFA + α-cellulose	173	18.4	99	0
TFA + glucose	174	—	102	—

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action of both acids on arabinose, galactose, and xylose is about the same, whereas TFA brings a higher yield for mannose, glucose, and 4-O-methylglucuronic acid because of a better splitting and/or a moderate action on these sugars.

During the dissolution, an intermediate compound between cellulose and TFA is formed, which is described in the literature as an addition compound (*11*) and as a monoester (*12*). These compounds should give different theoretical yields—170% for the addition compound and 159% for the monoester (based on cellulose charge)—and different fluorine contents—20.6% and 22.1%, respectively. To check this, α-cellulose and glucose were treated with concentrated TFA at 120°C. After removing the TFA by evaporation, the reaction products were dried carefully at 40°C in vacuo over KOH pellets for 44 hr. The products were weighed and their fluorine contents determined. Another part of the reaction product was treated with water, dried, weighed, and analyzed for fluorine.

The data obtained for yield and fluorine content (Table III) are more in accordance with an addition compound than with a monoester. Therefore, formation of such a compound containing one molecule of TFA per sugar unit during the treatment of polysaccharides with TFA is very probable. On the other hand, this result means for the hydrolysis method that after evaporating the TFA, the hydrolysate should be treated with water to decompose the addition compound.

The method described for the hydrolysis of cellulose-containing material does not result in sufficient sugar values if applied to wood, as lignin impedes the hydrolysis of polysaccharides. For substances with higher lignin contents, the time of swelling and hydrolysis must be prolonged. During such an intensive process, some of the sugars, mainly the pentoses, are decomposed, and their losses are up to 32% of the original individual sugar. So for this method, correction values had to be evaluated to correct the yields of the single sugars. These values were obtained by determination of the losses of individual sugars during the treatment of monosaccharides with TFA. Using the correction values, the intensive hydrolysis method is suitable for the determination of the sugar composition of wood (Table IV). The method was also applied to pulp.

The data of Table IV can be used for a complete wood or pulp analysis if some findings from the literature are considered which have reference to woods of temperate zones:

The main polyoses of softwoods are galactoglucomannan, with an average ratio of glucose to mannose of 1:3 and an average acetyl content of 1.3% (based on wood), and arabino-4-O-methylglucuronoxylan.

The main polyoses of hardwoods are 4-O-methylglucuronoxylan, with an average acetyl content of 4% (based on wood), glucomannan, with an average ratio of glucose to mannose of 1:2, and arabinogalactan.

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Table IV. Corrected Sugar Yields after Intensive Hydrolysis of Wood and Pulp with Trifluoroacetic Acid[a]

Sample	*Rha* (%)	*Man* (%)	*Ara* (%)	*Gal* (%)	*Xyl* (%)	*MGluU* (%)	*Glu* (%)
Picea abies	0.3	13.6	1.2	2.8	5.6	1.8	44.9
Larix decidua	0.0	11.5	2.0	6.1	5.1	2.2	43.3
Fagus sylvatica	0.5	0.9	0.7	1.4	19.0	4.8	43.8
Betula verrucosa	0.6	3.2	0.4	0.7	24.9	3.6	35.8
Fraxinus excelsior	0.5	3.8	0.6	0.9	18.3	6.0	39.8
Spruce sulphite pulp (unbleached)	0.0	6.9	0.0	0.0	3.6	0.0	80.4
Beech sulphite pulp (bleached, chem. grade)	0.0	1.2	0.0	0.0	2.6	0.0	97.9

[a] According to Method 3.

The average ash content of wood is 0.5%.

Furthermore it must be considered that parts of the lignin are dissolved during the hydrolysis, and therefore the lignin content must be determined indirectly from the difference of polysaccharide and ash content to 100%. Table V shows the data of wood and pulp analysis based on intensive hydrolysis with TFA and calculated under consideration of the mentioned facts.

For avoiding high losses in pentoses, another, more moderate hydrolysis method for lignin-containing material was developed. The method is proposed only if arabinose, xylose, galactose, and rhamnose are of interest. In this case, the material is swollen in 100% TFA and boiled with 20% TFA. The result of such a moderate hydrolysis, compared with that of an intensive hydrolysis, is demonstrated in Figure 7. It can be seen that after careful hydrolysis the areas of the peaks of rhamnose

Table V. Wood and Pulp Composition

Sample	*Mannan*[a] (%)	*Xylan*[b] (%)
Picea abies	22.2	8.9
Larix decidua	22.7	9.3
Fagus sylvatica	1.4	27.8
Betula verrucosa	4.8	32.5
Fraxinus excelsior	5.7	28.3
Spruce sulphite pulp (unbleached)	9.2	3.6
Beech sulphite pulp (bleached, chem. grade)	1.8	2.6

[a] Softwoods: galactoglucomannan with 1.3 (%) acetyl; hardwoods: glucomannan.
[b] Softwood: arabino-4-O-methylglucuronoxylan; hardwoods: 4-O-methylglucuronoxylan with 4 (%) acetyl.

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(Rha), arabinose (Ara), galactose (Gal), xylose (Xyl), and 4-O-methylglucuronic acid (M-GluU) are the same or larger than after intensive hydrolysis, evidence for a more moderate action of the acid. But for a complete hydrolysis of cellulose and glucomannan, the moderate method is not sufficient; therefore, the peaks for mannose (Man) and glucose (Glu) are much smaller after this treatment.

Finally, the TFA method can also be applied to materials with a very low polysaccharide content. One example is milled wood lignin (MWL) containing about 1.5% polysaccharides (Figure 8). Another example is fossil wood—a protopinacea, about 180 million years old, which contained about 0.02% polysaccharides, detected by the aid of TFA hydrolysis (Figure 8).

TFA has been used for about three years in our laboratory, and it has become our exclusive reagent for the total hydrolysis of polysaccharides. Moreover, dilute TFA is applied to partial hydrolysis of lignin–polysaccharide complexes (*13*).

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Methods

Preliminary Remarks. The material to be hydrolyzed should be in a milled or pulverized condition or reduced to minute pieces. Wood and pulp samples should be extracted with ethanol–benzene and ethanol according to TAPPI-Standard T6m-59. Each method described below can be applied to 2–50 mg of material. The evaporator for removing the TFA should be kept in a hood. Contact of the acid with skin and eyes must be avoided.

Soluble Polysaccharides. The sample is weighed into a round-bottomed flask and 3–6 g TFA is added. The mixture is refluxed for 1 hr. If an unhydrolyzable residue remains, the solution is filtered through a microcrucible. The solution is evaporated in a rotatory vacuum evaporator. About 10 mL of water is added to the dry matter and the solution

Based on Intensive Hydrolysis with TFA

Galactan[c] *(%)*	*Polyoses (%)*	*Cellulose (%)*	*Ash (%)*	*Lignin (%)*
0.0	31.1	40.4	0.5[d]	28.0
0.0	32.0	39.5	0.5[d]	28.0
2.6	31.8	43.3	0.5[d]	24.4
1.7	39.0	34.2	0.5[d]	26.3
2.0	36.0	37.9	0.5[d]	25.6
0.0	12.8	78.1	0.8[e]	8.3
0.0	4.4	97.3	0.0	0.0

[c] Hardwoods: arabinogalactan.
[d] Assumed average content.
[e] Determined.

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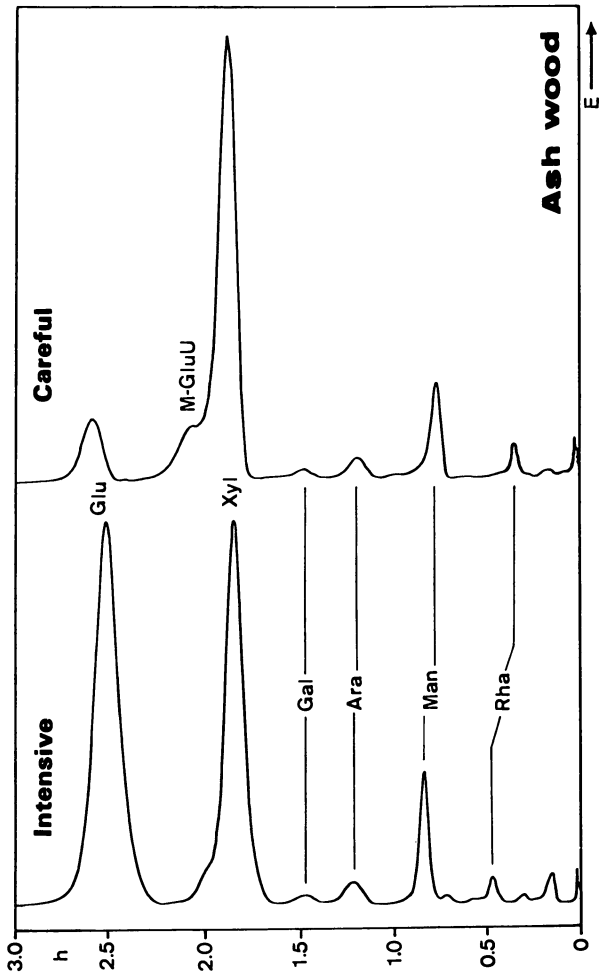

Figure 7. Comparison of the chromatograms of the hydrolysates of ashwood after the intensive and more careful hydrolysis with TFA

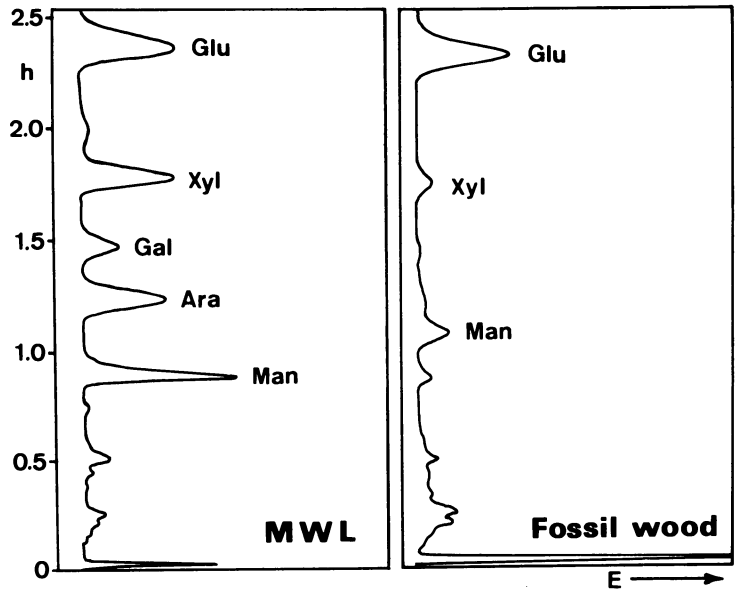

Figure 8. Chromatograms of the hydrolysates of materials with low polysaccharide content: milled wood lignin (MWL) from sprucewood and a 180-million-year-old protopinacea

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is evaporated again; this procedure is repeated once more. The dry hydrolysate can be dissolved in a defined volume of water and analyzed by thin-layer chromatography or in a sugar analyzer.

Cellulose-Containing Material (Low Lignin Content). About 3–6 g of concentrated TFA (100%) is added to the weighed sample in a round-bottomed flask. The mixture remains for at least 15 min at room temperature for swelling. Subsequently, the mixture is refluxed until the solution is clear (normally during about 15 min). Thereupon the TFA is diluted to 80% by addition of water, and the solution is refluxed again for 15 min. Then the TFA is diluted to 30% by adding water. After refluxing for 30 min, the TFA is evaporated. The residue can be analyzed. It is possible that material containing high-crystalline cellulose makes an application of the more intensive method, described below, necessary.

Cellulose-Containing Material (High Lignin Content). About 3–6 g of concentrated TFA (100%) is added to the weighed sample in a round-bottomed flask. The mixture remains overnight at room temperature for swelling, or it is heated at 60°C for 2 hr. Subsequently, it is refluxed for 1 hr. The solution is diluted to 80% TFA. After refluxing for 15 min, the solution is diluted to 30% TFA and refluxed for 2 hr. After filtration through a filter crucible, the TFA is evaporated. The dry matter is dissolved at least twice in water, evaporated again, and finally analyzed. The data for the single sugars received by thin layer chromatography or with the sugar analyzer must be corrected by dividing them by the following correction values:

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rhamnose	0.68	galactose	0.89
arabinose	0.80	mannose	0.85
xylose	0.70	glucose	0.95

If only rhamnose, arabinose, xylose, and galactose are to be analyzed in a sample, the mixture with TFA can be diluted after swelling to 20% TFA and refluxed for 3 hr. Then the solution is filtered and the TFA is evaporated. After the dissolution in water and repeated evaporation, the residue is analyzed. The correction values for the single sugars after this more moderate acid treatment are as follows:

rhamnose	0.95	xylose	0.85
arabinose	0.88	galactose	0.92

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8

Rapid Hydrolysis of Celluloses in Homogeneous Solution

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A rapid and complete hydrolysis sequence for celluloses including those of highest crystallinity, was developed. It consists of dissolving the cellulose in a mixture of acetic acid–acetic anhydride–dimethyl formamide–sulfuric acid at 120° to 160°C and decomposing the cellulose acetate sulfate formed by gradual addition of aqueous acid. Whereas glucose was formed quantitatively, the hydrolysis of various carbohydrate-containing material gave decomposition products in addition to monosaccharides.

The determination of the sugar units of a carbohydrate-containing material consists of (a) complete acid hydrolysis and (b) chromatographic separation and quantitative analysis of the monomeric sugars. The latter part has been greatly improved with the advances of modern and automatic techniques, notably ion exchange chromatography. The first part is still a time-consuming and not always reproducible process because of its heterogeneous nature.

The standard procedure by Saeman et al. (*1*) involves manual stirring of the polysaccharide with 72% H_2SO_4, standing at 30°C, and "secondary hydrolysis" at 100° or 120°C in a steam autoclave. While certain resistant polysaccharides are still incompletely depolymerized, decomposition of the more sensitive monosaccharides formed cannot be avoided. An alternative method by using trifluoroacetic acid was applied successfully for plant cell wall polysaccharides by Albersheim et al. (*2*) and for dissolving pulps and hemicelluloses by Fengel et al. (*3*). Highly crystalline cellulose was not well dissolved and not completely hydrolyzed by CF_3COOH.

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Another approach to achieve a fast and total breakdown of cellulose could be the preparation of a soluble derivative and its homogeneous hydrolysis. An example of an acetylation–acetolysis–hydrolysis sequence is given by Hestrin (*4*). Decomposition and darkening is considerable.

The observation of Blume and Swezey (*5*) that cellulose dissolves extremely rapidly in acetic anhydride–dimethylformamide, containing sulfuric acid catalyst, suggested that we investigate the possibilities of this reaction for hydrolyzing celluloses. The cellulose acetate formed was not degraded, whereas our purpose was complete degradation. Accordingly, the depolymerization of the cellulose derivative in solution had to be examined.

Among numerous studies by Malm and co-workers on cellulose acetate, a paper on "chain-length breakdown of cellulose by acetic acid solutions of water and sulfuric acid" (*6*) describes the importance of small concentrations of H_2SO_4 and H_2O for the reaction rates. These investigations were continued (*7*) in the range of 0.3 to 20% water in the solvents.

We started a series of experiments to dissolve celluloses, pulps, and samples of wood or bark in acetylating media containing dimethylformamide, followed by hydrolysis to achieve rapid and complete formation of monomeric sugars. The main purpose was to develop a rapid and reproducible method for the determination of the sugar units present in polysaccharides, particularly those resistant to milder treatment.

Table I. Dissolution

No.	*Cellulose*[b]	*Acid*	*% Acid on Cellulose*	*Temp. (°C)*
1	A	$HClO_4$	50	140
2	C	HCl	50	120
3	A	CF_3COOH	200	100
4	B	HCOOH	750	100
5	B	CF_3SO_3H	50	140
6	B	H_2SO_4	1.5	140
7	B	H_2SO_4	10	160
8	B	H_2SO_4	10	120
9	B	H_2SO_4	25	120
10	A	H_2SO_4	50	100
11	B	H_2SO_4	50	120
12	B	H_2SO_4	50	140
13	A	H_2SO_4	50	140
14	A	H_2SO_4	50	160

[a] 0.40 g in 1–20 mL DMF, 0–10 mL CH_3COOH, 2 mL $(CH_3CO)_2O$.
[b] A = cotton, B = cotton linters, C = MN cellulose HR.

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Results and Discussion

Most experiments were performed with cotton or cotton linters as highly crystalline celluloses. Table I shows conditions leading to complete dissolution. A minimum amount of an acid which forms a cellulose ester (sulfuric or trifluoromethylsulfuric acid) (Entries 5; 7–14) is necessary for the reaction. The dissolution is accelerated by a temperature increase (Entries 10–12; 13, 14) and leads to water-soluble cellulose acetate hydrogensulfate. Whereas this "primary hydrolysis" can be achieved within 1–5 min, the deesterification and complete hydrolysis of the soluble cellulose derivative proved to be much more difficult. This is in contrast to the generally accepted view that the main resistance to the hydrolysis of cellulose lies in the crystalline nature or low accessibility determining the heterogeneous first step of the reaction.

The complete hydrolysis of the soluble cellulose derivative required gradual addition of water and, preferably, acid. Water can act on cellulose acetate hydrogensulfate in three ways: (a) saponification of sulfate groups, (b) saponification of acetate groups, and (c) hydrolysis of glycosidic bonds. Reaction (a) is most rapid and occurs under the mildest conditions. As Table II shows, the product is not any more water soluble (Entries 1–4). Under more severe conditions, the deacetylation (b) has proceeded to water-soluble products, the total amount of dissolved carbohydrates corresponding to theory (Entries 5–10). How-

Experiments of Cellulose[a]

Time (min)	*Dissolution*	*Time (min)*	*Precipitate on Addition to Water*	*Total Carbohydrate in Solution[a]*
10	none			
25	none			
60	none			
20	none			
1	complete	20	none	
60	little			
4	complete	20	none	108
3	complete	5	yes	
3	complete			
20	complete	30	trace	99
5	complete	5	none	98
2	complete	2	none	
3	complete	3	none	
1	complete	1	trace	94

[a] Determined by Orcinol–H_2SO_4 and calculated on theoretical yield of glucose.

In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

Table II. Secondary Hydrolysis of Cellulose Acetate Hydrogensulfate

No.	*% Cellulose in Solvent*[b]	*% H_2SO_4 in Solvent*[b]	*DMF (mL)*	*HOAc (mL)*
1	1.4	0.7	20	4
2	2.5, . . . , 1	1.3, . . . , 0.6	10	4
3	2, . . . , 1	2, . . . , 0.8	10	8
4	4, . . . , 3	2, . . . , 17	1	7
5	2, . . . , 1	4, . . . , 10	20	0
6	3, . . . , 2	8, . . . , 4	5	5
7	3, . . . , 2	8, . . . , 4	2	8
8	3, . . . , 1	8, . . . , 4	0.5	10
9	3, . . . , 1	16, . . . , 4	1	10
10	3, . . . , 1	16, . . . , 4	0.5	10
11	4, . . . , 1	8, . . . , 14, . . . , 9	1	7

[a] 0.4 g linters in 0.5–20 mL DMF, 0–10 mL HOAc, 2 mL $(Ac)_2O$.
[b] Varying concentrations are a consequence of the addition of water or sulfuric acid after 5 and 10 min.

ever, the smaller value for the reducing carbohydrates in solution indicates incomplete hydrolysis to monosaccharides (Reaction (c)).

To achieve total breakdown of the polymeric carbohydrates, the gradual addition of aqueous acid was convenient (Entry 11). Sulfuric acid or perchloric acid provided the high acid strength necessary. Table III (Entries 2–5) indicates that a high acid concentration leads to a complete depolymerization of glucose oligomers as shown by ion exchange chromatography. Dimethylformamide, although necessary to obtain fast and colorless dissolution of cellulose, retards the hydrolysis reaction. Most of it could be replaced by acetic acid.

Table III. Secondary Hydrolysis of Cellulose Acetate

No.	*% Cellulose in Solvent*[a]	*% H_2SO_4 in Solvent during Dissolution*	*% $HClO_4$ in Solvent*[a]
1	5, . . . , 2	0.5[d]	7, . . . , 10, . . . , 7
2	4, . . . , 1	8[e]	7, . . . , 10, . . . , 7
3	4, . . . , 2	8[e]	6
4	4, . . . , 1	8[f]	7, . . . , 12, . . . , 10
5	4, . . . , 1	8[g]	7, . . . , 12, . . . , 10

[a] Varying concentrations are a consequence of the addition of perchloric acid and water after 5, 10, and 15 min.
[b] Determined according to Sumner and calculated on theoretical yield of glucose.
[c] Determined by ion exchange chromatography.

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in DMF–HOAc by Gradual Additions of Water or Aqueous H_2SO_4[a]

% Water in Solvent after Addition[b]	*Time (min)*	*Precipitate on Addition to Water*	*Total Carbohydrate in Solution*[c] *(%)*	*Reducing Carbohydrates in Solution*[d] *(%)*
3.5	60	yes		
10, . . . , 50	45	yes		
10, . . . , 60	60	yes		
17	10	yes		
20, . . . , 50	85	trace	91	29
20, . . . , 50	30	trace	103	36
20, . . . , 40	15	none	104	42
20, . . . , 60	20	none	99	62
40, . . . , 70	30	none	105	76
30, . . . , 70	30	none	106	88
30, . . . , 70	30	none	—	102

[c] Determined by Orcinol–H_2SO_4 and calculated on theoretical yield of glucose.
[d] Determined according to Sumner on theoretical yield of glucose.

The course of the reaction was proved by isolating intermediate stages of the sequence either by precipitation or by dialysis: (1) After 20 minutes in a solution containing 0.8% H_2SO_4, the precipitated cellulose acetate hydrogensulfate was obtained in a 130% yield on cellulose. The degree of substitution for acetyl was 2.24; for hydrogensulfate it was 0.03. (2) From a solution with 8% H_2SO_4, the yield of cellulose derivative after dialysis was 85% with $DS_{OAc} = 1.3$; $DS_{OSO_3H} = 0.01$ after 3 min. After 9 min, 48% could still be isolated by dialysis, and after 15 min, 20% was not yet sufficiently degraded to escape by dialysis.

Hydrogensulfate by Aqueous Perchloric Acid in DMF–HOAc

% Water in Solvent after Additions[a]	*Time (min)*	*Reducing Carbohydrate in Solvent*[b] *(%)*	*Glucose % of Reducing Carbohydrate*[c]
30, . . . , 65	30	63	7
30, . . . , 65	30	95	71
30	30	92	72
30, . . . , 65	30	102	99
30, . . . , 65	30	102	97

[d] 0.4-g linters and 3-mL DMF.
[e] 0.4-g linters and 1-mL DMF.
[f] 0.4-g linters and 0.5-mL DMF.
[g] 0.4-g linters and no DMF; very dark solution.

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The conditions necessary for a complete and fast breakdown of cellulose to glucose in solution were more severe than expected: acid concentrations of 8 to 15.5% at 110°C for 30 min. Monosaccharides more sensitive to such an environment could therefore be expected to undergo considerable deterioration. Also, some of the noncarbohydrate components in wood and bark are sensitive to acid-catalyzed condensation and decomposition reactions. Accordingly, it was no surprise to obtain rather dark solutions on applying the experimental conditions of Table III to pulps, holocelluloses, and samples of wood and bark. The content of reducing substances in the solutions was frequently higher than expected from the sugar composition, which also points to decomposition products, yielding color-intensive compounds with Sumner's reagent. Analysis by ion exchange chromatography showed overall sugar yields higher than obtained by sulfuric acid hydrolysis, but the relative amounts of monosaccharides were not reproduced due to uneven destruction.

Fir bark, extracted with ethanol, may serve as an example:

Hydrolysis with	*Residue*	*Red. Sub.*	*Sugar Units (Rel. %)*						
			Oligo. sacch.	*Rha*	*Ara*	*Man*	*Gal*	*Xyl*	*Glc*
DMF–HOAc–H_2SO_4 ($HClO_4$)	29%	72%	1	2	8	3	5	7	74
72% H_2SO_4 (H_2O)	37%		0	1	10	8	5	10	65

The residues left after the treatment were always lower than after applying "regular" hydrolysis conditions and depended on the acid strength. Fir wood could be dissolved to 95%.

From these experiments we conclude that for pure and highly crystalline celluloses, a fast decomposition to glucose with minimum by-products is possible. Carbohydrates, containing other sugar units than glucose, are hydrolyzed accompanied by destruction of monosaccharides. The conditions for complete breakdown of cellulose are too severe for other polysaccharides. The problem of achieving optimum hydrolysis conditions is, of course, not a new one and must be solved for each polysaccharide or polysaccharide-containing material. The problem was not pursued further.

To determine the velocities of the aforementioned Reactions (a), (b), and (c), products will be isolated after short reaction times and their degrees of substitution and polymerization will be determined. By selecting proper experimental conditions, we expect to be able to prepare solutions of cellulose with various desirable DS's and DP's. The preparative possibilities of these rapidly obtainable solutions of cellulose, wood, or other cellulosic substances will be investigated.

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In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

Experimental

Cotton linters (DP = 2360) were obtained from Temming AG; MN cellulose was obtained from Macherey Nagel; cotton was of Israeli origin and bleached. Wood and bark samples were obtained from freshly cut trees. Pulps and holocelluloses were prepared in the Institut für Holzchemie. Other chemicals were reagent grade. In the dissolution experiments, the solvents were added to the cellulosic material and shaken in an oil bath at 120° to 160°C. Dissolution was accompanied by boiling and a color change (more or less) to yellow or brown. This was intensified by addition of acid or decreasing the content of DMF. Twenty percent aqueous H_2SO_4 or $HClO_4$ was added in two or three portions after 5, 10, and 15 min through a reflux condenser. Generally, after 30 min, the contents of the flask were poured into an excess of water. After filtration of the residue, if left, the total carbohydrate content of the solution was determined with Orcinol–sulfuric acid (*8*) and the content of reducing sugars according to Sumner (*9*). The sugar composition was analyzed quantitatively by the method of Sinner et al. (*10*). The determinations of the degree of substitution were performed by standard procedures for acetyl content (*11*) and sulfur content (*12*), respectively.

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9

New Catalysis Systems for Hydrolysis of Model Substances of Cellulose

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Polymer catalysts showing interactions with the substrate, similar to enzymes, were prepared and their catalytic activities on hydrolysis of polysaccharides were investigated. Kinetical analyses showed that hydrogen bonding and electrostatic interactions played important roles for enhancement of the reactions and that the hydrolysis rates of polysaccharides followed the Michaelis–Menten type kinetics, whereas the hydrolysis of low-molecular-weight analogs proceeded according to second-order kinetics. From thermodynamic analyses, the process of the complex formation in the reaction was characterized by remarkable decreases in enthalpy and entropy. The maximum rate enhancement obtained in the present experiment was fivefold on the basis of the reaction in the presence of sulfuric acid.

Recently, designs and preparations of new catalysis systems with high activity and selectivity, resembling enzymes, have been a subject of numerous investigations. Some reviews are available for these studies (*1, 2, 3, 4, 5*). The enzyme molecule contains active centers which directly catalyze the reaction and substrate binding sites which distinguish the substrate and selectively bind it. In the attempts to synthesize new effective catalysts, these functions also have been investigated, and the substrate binding effect has been discussed in numerous papers. The effects have ranged from those showing only the "concentration effect" to those containing selectivity for the substrate and/or multifunctional interactions in addition to the concentration effect. In general, interactions

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of hydrophobic, electrostatic, hydrogen bonding, and charge transfer nature are important for substrate binding. In the synthesized catalyst systems, however, hydrophobic and electrostatic interactions predominantly play important roles, especially in an aqueous solution.

We have investigated the hydrolysis of polysaccharides such as cellulose and its analogs in the presence of newly designed catalysts, applying the information obtained so far with enzymatic and chemical catalyses to our reaction systems. The information, however, could not be applicable directly to the hydrolysis of cellulose because the information was mainly concerned with the hydrolysis of more labile linkages such as phenyl ester bond and with interactions of hydrophobic and electrostatic nature. Glucoside linkage is more stable, and cellulose has neither a hydrophobic group nor an electric charge but has only a hydroxyl group as a functional one. In this chapter, discussions are narrowed down to the hydrolysis in the presence of soluble polymer catalysts although other catalysis systems such as those in the presence of an insoluble polymer catalyst (*6, 7, 8*) or reversed micelle (*9, 10*) are also interesting. Some attempts already have been made to use polymer catalysts to the hydrolysis of carbohydrates. In most cases, however, the catalytic activities were comparable with or less than that of mineral strong acids. Kern et al. (*11*) investigated the hydrolysis of sucrose catalyzed by poly(vinylsulfonic acid). They found that the catalytic activity of the polymer was comparable with that of sulfuric acid. Hartler et al. (*6*) found that in the hydrolysis of sucrose, catalyzed by a poly(styrenesulfonic acid) type cation exchanger, the reaction rate was 1.25 times greater than that in the reaction catalyzed by sulfuric acid and that in the case of cotton the reaction practically did not proceed. Paiter et al. (*12*) reported that in the hydrolysis of starch the catalytic activity of poly(styrenesulfonic acid) was about 30% less than that of hydrochloric acid. These results seemed to be related to the fact that these polymers had no functional groups which would interact with the substrate.

Then, as a first step, we designed some polymer catalysts containing polyalcohol sequences which were expected to form hydrogen bridges with hydroxyl groups of cellulose (Figure 1). Besides enzymatic reactions, there is little information on hydrogen bonding interactions in aqueous solutions. However, the hydrogen bonding interactions are believed to be rather strong even in water. For example, cellulose is insoluble in water, and even the solution of amylose or poly(vinyl alcohol) with high degree of saponification is not sufficiently stable, and the solute can associate to precipitate depending on the concentration and the temperature. In addition, Toyoshima et al. (*13*) reported compatibility between poly(vinyl alcohol) and starch. Therefore, we expected

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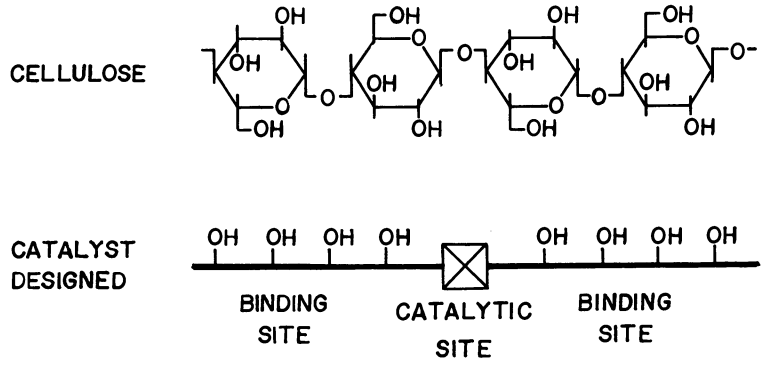

Figure 1. Drawing of polymer catalyst designed. Catalytic site is $-SO_3^-H^+$ in this chapter.

that the substrate binding attributable to hydrogen bonding interactions between hydroxyl groups of the substrate and vinyl alcohol sequences in the catalyst might be useful for enhancement of the reaction.

Acid hydrolysis of cellulose is considered to be a specific catalysis and the reaction rate depends on the concentration of proton. The addition of the proton to the oxygen atom of glucoside likage is preequilibrium state of the reaction (*14*). Some polymers containing sulfonic acid groups as catalytic sites have been prepared in the present investigation.

Experimental Section

Preparation of Polymer Catalysts. The preparation methods of two copolymer catalysts are shown in Figure 2. In the first method (*15*), butyl vinylsulfonate was prepared from ethane disulfonyl dichloride according to Whitmore (*16*), copolymerized with vinyl acetate, and then saponified. According to Overberger et al. (*17*), this copolymerization proceeds nearly alternately. This copolymer was designated as the random copolymer catalyst. In the second method (*18*), first styrene was polymerized with tetraethyl thiuram disulfide as an initiator according to Otsu et al. (*19, 20, 21*). The polystyrene molecule obtained has two diethyl dithiocarbamate groups at both ends of the chain. To the polystyrene, vinyl acetate was block copolymerized under UV-light irradiation, sulfonated, and then saponified. This copolymer was designated as the block copolymer catalyst. These copolymers were passed through a cation exchange column to obtain free acid catalysts. Both copolymers thus obtained have vinyl alcohol sequences as substrate binding sites and sulfonic acid groups as catalytic sites.

Estimation of Catalytic Activity. The catalytic activities of the copolymers on the hydrolysis of polysaccharides were estimated, with the measurement of increase in reducing sugar in the reaction mixture with reaction time according to Somogyi method (*22*). The hydrolysis rates of sucrose were determined from the measurement of optical rotatory power

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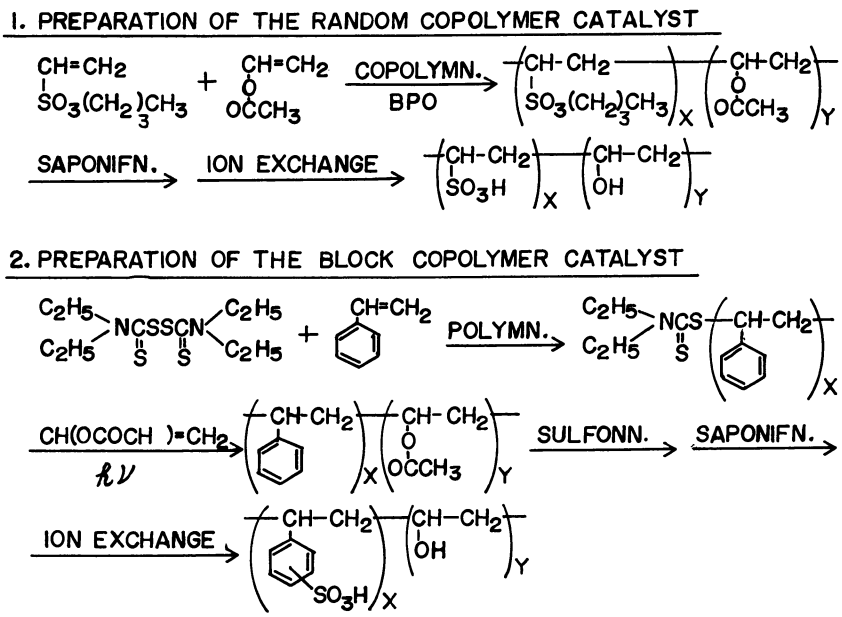

Figure 2. Preparation method of copolymer catalysts

of the reaction mixture with an automatic digital polarimeter. The concentration of the polymer substrate and catalyst were expressed as concentration of the monomeric unit in moles and as normality of acid, respectively.

Results and Discussion

The catalytic activities of the copolymers on the hydrolysis of soluble polysaccharides were investigated and kinetically analyzed. Figure 3 shows the plots of relative hydrolysis rates of dextrin in the presence of the random copolymer catalyst and in the presence of poly(vinylsulfonic acid)–poly(vinyl alcohol) mixture, against the mole ratio of vinyl alcohol to vinylsulfonic acid repeating units, keeping the concentration of vinylsulfonic acid constant. In the figure the catalytic activity of the copolymer increases proportionally to the ratio of vinyl alcohol to vinyl sulfonic acid repeating units up to 80. On the other hand the poly(vinylsulfonic acid)–poly(vinyl alcohol) mixture causes decrease of its catalytic activity with increasing ratio. These results may suggest that the vinyl alcohol sequences in the copolymer play important roles for enhancement of the reaction.

The effect of salt addition on the hydrolysis rate of dextrin in the presence of the random copolymer catalyst were investigated. The results are summarized in Table I. The catalytic activity of the copolymer is

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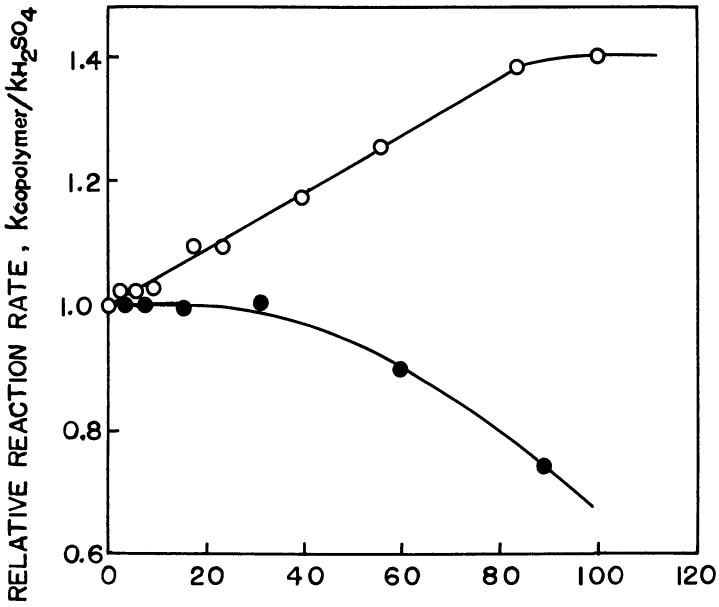

Figure 3. Change in the relative hydrolysis rates (based on sulfuric acid) of dextrin with the mole ratio of vinyl alcohol to vinylsulfonic acid repeating units at 80°C, keeping the concentration of vinylsulfonic acid unit constant at 5.00 × 10^{-3}N. (○) In the presence of the random co-poly(vinyl alcohol–vinylsulfonic acid); (●) in the presence of poly(vinyl alcohol)–poly(vinylsulfonic acid) mixture. [Substrate] = 2.00 × 10^{-2}M.

Table I. Effect of Salt Addition on the Hydrolysis Rate of the Dextrin at 80°C[a]

No.	*Salt Added*	*Catalyst*	$k_{obs}/k°_{sulfuric\ acid}$[b]	*pH*
1	—	sulfuric acid	1.00	2.4
2	—	copolymer[c]	1.07	2.4
3	NaCl	sulfuric acid	1.60	2.0
4	NaCl	copolymer	1.60	2.0
5	Na_2SO_4	sulfuric acid	0.73	3.3
6	Na_2SO_4	copolymer	0.72	3.3
7	KNO_3	sulfuric acid	1.33	2.4
8	KNO_3	copolymer	1.31	2.4
9	K_2SO_4	sulfuric acid	0.65	3.3
10	K_2SO_4	copolymer	0.65	3.3

[a] Ion strength, 1.00*M*. [Catalyst] 5.00 × $10^{-3}M$.
[b] $k_{obs}/k°_{sulfuric\ acid}$ is the ratio of the pseudo-first-order rate constant of each reaction to that of the reaction catalyzed by sulfuric acid without salt.
[c] The random copolymer (ratio of vinyl alcohol to vinylsulfonic acid units, 22.0).

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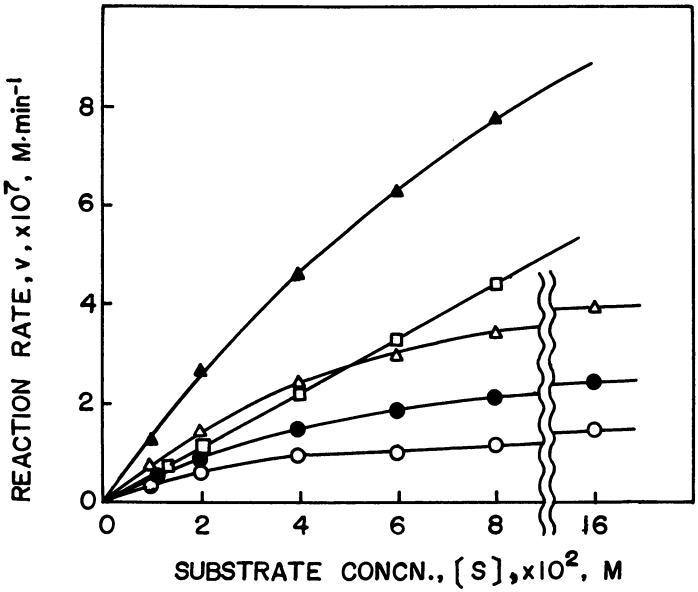

Figure 4. Dependence of amylose hydrolysis rates (v) on the substrate concentration in the presence of the random copolymer (▲,△,●,○) and sulfuric acid (□) at various temperatures. Temp: (▲) 85°, (△,□) 80°, (●) 75°, and (○) 70°C. [Catalyst] = 2.00 × 10^{-3}N.

clearly higher than that of sulfuric acid in the salt-free system, No. 1 and 2. Although the reaction rate was changed depending on the salt added, probably attributable to the change in pH, the difference in the catalytic activities between the copolymer and sulfuric acid completely diminished with addition of the salt, no matter how the reaction was enhanced or retarded. These phenomena principally may be caused by the weakening of the electrostatic interactions between the catalyst polyanions and protons, which is expected to play a significant role in the rate enhancement.

The substrate concentration dependence of the reaction rates was investigated kinetically to analyze the substrate binding effect. Figure 4 shows the relationships between the hydrolysis rate of amylose in the presence of the random copolymer catalyst and the concentration of the substrate at some reaction temperatures. The reaction rate clearly showed the saturation phenomenon at each reaction temperature. If the reaction proceeds via complex formation between catalyst and substrate, the elementary reaction could be described in the most simplified form as

$$S + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} SC \xrightarrow{k_c} C + P \qquad (1)$$

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in Equation 1 where S, C, SC, and P are substrate, catalyst, complex, and product, respectively. Values of k are the rate constants of the corresponding steps. If k_{-1} is far larger than k_c, the reaction rate could be expressed with the Michaelis–Menten type kinetics. If these assumptions are acceptable, linear relationships must be obtained in the plots of the reciprocals of the reaction rate vs. the initial concentration of substrate, so-called Lineweaver–Burk plot.

In practice, as shown in Figure 5, the corresponding plots gave fairly good straight lines. Therefore we concluded that reaction kinetically can proceed via complex formation between the catalyst and the substrate. Then the reaction rate (v) is expressed with Equation 2 where K_m, $[C]$,

$$v = \frac{k_c \cdot [C] \cdot [S]}{K_m + [S]} \tag{2}$$

and $[S]$ are apparent Michaelis constant and concentrations of catalyst and substrate, respectively.

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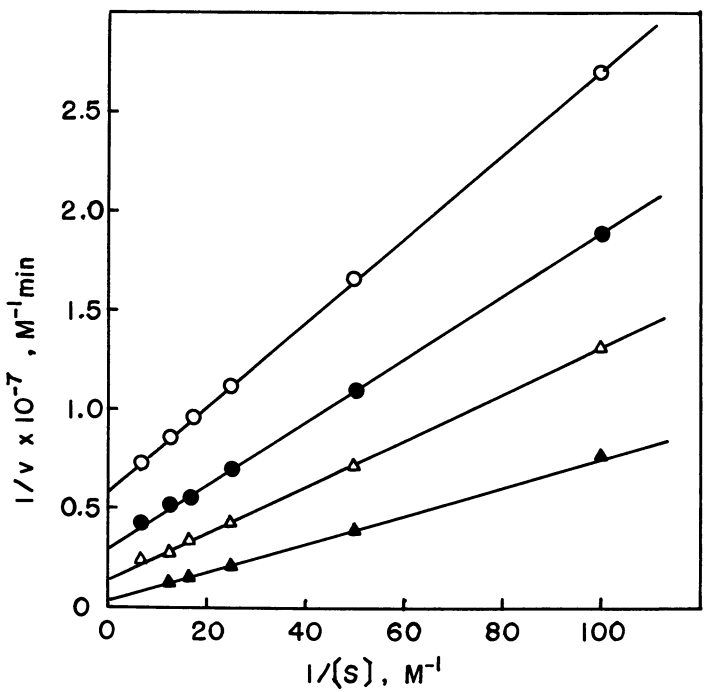

Figure 5. Lineweaver–Burk plots of amylose hydrolysis rates in the presence of the random copolymer at various temperatures. Temp: (▲) 85°, (△) 80°, (●) 75°, and (○) 70°C. [Catalyst] = 2.00 × 10⁻³N.

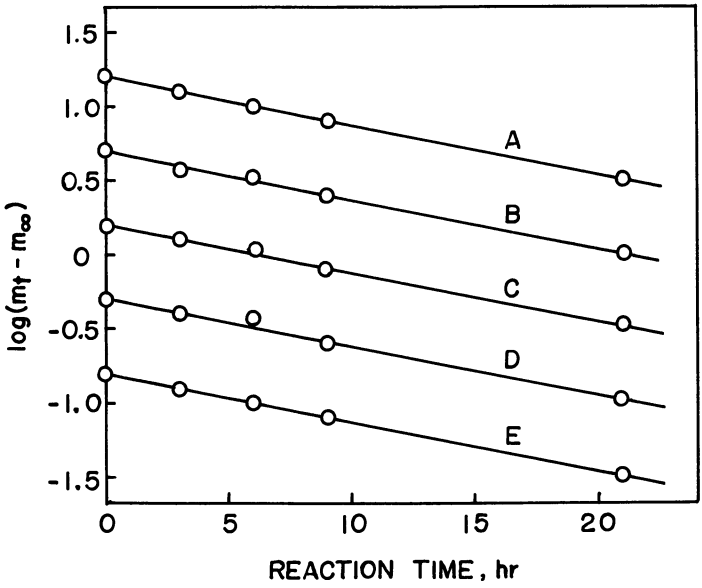

Figure 6. Pseudo-first-order plots for the hydrolysis of sucrose in the presence of the random copolymer at 50°C. [Catalyst] = 5.00 × 10^{-3}N. [Substrate] = (A) 5.00 × 10^{-1}M; (B) 1.5 × 10^{-1}M; (C) 5.00 × 10^{-2}M; (D) 1.5 × 10^{-2}M; (E) 5.00 × 10^{-3}M. Variables m_t *and* m_∞ *are optical rotatory power at reaction times* t *and* ∞*, respectively.*

Similar investigation was made for sucrose as a substrate. The reaction rate, however, did not show saturation phenomenon, and as shown in Figure 6, the first-order plots of the reactions gave fairly good straight lines parallel with each other. This result suggests that the hydrolysis of low-molecular-weight substrate follows ordinary second-order kinetics.

The inhibition effect of poly(vinyl alcohol) on the amylose hydrolysis was investigated. Figure 7 shows Lineweaver–Burk plots of the amylose hydrolysis rates catalyzed by the random copolymer in the presence of poly(vinyl alcohol). The reaction rate is found to decrease with increasing the concentration of poly(vinyl alcohol), and all of the straight lines obtained in the plots cross with each other at a point on the ordinate. This is a feature of the competitive inhibition in the enzymatic reactions. In the present reaction system, however, it is inferred to suggest that the copolymer and poly(vinyl alcohol) molecules competitively absorb the substrate molecules. The elementary reaction can be described in the most simplified form as in Equation 3 where *I*, *SI*, and K_I are inhibitor, nonproductive complex, and inhibitor constant, respectively. Then the reaction rate is expressed with Equation 4.

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$$SI \underset{K_I}{\overset{I}{\leftrightarrows}} S \underset{K_m}{\overset{C}{\rightleftarrows}} SC \xrightarrow{k_c} P + C \tag{3}$$

$$v = \frac{k_c \cdot [C] \cdot [S]}{K_m \left(1 + \frac{[I]}{K_I}\right) + [S]} \tag{4}$$

The inhibition effect also was investigated for some low-molecular-weight analogs as inhibitors. However, changes in the reaction rate with addition of these substrates were not found. This result and the second-order kinetics of the hydrolysis of sucrose indicate that the interactions between the substrate and the catalyst polymer depend on the molecular weight of the substrate. This effect can be considered as a sort of so-called "polymer effect" and is advantageous for the hydrolysis of high-molecular-weight substrates such as cellulose and starch; that is, in those cases the substrate is absorbed around the copolymer and hydrolyzed, whereas the products are scarcely absorbed and readily diffuse away.

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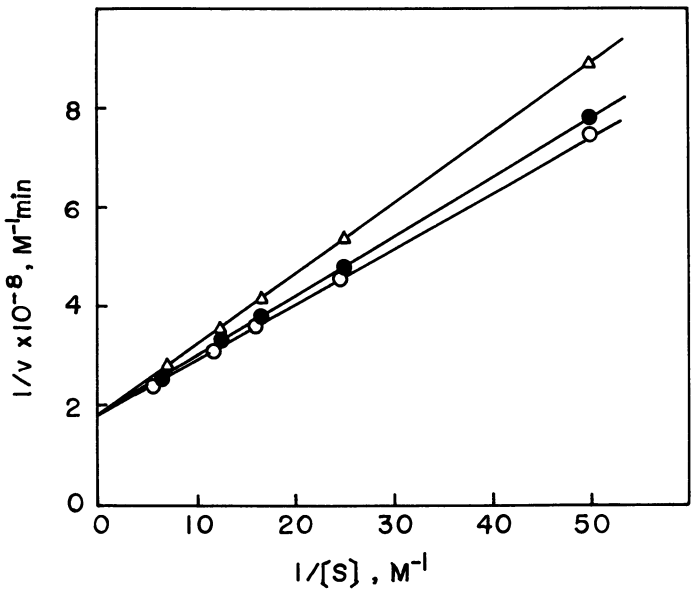

Figure 7. Lineweaver–Burk plots of amylose hydrolysis rates catalyzed by the random copolymer in the presence of poly(vinyl alcohol). [Catalyst] = 2.00 × 10⁻³N. [Poly(vinyl alcohol)] = (○) 0; (●) 4.0 × 10⁻²M; (△) 8.0 × 10⁻²M. K_m and K_I calculated according to Equation 4 are 0.083 and 0.22 M, respectively.

The catalytic activities of the block copolymer on the hydrolysis of dextrin also were investigated. Figure 8 shows the plots of reaction rate against the substrate concentration. Similar tendency, but larger rate enhancement of the reaction are found compared with that in the presence of the random copolymer catalyst (Figure 4).

Figure 9 shows Lineweaver–Burk plots of dextrin hydrolysis rates in the presence of the block copolymer. Again, fairly good straight lines are obtained. Some other kinetical investigations also were made for the catalytic activity of the block copolymer, and similar tendencies of catalytic behavior were found compared with that of the random copolymer.

The parameters of the Michaelis–Menten type kinetics were calculated for the reactions and are summarized in Table II. The apparent Michaelis constant values (K_m) are rather large, indicating that the concentration of the complex at the equilibrium state is not high, unlike ordinary enzymatic reactions. The ratio of k_c/K_m against the second-order rate constant with sulfuric acid (k_2) can be considered to be an indication of the rate enhancement. The ratio increased with increasing mole fraction of the vinyl alcohol repeating unit in the copolymer and with

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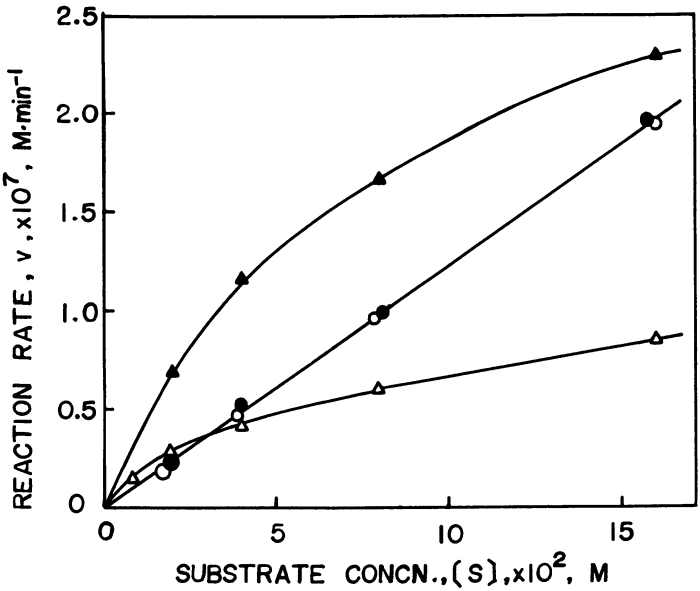

Figure 8. Dependence of dextrin hydrolysis rates (v) on the substrate concentration in the presence of the block copolymer at 70°C. [Catalyst] = 1.00 × 10^{-2}N. Catalyst (mole ratio of vinyl alcohol to styrenesulfonic acid units in the copolymer): (○) sulfuric acid; (●) block copolymer No. 1 (1.4); (△) block copolymer No. 2 (9.8); (▲) block copolymer No. 3 (22.1).

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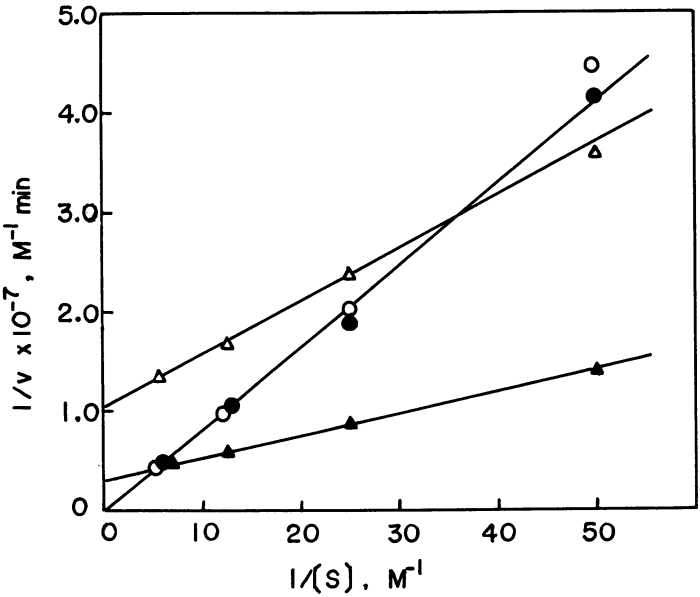

Figure 9. Lineweaver–Burk plots of dextrin hydrolysis in the presence of the block copolymer at 70°C. [Catalyst] = 1.00 × 10⁻²N. Catalyst: (○) sulfuric acid; (●) block copolymer No. 1; (△) block copolymer No. 2; (▲) block copolymer No. 3.

Table II. Parameters of Michaelis–Menten Type Kinetics at Various Temperatures

Catalyst (VA/VS[a])	*Parameter*	*Reaction Temperature (°C)* 85	80	75	70	65
Random copolymer (10.9)	$K_m \times 10$ (M)	1.18	0.83	0.53	0.36	—
	$k_c \times 10^4$ (min^{-1})	10.91	4.86	2.69	1.42	—
	$k_c/K_m \times 10^3$ (M^{-1} min^{-1})	9.25	5.86	5.08	3.94	—
Block copolymer No. 2 (9.8)	$K_m \times 10$ (M)	—	1.74	1.39	1.09	0.94
	$k_c \times 10^4$ (min^{-1})	—	4.57	2.56	1.45	0.80
	$k_c/K_m \times 10^3$ (M^{-1} min^{-1})	—	2.63	1.84	1.33	0.85
Block copolymer No. 3 (22.1)	$K_m \times 10$ (M)	—	1.32	0.93	0.63	0.45
	$k_c \times 10^4$ (min^{-1})	—	9.77	5.37	2.95	1.58
	$k_c/K_m \times 10^3$ (M^{-1} min^{-1})	—	7.40	5.76	4.68	3.53
Sulfuric acid	$k_2 \times 10^3$ (M^{-1} min^{-1})	—	3.72	2.18	1.24	0.69

[a] VA/VS = mole ratio of vinyl alcohol to vinyl- or styrenesulfonic acid units in the copolymer.

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Table III. Thermodynamic Parameters[a]

Catalyst	$1/K_m$ ΔH	ΔS	ΔG
Random copolymer	−20.8	−54	−1.8
Block copolymer No. 2	−9.8	−24	−1.5
Block copolymer No. 3	−17.2	−45	−1.9
Sulfuric acid	—	—	—

[a] ΔH and ΔG are in kcal · mol^{-1}, and ΔS is in entropy unit.

decreasing reaction temperature. Also the catalytic activity of the block copolymers is larger than that of the random copolymers. The maximum rate enhancement obtained in the present experiment was about fivefold on the basis of the reaction in the presence of sulfuric acid at 65°C, and it was predicted to increase with increasing reaction temperature.

The thermodynamic parameters were calculated and are summarized in Table III. Both enthalpy and entropy decrease considerably with complex formation. Such a large decrease in enthalpy and entropy has not been found in other complex formation systems. For example, the change in enthalpy with the complex formation because of hydrophobic interactions generally is not so large and change in entropy is positive (*23, 24*) and change in enthalpy with the complex formation in enzymatic hydrolysis of cellulose is slightly positive (*25*). These unusual decreases in enthalpy and entropy are inferred to be characteristics of the present complex formation system.

The activation enthalpy of the overall reaction in the presence of the copolymer is smaller than that in the presence of sulfuric acid. However, the activation entropy is also smaller. As the result, activation free energy of the overall reaction in the presence of the copolymer is only slightly smaller than that in the presence of sulfuric acid.

Conclusion

From these results, it can be concluded that the rate enhancement of polysaccharide hydrolysis obtained with the present copolymer catalyst was attributable to the hydrogen bonding interactions between the substrate and the catalyst and to the electrostatic interactions between the catalyst polyanions and protons. A drawing of this concept is shown in Figure 10. A polymer molecule is surrounded by a proton atmosphere. The substrate molecules are pulled into the atmosphere by hydrogen bonding interactions and hydrolyzed in the presence of a high concentration of proton.

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of $1/K_m$, k_c, k_c/K_m, and k_2 at 70°C

k_c			k_c/K_m, k_2		
$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta G^{\ddagger}$
38.4	26	29.2	17.6	−28	27.4
26.8	−6	29.0	17.0	−30	27.4
27.9	−2	28.5	10.8	−43	26.6
—	—	—	25.8	−5	27.6

The hydrogen bonding interactions between the substrate and the copolymer were the results of kinetic and thermodynamic analyses and other information, although it has not been verified directly.

Some factors such as the length of vinyl alcohol sequences, charge density in the polymer domain, and conformation of the copolymer also are supposed to be important, in addition to the mole ratio of vinyl alcohol unit to sulfonic acid group, for enhancement of the reaction, and influences of these factors on the reaction are currently under investigation.

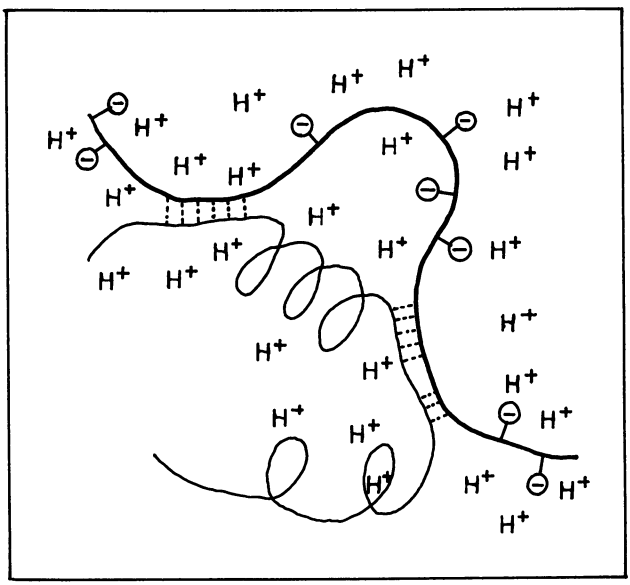

Figure 10. Drawing of hydrogen bonding interactions between polysaccharide and copolymer molecules, and electrostatic interactions between the polyanion and protons. (⊖) polyanion; (——) polysaccharide molecule; (H^+) proton; (– – –) hydrogen bonding interactions.

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10

Synergism Between Enzymes Involved in the Solubilization of Native Cellulose

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Although it is well established that certain cellulolytic fungi hydrolyze native cellulose with enzyme systems containing endo- *and* exo-*β-1,4-glucanase, and β-glucosidase activities, the actual mechanism by which soluble fragments are removed from the more crystalline areas is as yet unknown. Synergism between endoglucanases (so-called* C_x*) and certain exoglucanases (so-called* C_1*) is clearly established, and many believe that attack is initiated by the* C_x *and that the new chain ends generated are then hydrolyzed by* C_1*. However, the discovery that* C_1 *acts synergistically only with certain* C_x *components suggests that the proposed mechanism is an oversimplification. Possibly the more crystalline areas are effectively solubilized only by the rapid sequential action of those endo- and exoglucanases capable of forming an enzyme–enzyme complex on the surface of the cellulose chains.*

Synergism between Enzymes Involved in the Solubilization of Native Cellulose

The study of the synergism shown by enzymes of the cellulase complex in solubilizing highly ordered native cellulose has now progressed to a stage where it is possible to state with some certainty the principal enzyme components involved. Not everyone agrees, however.

As currently understood, cell-free enzyme preparations that can solubilize native cellulose contain at least two types of enzyme, so-called C_1 and C_x (*1*). A third enzyme, a β-glucosidase or cellobiase, is normally,

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but not always, present as an extracellular enzyme. There may be other enzymes in some cellulase systems (*2*).

C_x itself is a complex of enzymes that hydrolyze β-1,4-glucosidic linkages in the cellulose molecule. C_x enzymes are normally randomly acting (endoglucanases), but they may occasionally be endwise acting (exoglucanases), removing glucose (*3*) or even cellobiose (*4*) successively from the nonreducing end of the cellulose molecule.

C_x enzymes can, when acting in isolation, hydrolyze soluble cellulose derivatives (CM-cellulose, hydroxyethylcellulose) or cellulose that has been modified by grinding or swelling in concentrated acid (H_3PO_4-swollen cellulose) or alkali, but they are unable to attack highly ordered crystalline cellulose. Attack on crystalline cellulose requires the presence of the C_1 component. That, however, is as far as the agreement goes; although it is generally accepted that C_1 is essential, there is still some debate regarding the mode of action.

With the discovery in the last few years that the C_1 component is associated with exoglucanase activity (*5–10*), the debate has been resolved to some extent, at least to the satisfaction of some, but there are still many unanswered questions regarding the synergistic action between C_1 and C_x in solubilizing highly ordered cellulose. It is hoped that the results presented in this chapter will go some way toward providing some of the answers; at worst they will help to formulate the questions that need answering.

Proposed Mechanisms of Synergistic Action

An enzymatic mechanism involving a C_1 component for the hydrolysis of cellulose was first proposed in 1950 (*1*). It was suggested that C_1 was the first step in a two-step process involving a disruption of hydrogen bonds followed by hydrolysis of "swollen" cellulose by endoglucanases (C_x). The existence of C_1 was postulated, however, purely on the basis of indirect evidence, for no component of this type was isolated at that time. Some organisms that were able to degrade amorphous cellulose were unable to degrade crystalline cellulose. Some organisms that degraded highly ordered cellulose produced culture filtrates that were incapable of degrading the more highly ordered material, but the same culture filtrates could degrade cellulose that had been made accessible by derivatization (CM-cellulose), swelling in phosphoric acid, or grinding. Swelling, grinding, or derivatization, it was argued, were simulating C_1 action by producing hydrated cellulose chains. Thus C_1 was envisaged to be a prehydrolytic factor (a hydrogen bondase (*11*)) producing an "activated" cellulose which was capable of attack by the hydrolytic enzymes of the complex (Scheme 1).

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Scheme 1. Original C_1–C_x hypothesis

Crystalline cellulose	$\xrightarrow{C_1}$	reactive cellulose	$\xrightarrow{C_x}$	β-glucosidase $[\text{glucose}]_{n=1-6} \rightarrow$ glucose

But the facts as presently established do not fit this hypothesis. Indeed, now that C_1 has been isolated from cell-free culture filtrates from a number of fungi, detailed studies on the purified enzyme are interpreted to indicate that C_1 is an exoglucanase (*5–10*); in some cases it is specifically a cellobiohydrolase (*5, 6, 8, 9, 10, 12, 13*). But it is an exoglucanase or cellobiohydrolase with the remarkable property of being able to act in synergism with the randomly acting C_x type of enzyme to solubilize crystalline cellulose, and it must be distinguished from other exoglucanases, such as the cellobiohydrolase produced by the bacterium *Cellvibrio gilvus* (*4*) and the glucohydrolase of the fungus of *Trichoderma viride* (*3*), which do not possess this capability. The best method of distinguishing it from these enzymes is, in our view, to retain the name C_1, but this too is a controversial point.

In an enzyme system containing endo- and exoglucanases both of which must be present in order to effect degradation of crystalline cellulose, it seems logical to assume that it is the randomly acting C_x enzymes that initiate the attack (Scheme 2). Synergism shown by mixtures of C_1 and C_x when solubilizing highly ordered cellulose can then be explained in terms of the new chain ends generated by C_x being hydrolyzed by C_1, for in this respect, the process will be synergistic. This hypothesis, which reverses the order of action of C_1 and C_x as originally postulated, enjoys a high degree of support (*5, 6, 8, 9, 10, 12–17*).

Scheme 2. Hypothesis involving sequential action: endoglucanase (C_x) followed by cellobiohydrolase (C_1)

Crystalline cellulose	$\xrightarrow{C_x}$	reactive cellulose	$\xrightarrow{C_1}$	β-glucosidase cellobiose $\longrightarrow$ glucose

Reese (*18*) is not convinced. He argues that the first step in the enzymatic hydrolysis of other highly ordered structures such as DNA and collagen is affected by enzymes whose function it is to produce some disorder in the structures as a preliminary to hydrolysis by other enzymes. Such enzymes are highly specific for ordered molecules, and they are unable to act on the disordered structures that they produce. Thus Reese rationalizes that the first step in the hydrolysis of crystalline cellulose involves a similar type of enzyme.

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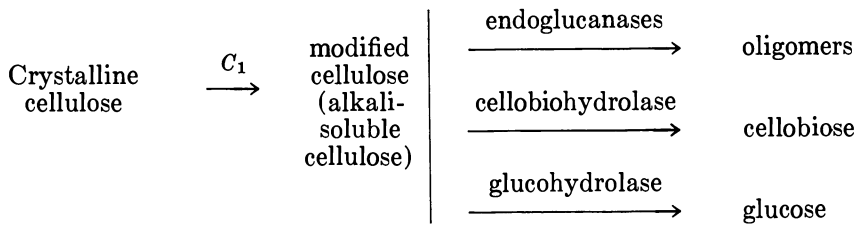
Scheme 3. Modified C_1–C_x hypothesis



As a result of these and other observations, Reese (*18, 19*) now presents a modified C_1–C_x hypothesis (Scheme 3). This new concept retains the idea that C_1 causes some "swelling" of the crystalline cellulose, but it is now suggested that this is preceded by cleavage of some covalent linkages on the surface of the crystallite. The cellobiohydrolase that is identified with C_1 in the C_x–C_1 hypothesis (Scheme 2) is relegated to the role of hydrolyzing an "activated" cellulose (Scheme 3) along with the endoglucanases and glucohydrolase. Thus the mechanism by which crystalline cellulose is converted to soluble fragments remains a controversial one.

Synergistic Activity between "Purified" Enzymes

High levels of C_1 are found in culture filtrates of only a few cellulolytic microorganisms; high levels of C_x are found in many. Many culture filtrates rich in C_x contain no C_1, but the converse is not true. Good examples of culture filtrates rich in C_1 are those from the fungi *Fusarium solani* (*12, 20, 21*), *Penicillium funiculosum* (*13, 21*), *Sporotrichum pulverulentum* (*23, 24*), and *Trichoderma koningii* (*25, 26, 27*), but culture filtrates of *T. viride* (*28–32*), particularly *T. reesei* (*33*) (formerly *T. viride* QM 6a) and derived mutants (*34*), appear to be the best.

C_1, C_x, and β-glucosidase components have been isolated from culture filtrates from each of these fungi and synergistic activity has been demonstrated on a variety of substrates. Separation normally involves chromatography on Sephadex gels, DEAE-Sephadex, ion exchange resins, electrophoresis on polyacrylamide gels, or electrofocusing in a stabilized pH gradient. The best separations are normally achieved by a combination of these procedures.

The most intensive fractionation studies have been performed on the cellulase elaborated by *T. koningii* (*5, 6, 9, 16, 26, 27*) and *T. viride* (*8, 10, 28–32*). In a typical separation, normally by chromatography on DEAE-Sephadex, three protein peaks are obtained (Figure 1). The first and second contain C_x; C_1 is in the third. C_x and β-glucosidase are separated by chromatography on SE-Sephadex and C_1 is purified by repeated chromatography on DEAE-Sephadex and by isoelectric focusing.

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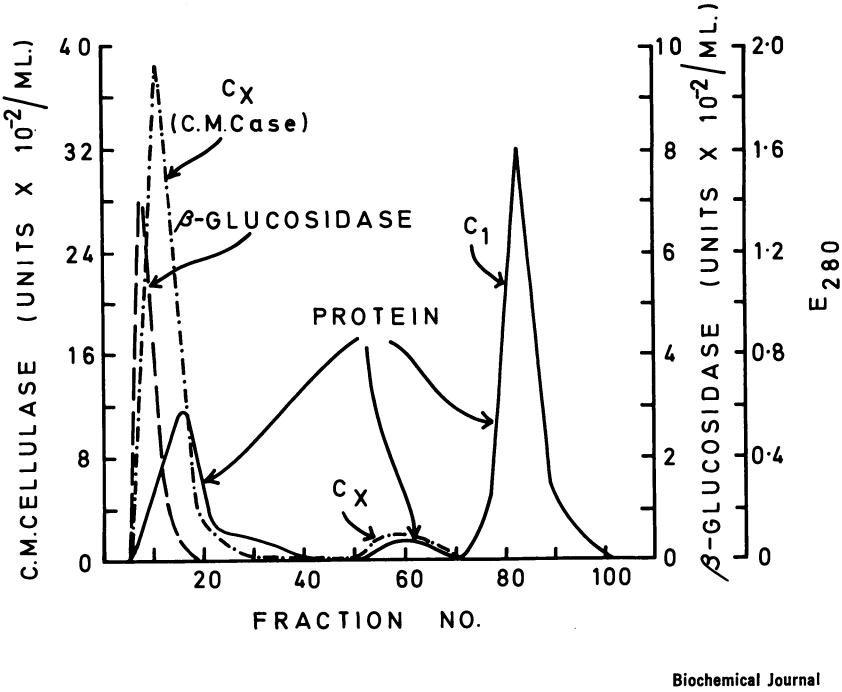

Figure 1. Separation of C_1 from C_x and β-glucosidase components of T. koningii *cellulase on DEAE-Sephadex. Protein 1 eluted under starting conditions; proteins 2 and 3 with linear salt gradient* (26).

Purified in this way, C_1 has little action on CM-cellulose (the substrate normally used to estimate C_x activity), but it can still attack cellulose that has been swollen in H_3PO_4 or a never-dried bacterial cellulose (9).

In respect to its ability for degrading H_3PO_4-swollen cellulose, C_1 is similar to C_x, but the mode of attack clearly is different (Figure 2). Whereas C_1 attacks from the end of the chain and produces little change in degree of polymerization, C_x attacks at random. Glucose, cellobiose, and cellotriose are found in the products of the action of C_x; C_1 produces principally cellobiose, as would be expected from a cellobiohydrolase.

When purified to a high degree, neither C_1, C_x, nor β-glucosidase enzymes in *T. koningii* cellulase show much capacity for degrading a highly ordered cellulose, such as the archetypal cotton fiber, but the activity that is lost on separation is recovered when the enzymes are recombined. Where losses of activity during the fractionations are low, and when enzymes have been recombined in their original proportions, complete recovery of cotton-solubilizing activity is observed (Table I).

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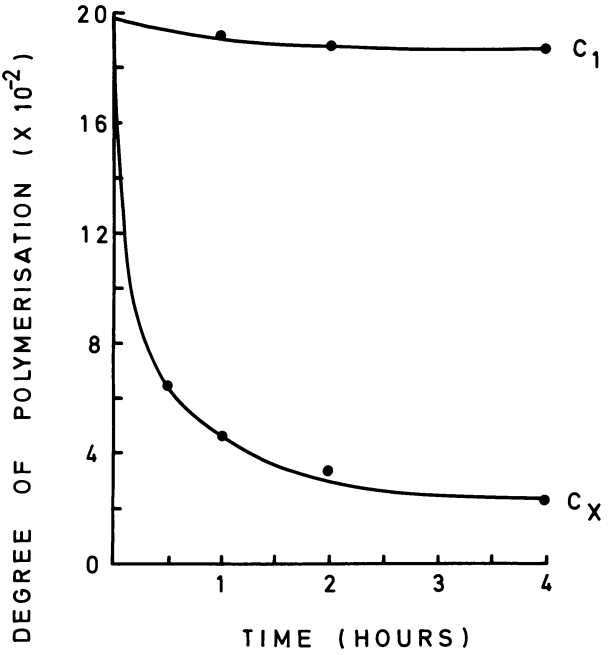

Figure 2. Rate of change of degree of polymerization of H_3PO_4-swollen cellulose by C_1 and C_x enzymes of T. koningii *cellulase. Residual cellulose was dissolved in Cadoxen (46) and the degree of polymerization determined viscometrically (46).*

Table I. Cellulase (Cotton-Solubilizing) Activity of C_1, C_x, and β-Glucosidase of *T. koningii* and *F. solani*, when Acting Alone and in Combination[a]

Enzyme	*Cotton Solubilization (%)*	
	F. solani	T. koningii
C_1 (cellobiohydrolase)	2	1
C_x (endoglucanases)	1	1
β-glucosidase	1	nil
C_1 and C_x	58	53
C_1 + β-glucosidase	18	20
C_1 + C_x + β-glucosidase	71	72
Original culture filtrate	71	71

[a] All enzymes were present in the same proportions in which they were present in 1 mL of original cell-free culture filtrates. Incubations were for 7 days at 37°C (*21*).

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Results similar to those obtained with *T. koningii* cellulase have been obtained in our laboratory (using improved fractionation procedures (*12, 13*)) with the components of *F. solani* cellulase (Table I), and Selby and Maitland have reached identical conclusions as a result of their studies on *T. viride* (*30*) and *P. funiculosum* (*22*) cellulases. In all four cases, reconstitution experiments for cellulase (cotton-solubilizing) activity show that much of the activity is dependent on the synergistic interaction of C_1 and C_x enzymes in particular, but complete recovery of activity is achieved only when β-glucosidase is present to remove the inhibitory effects of cellobiose (*9, 17*). A suggestion (*4*) that C_1 and β-glucosidase are the only two components required to produce extensive hydrolysis has been challenged (*16*).

Thus the mechanism of cellulase action is a complex one, involving three different types of enzyme and two types of synergism. In the first stage of the reaction, an enzyme *A* is producing a reactive product for enzyme *B*, while in the second stage an enzyme *C* is removing an inhibitory product of the action of the other enzyme. Only synergism of the first type will be discussed further.

Variations in Synergistic Activity

Synergism that is high on cotton with mixtures of highly purified C_1, C_x, and β-glucosidase components of *T. koningii, F. solani,* and *P. funiculosum* cellulase is low on less well ordered cellulose (H_3PO_4-swollen cellulose, Figure 3) and absent on soluble derivatives of cellulose such as CM-cellulose. In other fractionation studies reported in the literature, one can find general support for the observation with respect to CM-cellulose, but synergism toward H_3PO_4-swollen cellulose is variable: Eriksson (*14*), in the extreme, could find no synergism between C_1 and C_x types of enzymes from *Sporotrichum pulverulentum* cellulase when using H_3PO_4-swollen cellulose.

Results reported for the potentiation in activity shown by recombined fractions toward highly ordered substrates are the most varied of all. In many of these reports (*3, 28, 31, 35–39*), however, some of the fractions isolated differed only in their relative activities toward substrates such as CM-cellulose, Avicel, cotton, filter paper, and hydrocellulose, and, not surprisingly, synergism between the fractions, where tested, was not particularly high. Such fractionation studies have produced completely different conclusions as to the number of components involved and for the substrate specificities of the individual components. Comparing the results obtained with C_1, C_x, and β-glucosidase components of *T. koningii* and *F. solani* (Table I), *T. viride* (*30*), and *P. funiculosum* (*22*) cellulases, one might be justified in suggesting that in many of these other

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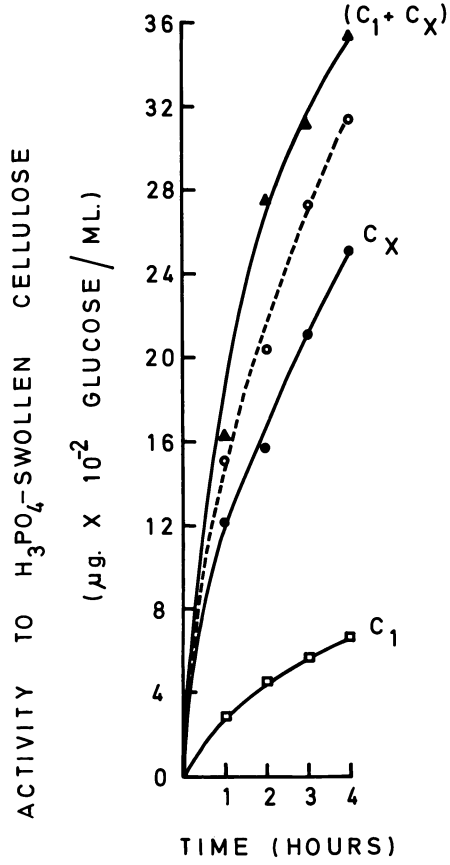

Figure 3. Activity of the components of F. solani *cellulase to H_3PO_4-swollen cellulose, when acting alone and in combination. Broken line indicates activity expected from a simple addition of the activities of* C_1 *and* C_x.

reports the components have been incompletely separated. However, it is also possible that enzymes from different sources may exhibit quite different substrate specificities.

With regard to the specificity of the C_1 component, there is general agreement that when highly purified it shows little or no capacity for attacking CM-cellulose, but there is some disagreement in respect to the ability to degrade highly ordered cellulose. In the extreme, a C_1 (Avicelase) component isolated from a commercial preparation of *T. viride* cellulase could produce 45% degradation of Avicel when acting alone (*15*) and 79% when the incubation was performed in an ultrafiltration cell under conditions that permitted the complete removal of the inhibitory products of the reaction.

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Comparative studies done in our laboratory with the C_1 component of *T. koningii*, *F. solani*, and *P. funiculosum* show that Avicel is certainly more susceptible to hydrolysis than either cotton fiber or Whatman cellulose powder, but extensive hydrolysis has never been observed. Indeed, 7% degradation is the maximum obtained, and this was not significantly increased using a dialysis arrangement similar to that used with *T. viride*. Clearly, the observations made with *T. viride* C_1 (Avicelase) are consistent with the hypothesis that the C_1 component is the only component capable of hydrolyzing crystalline cellulose when acting alone, which is how Li, Flora, and King (*3*) interpreted their data obtained with a C_1 (hydrocellulase) that was also isolated from a commercial preparation of *T. viride* cellulase. Yet all C_1 components isolated from *T. viride* cannot be so described: the C_1 component by Selby and Maitland (*30*) could not degrade cotton, while the three C_1 components (isoenzymes) purified by Brown and his colleagues (*8*) "had little activity towards CM-cellulose or crystalline cellulose."

In the same context, results from cotton vary, too. Halliwell and Griffin (*9*), for example, report that cotton was solubilized to the extent of 20% by a C_1 component from *T. koningii* cellulase, despite the fact that it was free from contaminating C_x (CM-cellulose) activity. Since this compares with a value of between 2 and 4% for the C_1 components of *F. solani*, *T. koningii*, and *P. funiculosum* isolated in our laboratory, it seems reasonable to assume that there is some fundamental difference between the two components, bearing in mind that the substrates used in both studies were identical, as far as one can ascertain.

Clearly, there are significant differences in the extent to which the various purified C_1 components can degrade highly ordered substrates, even when the enzymes are free or apparently free of contaminating C_x activity. The reason for these differences is not immediately apparent, for there is no obvious common denominator when one compares the source of the enzymes, the method of isolation, or the substrates.

Despite the variation in substrate specificity, there is general, but not absolute, agreement that C_1 is an exoglucanase. C_1 of *T. koningii* (*5, 6, 9, 16*), *F. solani* (*12, 13*), or *P. funiculosum* (*13*) is highly specific for the penultimate glucosidic linkage on the nonreducing end of the chain; C_1 of *T. viride* is less so in some cases (*8, 15*). Of the C_1 components isolated, that of *S. pulverulentum* (*14*) is the least specific, glucose, cellobiose, and even cellotetraose being found in the products of hydrolysis. Similar products would be expected from an endoglucanase, but the inversion of configuration of the anomeric hydroxyl on the soluble fragments released is a characteristic property of an exoglucanase: the cellobiohydrolase of *Cellvibrio gilvus* (*4*) cellulase and the glucohydrolase from *T. viride* (*3*) cellulase both act by inversion of configuration.

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Thus it would appear that endoglucanases and cellbiohydrolases or exoglucanases are involved in the first phase of the breakdown of cellulose. But can the mechanism be explained simply in terms of the sequential action of these enzymes or are there other enzymes involved? Reese (*1*) and Eriksson (*2*) think there are, but for quite different reasons: one (*1*) envisages degradation as involving a nonhydrolytic chain-separating enzyme; the other (*2*), an oxidase that oxidizes some of the glucose residues in cellulose to uronic acids which in turn cause the breaking of hydrogen bonds between cellulose chains as a preliminary to hydrolysis by endo- and exoglucanases. We can examine both hypotheses.

Are the C_1 and Cellobiohydrolase Activities Properties of the Same Protein?

The evidence that the component that acts in synergism with the randomly acting C_x components is an exoglucanase is not compatible with Reese's conception of C_1 (Schemes 1 and 3). To circumvent this problem, he has suggested that C_1 and cellobiohydrolase may be two different enzyme components that co-chromatograph under all conditions tested so far (*40*). Such a possibility would seem extremely unlikely in view of the large number of C_1/cellobiohydrolase components that have been isolated under quite different conditions, but it is a possibility that has also occurred to us. The logic behind the hypothesis that C_1 is a chain-disaggregating enzyme is so attractive that the concept cannot be discarded lightly, as some would do.

We have spent some time investigating possible heterogeneity in the C_1 components of *F. solani, T. koningii,* and *P. funiculosum.* This has been done by testing for both C_1 activity (defined as the enzyme that acts in synergism with the C_x enzymes to solubilize cotton cellulose or other crystalline cellulose) and cellobiohydrolase (release of cellobiose from H_3PO_4-swollen cellulose) during different fractionation studies on the various C_1 components.

The C_1 component of *T. koningii* isolated as a single component by chromatography on DEAE-Sephadex (Figure 1) is certainly heterogeneous when examined qualitatively by isoelectric focusing in a polyacrylamide gel (Figure 4): when ampholyte covering the pH range 3.5–5.0 is used, a major and a minor protein component are separated. Quantitative data can be obtained when the separation is carried out in an isoelectric-focusing column, where the pH gradient is stabilized in a sucrose density gradient. However, under these conditions, in an ampholyte solution covering approximately the same pH range as that used in the gel, only a partial resolution is obtained (*6*). It is necessary to use an ampholyte solution covering only half of a pH unit to achieve complete separation (*6*) (Figure 5).

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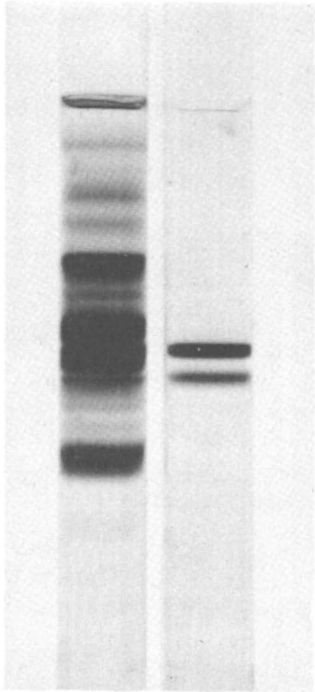

Figure 4. Gel electrofocusing of T. koningii *cellulase (A,* left*), and* C_1 *(B,* right*) in an ampholyte covering the pH range 3.5–5.6.* C_1 *was isolated as in Figure 1 and purified by further chromatography on DEAE-Sephadex (6).*

Both major and minor protein components separated in this way were C_1 types. This was established when it was found that either acted in synergism with a mixture of the C_x and β-glucosidase activities to the same extent in solubilizing cotton cellulose (Table II). Furthermore, both components were cellobiohydrolases in that they could degrade H_3PO_4 cellulose to cellobiose.

Table II. Cellulase (Cotton-Solubilizing) Activity of the C_1 Component of *T. koningii* Separated by Isoelectric Focusing, when Acting in Synergism with C_x and β-Glucosidase[a]

C_1 *Component*	*Solubilization (%)*
C_1 (Minor)—Figure 5	59
C_1 (Major)—Figure 5	60
Original—C_1 before electrofocusing	66

[a] Cellulase (cotton-solubilizing) activity was determined by mixing the respective C_1 components with an excess of C_x and β-glucosidase and then incubating for 7 days at 37°C (*21*).

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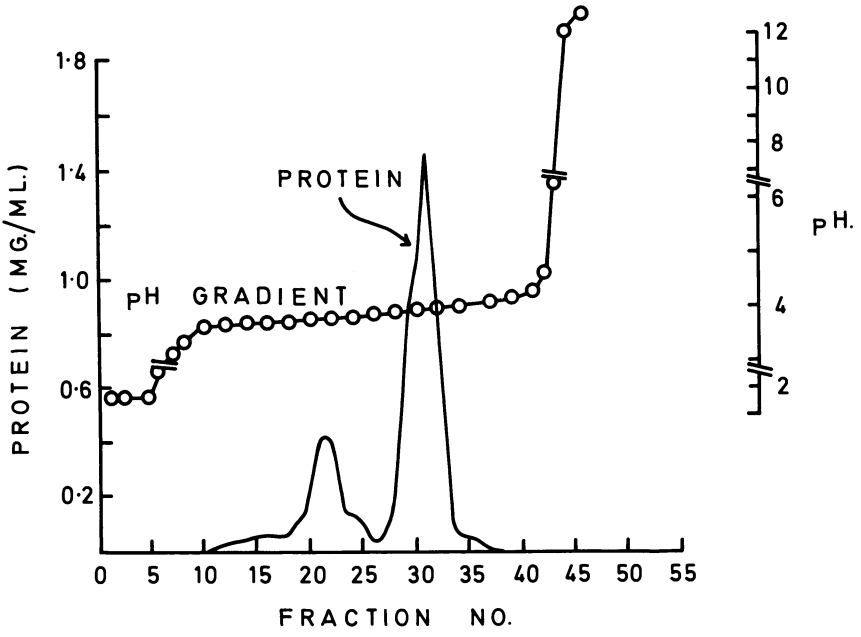

Figure 5. Isoelectric focusing of the C_1 *component of* T. koningii *in an ampholyte gradient covering the range pH 3.72–4.25. The ampholyte was supported in a sucrose density gradient in a 110-mL LKB electrofocusing column. The* C_1 *component used was similar to one eluted as a single component from DEAE-Sephadex (Figure 1) and purified further by chromatography on DEAE-Sephadex (6). From Ref. 6, in part.*

With the C_1 component of *F. solani,* which, like *T. koningii* C_1, was isolated as a single component by chromatography on DEAE-Sephadex, the results were even more convincing. In an ampholyte solution covering only half of a pH unit, C_1 of *F. solani* was resolved into four components (Figure 6), each with identical hydrolytic properties (cellobiohydrolase) and similar capacities for solubilizing cotton cellulose when acting in synergism with a mixture of C_x and β-glucosidase (Table III). Particularly noteworthy is the fact that the peaks of both types of activity were coincident in each of the four separated components.

In support of his belief that cellobiohydrolase cannot be identified with C_1, Reese also argues that the hydrolytic property (cellobiohydrolase) normally manifested by C_1 is of relatively low concentration (*18*); which is true. Figure 7 shows the fractionation of *P. funiculosum* on DEAE-Sephadex into C_1, C_x, and β-glucosidase activities (*13*); it also shows the fact that the specific activity of the C_1 component (peak 2) in terms of its activity on H_3PO_4-swollen cellulose is very low in comparison

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Table III. Cellulase (Cotton-Solubilizing) Activity of the C_1 Component of *F. solani* Separated by Isoelectric Focusing, when Recombined with C_x and β-Glucosidase[a]

Sources of C_1 *Component*	*Solubilization (%)*
Fraction *A* (Figure 6)	59
Fraction *B* (Figure 6)	60
Fraction *C* (Figure 6)	67
Fraction *D* (Figure 6)	68
C_1 before isoelectric focusing	71

[a] All assays contained the same amount of C_1, C_x (CM-cellulase), and β-glucosidase. Incubation was for 7 days at 37°C (*21*). From Ref. *12*.

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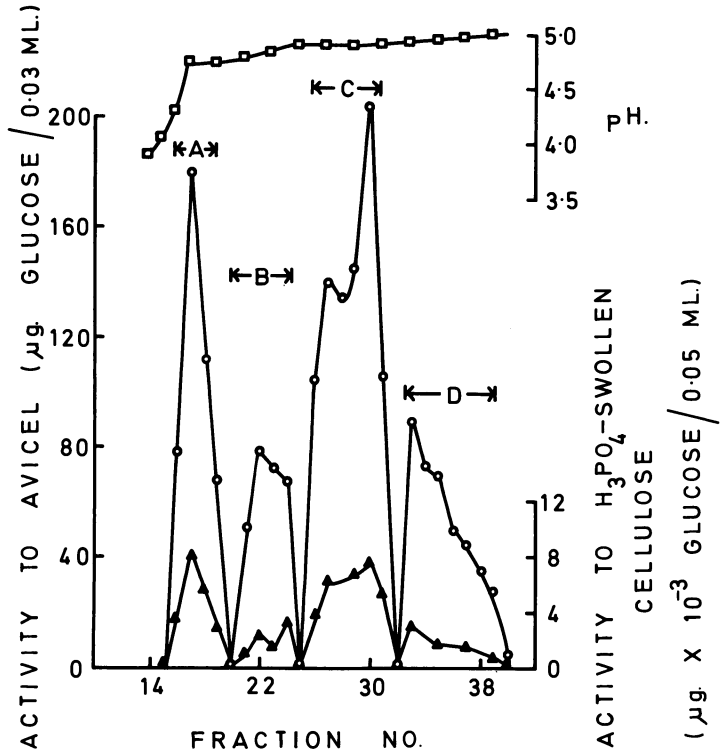

Figure 6. Isoelectric focusing of the C_1 *component of* F. solani *cellulase in an ampholyte solution covering the pH range 4.6–5.1 in an LKB 110-mL column. The* C_1 *component used was that which was eluted from a DEAE-Sephadex column as a single component* (12). *Activity to* H_3PO_4*-swollen cellulose (– ▲ –); activity to Avicel with* C_x *and β-glucosidase added to show synergistic activity (– ○ –); pH gradient (– □ –). From Ref.* 12, *in part.*

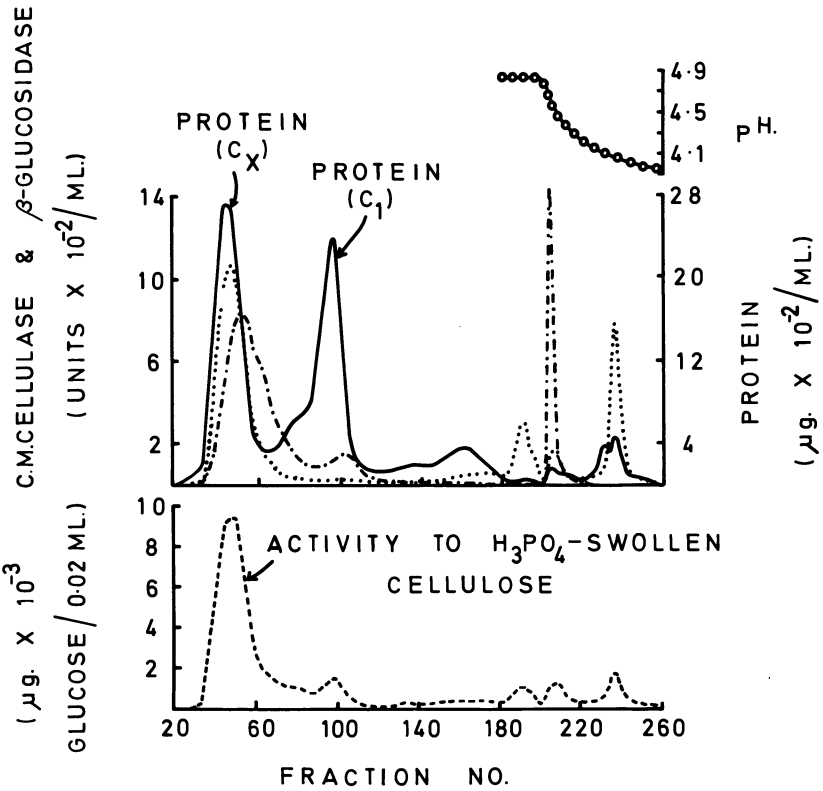

Figure 7. Separation of C_1, C_x, *and β-glucosidase components of* P. funiculosum *cellulase on DEAE-Sephadex. Protein (——); C_x (CMCase) (· · ·); β-glucosidase (– · –); activity to H_3PO_4-swollen cellulose (– – –); pH gradient (○ — ○). From Ref.* 13, *in part.*

with the same activity shown by C_x (peak 1). We have found *T. koningii* C_1 to be similar in this respect; indeed, by using only a short incubation period and low enzyme concentrations, no capacity for hydrolyzing H_3PO_4-swollen cellulose was recorded at one stage (*41*).

If the specific activity of the cellobiohydrolase of *P. funiculosum* was low after chromatography on DEAE-Sephadex, it was even lower after electrofocusing (Figure 8), much of the activity toward H_3PO_4-swollen cellulose being resolved along with the β-glucosidase which was also associated with the cellobiohydrolase in Figure 7. At this stage of purification, the specific activity of the cellobiohydrolase is clearly very low indeed, but it is still associated with C_1 activity in that it can still act synergistically in a reconstituted mixture containing the five C_x-β-glucosidase fractions (Figure 7) to solubilize cotton fiber.

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On the basis of all this evidence, the possibility must be remote that C_1 and cellobiohydrolase activities do not reside in the same protein. The separation of so many different components all with C_1/cellobiohydrolase activity must be good evidence that C_1 can be identified with cellobiohydrolase. Clearly, there are good grounds for questioning the basic tenet of Reese's hypothesis, namely, that the function of C_1 is to produce some disorder between the cellulose chains: only the prediction that two components are required for hydrolysis of highly ordered cellulose is confirmed.

Another interesting point emerges from the isoelectric focusing of the C_1 component of *P. funiculosum,* namely, the fact that the β-glucosidase separated from the C_1/cellobiohydrolase was capable of degrading a long-chain cellulose polymer such as that found in H_3PO_4-swollen cellulose. An enzyme with a capacity for attacking both a nitrophenylglucoside and a long polymer is, according to definition (*42*), more likely to be an exoglucanase (β-1,4-glucanglucohydrolase) than a β-glucosidase or cellobiase. If it is an exoglucanase, then an interesting question arises,

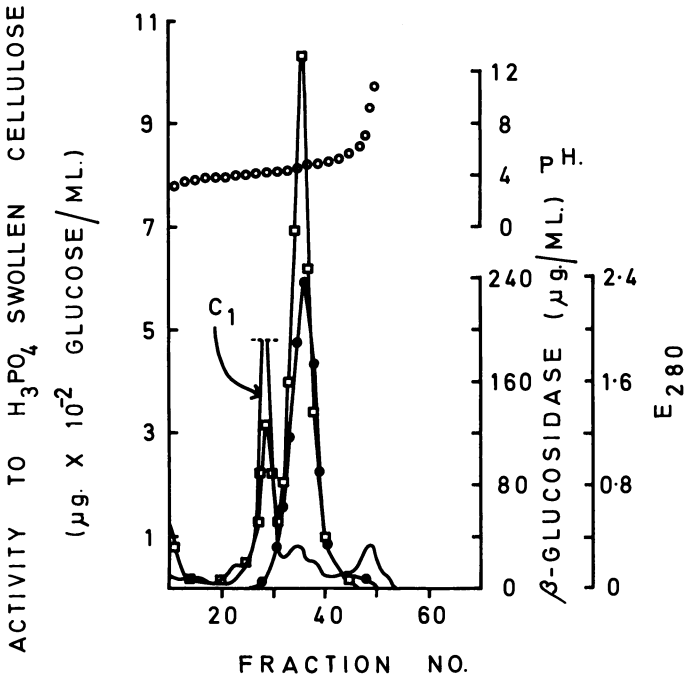

Figure 8. Isoelectric focusing of C_1 *component of* P. funiculosum *cellulase in a 110-mL LKB column.* C_1 *was from Figure 7. Ampholyte covering the pH range 4–6 was used. Protein (——); β-glucosidase (– ● –); activity to* H_3PO_4*-swollen cellulose (– □ –); pH gradient (○).* C_1 *was in fractions 25–30.*

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for we have found that, unlike the cellobiohydrolase, this enzyme is unable to act synergistically with C_x enzymes to solubilize cotton cellulose. A similar component has been isolated from *T. viride* (*3*).

Why is it that an enzyme that removes glucose units two at a time from the end of the cellulose chain (cellobiohydrolase) can act synergistically with C_x to solubilize highly ordered cellulose, and another enzyme that removes only one glucose unit at a time from the end of the chain (glucohydrolase) does not? Reese claims to have the answer, but as we have seen, there is no evidence to sustain his argument. An alternative speculation that recognizes that the conformation and steric rigidity of the anhydroglucose unit in the cellulose crystallite are important factors in a cellulase context has more appeal (*43*). As cellobiose is the repeating unit in the crystallite, one could imagine that for stereochemical reasons, only a C_1/cellobiohydrolase would be an effective catalyst. C_1/cellobiohydrolase, then, would be capable of attacking chain ends in the crystallites, while the glucohydrolase and other cellobiohydrolases that do not act synergistically with C_x (*3*) would be concerned only with attack of fully hydrated chain ends.

Oxidative Enzymes

The question of the involvement of oxidative enzymes in the breakdown of cellulose has been raised periodically, but now there is definite evidence that such enzymes occur in some culture filtrates. This was established by Eriksson and his colleagues (*2*) when they found that concentrated cell-free filtrates of the fungi *S. pulverulentum, Polyporus adustus, Myrothecium verrucaria,* and *T. viride* solubilized cellulose at a slower rate under anaerobic than under aerobic conditions.

In our laboratory, using a *T. koningii* cellulase preparation, we have also found some evidence for the presence of an enzyme that requires oxygen for its action (Table IV). The extent of the involvement of an oxidase in *T. koningii* cellulase is markedly less, however, than was found in culture filtrates of the other fungi mentioned, and this would suggest significant differences in the relative importance of the oxidizing enzyme in the hydrolysis of cellulose in some cellulase systems. Whereas the solubilization of cotton fiber by *S. pulverulentum* cellulase (*2*) was reduced from 52% to 22% when the incubation was done in an atmosphere of nitrogen, the activity of *T. koningii* cellulase was diminished by only 5%. There were, however, other points of difference, for we have found no evidence with *T. koningii* cellulase either for the consumption of oxygen when the incubation was carried out in an oxygen electrode, or for the presence of glucuronic or gluconic acids as products

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Table IV. Degradation of Cotton Cellulose by Cellulase Preparations from Three Cellulolytic Fungi in the Presence of Either Air, Nitrogen, or Oxygen[a]

	Cellulose Degradation (Wt loss %)		
Source of Cellulase	*Air*	*Nitrogen*	*Oxygen*
T. koningii	43.2	38.1	43.0
T. koningii[b]	43.6	38.0	—
P. funiculosum	43.5	43.0	42.9
F. solani	34.3	34.6	34.1
M. verrucaria	1.0	—	1.0

[a] Wt loss of 10 mg dewaxed cotton fiber incubated in a 5-mL reaction mixture for 3 days at 30°C with 0.05 mL of a 50-fold concentrate of culture filtrate (20–80% saturated $(NH_4)_2SO_4$ fraction). Only the oxygen and nitrogen were continuously bubbled through the incubation mixture.
[b] Reconstituted from purified C_1, C_x, and β-glucosidase.

in the hydrolysate. Perhaps the oxidase in *T. koningii* cellulase is present in too small an amount to be detected by these means, but it must be significant that only reducing sugars have been observed by other investigators in the products of hydrolysis of cellulase systems. Confirmation by other workers of the involvement of oxidative enzymes is necessary, particularly as the results obtained with *F. solani* and *P. funiculosum* (Table IV) show that oxidation is not always a factor in the enzymatic breakdown of cellulose.

Which Enzyme Initiates the Attack?

All manifestations of early attack on the cotton fiber are compatible with the interpretation that it is the C_x enzymes that initiate the attack. This is true whether it is fragmentation into short fibers (*16, 39*), increase in the uptake of alkali (*S* factor) (*16, 26*), or loss in tensile strength (*16*). It is possible that some of these early changes may reflect action only on a very small portion of the fiber, such as the amorphous or less orderly hydrogen bonded areas, the primary wall (*44*), or the areas of structural weakness that are reported to traverse the whole cell wall (*45*). At the same time, there must be some significance in the fact that it is, in the main, only the C_x enzymes that produce changes detectable by these methods.

Reese (*18*) attaches a great deal of significance to the increase in the fragment of cotton fiber which was soluble in alkali after treatment with a cellulase preparation ($C_1 + C_x +$ β-glucosidase) that contained methocel to inhibit C_x activity. He interprets this data to indicate that C_1 acts first.

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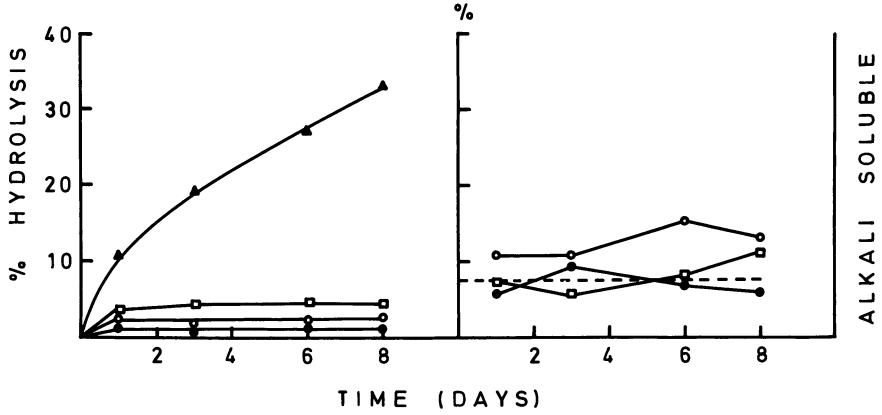

Figure 9. Effect of enzyme components from T. koningii *cellulase on the formation of alkali-soluble cellulose from cotton fiber. A reaction mixture consisting of 200 mg of cotton, 2 mL of 0.2 M acetate buffer, pH 4.5, enzyme, water, and NaN_3 solution to give a total volume of 10 mL, was incubated at 50°C for various times. Percentage hydrolysis was calculated from the loss in weight determined on weighed crucibles. The fraction soluble in alkali (10% NaOH) was determined in a similar fashion after incubating for 3 hr at 25°C. C_1 (● – ●); C_x + β-glucosidase (□ – □); low-molecular-weight C_x (○ – ○); control (– – –); reconstituted cellulase (i.e., C_1 + C_x + β-glucosidase + low-molecular-weight C_x) (▲ – ▲).*

We have repeated this work using purified C_1, C_x, and a low-molecular-weight C_x component (*26, 46*) isolated from *T. koningii,* but we can find no difference in the alkali-soluble fraction produced by C_1 or C_x (Figure 9). Indeed, of the three fractions tested, the low-molecular-weight C_x component produced the largest increase in the portion soluble in alkali.

In the absence of evidence for a chain-disaggregating enzyme that initiates the attack on the cotton fiber, or for more definite evidence for the involvement of oxidative enzymes, it seems reasonable to assume that the synergism between C_x and C_1 enzymes on highly ordered cellulose must be explained in terms of the sequential action of endo- and exoglucanases (cellobiohydrolase). Acceptable as this hypothesis is, however, it still does not explain why C_1 and C_x are individually both capable of degrading swollen cellulose but not highly ordered cellulose, yet when acting in concert, can solubilize highly ordered cellulose with ease. It is the nature of the event at the active site that still requires elucidation. Some observations made when a C_1 component from one source is acting in a mixture with C_x from another may be relevant in this context.

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Synergism between C_1 from One Fungus with C_x of Another

If the mechanism of cellulase action can be explained simply in terms of sequential action of endo- and exoglucanases, it is logical to expect that C_1 from one cellulase preparation should act synergistically with C_x from another, at least in those enzyme systems from which both C_x and C_1 have been isolated. Synergism between C_1 of *P. funiculosum* and C_x of *T. viride* has already been demonstrated (*22*), and the results in Table V show that "cross-synergism" of this type is shown by many different mixtures of the C_1 and C_x components of *F. solani, T, koningii,* and *P. funiculosum* cellulases. In each case, a marked potentiation in activity is observed.

In other culture filtrates where C_x is high but C_1 is low, the addition of C_1 also produces an increase in activity toward cotton (Table VI), but this, in a sense, is an extension of the results demonstrated in Table V, for here, too, C_1 was added to culture filtrates of organisms that released both C_1 and C_x into solution. All these results, then, are consistent in that either a high degree of synergism or a high cellulase activity resulted. When, however, C_1 is added to culture filtrates which are rich in C_x activity but have never at any time contained a C_1 component in active form (and there are many of these), a variation in synergistic activity is observed. Such culture filtrates as a rule showed only a slight potentiation in activity with C_1 (Table VII), but in a few cases, more significant increases in activity have been observed (*41*). Culture filtrates of the potent cellulose decomposers *Stachybotrys atra* and *M. verrucaria* have shown consistently small increases in capacity for degrading cotton when mixed with C_1 from either *T. koningii* (*41*) or *P. funiculosum* (Table VII), even when the incubation lasted up to 2 weeks. Culture filtrates

Table V. Synergistic Effects on Cellulase Activity Shown by Combination of C_1 and C_x from Various Fungal Sources[a]

Source of C_1 Component	*Source of C_x Component*	*Solubilization of Cotton (%)*
T. koningii	*T. koningii*	54
T. koningii	*F. solani*	79
F. solani	*F. solani*	59
F. solani	*T. koningii*	51
P. funiculosum	*P. funiculosum*	72
P. funiculosum	*T. koningii*	51
P. funiculosum	*F. solani*	45

[a] All assays contained the same number of C_x (CM-cellulase) units of activity (*26*) and 200 μg of C_1 protein. Neither C_1 nor C_x from either organism showed a significant capacity for solubilizing cotton fiber when acting in isolation.

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Table VI. Synergistic Effects on Cellulase (Cotton-Solubilizing) Activity Shown by Some Fungal Culture Filtrates, when Supplemented with C_1 from *T. koningii* or *P. funiculosum* Cellulases[a]

	Solubilization (%) of Cotton		
Culture Filtrate	*Alone*	+ T. koningii C_1	+ P. funiculosum C_1
F. moniliforme	41	63	61
F. oxysporum	35	46	48
F. avenaceum	20	59	51
S. pruinosum	35	58	64

[a] All assays contained the same number of C_x (CM-cellulase) units of activity (*26*). 200 μg of C_1 protein was added where applicable. Neither of the C_1 components could degrade cotton fiber significantly when acting alone.

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of some strains of *Memnoniella echinata* and *Gliocladium roseum*, on the other hand, have tended to give variable results. There is no doubt, however, that our results are consistent with the conclusion that "cross-synergism" is best between the respective C_1 and C_x components of fungi that freely release C_1 in active form into the culture medium.

Why is it that all endoglucanases cannot cooperate with C_1 to the same extent? Clearly there are several possible explanations. First, if as seems likely, it is the endoglucanases (C_x) that initiate the attack, it may be that the endoglucanases in some culture filtrates produce fragments

Table VII. Synergism between the C_1 Components of *T. koningii* and *p. funiculosum* and from C_x Culture Filtrates of Other Fungi, in Solubilizing Cotton Fiber

	Solubilization of Cotton Fibers (%)		
Source of Culture Filtrate	*Alone*	*with* T. koningii C_1	*with* P. funiculosum C_1
M. verrucaria IMI 45541	6	20	20
S. atra IMI 82021	4	21	11
G. roseum IMI 69027	4	15	14
M. echinata IMI 16201	5	21	18
G. roseum[a]	3	32	20
M. echinata[a]	5	28	—

[a] Unclassified strains: gifts from Dr. J. H. Walsh, Department of Biochemistry, University of Manchester, Institute of Technology. Cellulase assays (*21*) were carried out for 7 days at 37°C with 1 mL of cell-free culture filtrate. C_1 added amounted to 180–200 μg protein.

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that are too small or too large to be accommodated by the active site of C_1. Alternatively, it is possible that some fungi may use completely different mechanisms from the ones discussed here. But a third possibility, and one which we favor is that the two enzymes (C_1 and C_x) must interact in some way to form a complete cellulase complex. Some recent results obtained from a study of the synergism shown when C_1 induced on one carbon source is mixed with C_x induced on another makes this third suggestion a plausible one.

Synergism between C_1 Induced on One Carbon Source with C_x Induced on Another

T. koningii was grown in separate synthetic media containing as the sole carbon source either lactose, Avicel, Whatman cellulose powder, or the α-celluloses from oat straw, birch wood, or ryegrass. Each of the cellulase complexes induced when the fungus was grown in these media was fractionated on DEAE-Sephadex into C_1 and C_x activities (as in Figure 1), and the enzymes were compared with similar types of enzymes induced on cotton cellulose. The enzymes were compared by mixing the C_1 induced on one carbon source with the C_x induced on another and then measuring the capacities of the mixtures for solubilizing highly ordered substrates such as cotton and Avicel.

Table VIII shows that the individual C_1 components induced on the various carbon sources showed almost the same capacity for solubilizing highly ordered substrates when acting in admixture with the C_x which was induced on cotton. On this basis, at least, the C_1 components were

Table VIII. Cellulase Activity Shown by Mixtures of C_1 Induced on Various Carbon Sources with C_x Induced on Cotton[a]

Carbon Source on which C_1 Induced	*Carbon Source on which C_x Induced*	*Solubilization (%)* (a) Cotton	(b) Avicel
Oat straw α-cellulose	cotton	72	68
Birch wood α-cellulose	cotton	70	67
Ryegrass α-cellulose	cotton	73	68
Avicel	cotton	73	68
Whatman cellulose powder	cotton	73	63
Lactose	cotton	72	64
Cotton	cotton	73	69

[a] All assays (*21*) contained C_1 180 μg protein, 1000 units of C_x (CM-cellulose) activity (*26*), and 100 units of β-glucosidase; where necessary, β-glucosidase isolated from the cellulase of *F. solani* (*21*) was added. The α-celluloses were the residues left after delignification and extraction with 18% (w/v) NaOH of oat straw, birch wood, and ryegrass.

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similar. However, marked differences in the degree of solubilization of cotton were observed when the cotton-induced C_1 was acting in synergism with the C_x induced on either Avicel or lactose, or the α-cellulose from ryegrass or oat straw (Table IX). In the extreme C_1 induced on cotton and C_x induced on ryegrass α-cellulose showed 35% solubilization of cotton, while C_1 induced on cotton and C_x induced on birch α-cellulose showed 61%, and this compares with 72% solubilization when both C_1 and C_x were from the same source. The effect was not peculiar to cotton, for a similar range of synergistic effects was obtained with Avicel as the substrate (Table IX).

C_x enzymes occur in multiple forms in most cellulose systems, and they appear to be different enzymes with different relative activities on a number of cellulose substrates. It was always possible, then, but not necessarily likely, that a completely different C_x system was being synthesized in each case and that this would account for the difference in synergism. This possibility was excluded, however, when it was found that each C_x system was identical in terms of CM-cellulase activity: in each case the number of reducing end groups produced per unit decrease in viscosity was the same.

But CM-cellulose is not the only substrate for measuring C_x activity. H_3PO_4-swollen cellulose, as mentioned earlier, is also a subsratte for C_x enzymes, and various C_x enzymes have been isolated from *T. viride* with differing capacities for degrading this substrate per unit of CM-cellulase activity. Two of the C_x systems, that induced on Avicel and that on ryegrass α-cellulose, did indeed show a variation in the capacity for solubilizing H_3PO_4-swollen cellulose per unit of CM-cellulose activity, but when the C_x enzymes were recombined with C_1 such that they were all equivalent in terms of this activity, a variation in synergism similar to that shown in Table I was observed.

Table IX. Cellulase Activity Shown by Mixtures of C_x Induced on Various Carbon Sources with C_1 Induced on Cotton[a]

Substrate on which C_1 *Induced*	*Substrate on which* C_x *Induced*	*Solubilization (%)* (a) *Cotton*	*Solubilization (%)* (b) *Avicel*
Cotton	Oat straw α-cellulose	40	65
Cotton	Birch wood α-cellulose	61	64
Cotton	Ryegrass α-cellulose	35	47
Cotton	Avicel	30	65
Cotton	Whatman cellulose powder	72	73
Cotton	Lactose	38	49
Cotton	Cotton	72	77

[a] Identical conditions to those described in Table VIII were used.

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Clearly, the C_1 components synthesized on the various carbon sources are similar, but there are some unexplained differences in the C_x fraction that affect synergism with C_1. The reason for these unexpected results is not immediately apparent, but the answer may be connected with some earlier observations made by Rautela and King (*47*). Working with *T. viride,* Rautela found that when the fungus was cultivated in a medium containing any one of the four known crystal forms of cellulose as the carbon source, the organism produced enzymes that required minimal activation energy for hydrolysis of the crystal form on which it was cultivated. Rautela's results are remarkable in that they show that the enzyme-synthesizing mechanism of *T. viride* can alter the structure of the active site of the enzyme so as to accommodate a specific crystal lattice structure as its substrate (*43*); this may explain the differences in synergism observed in our laboratory, at least in some cases.

We envisage that any alteration in the active site would be accompanied by some modification of the tertiary structure of the enzymes (C_x), which, although possibly only very minor, may be sufficient to prevent the cooperation of C_x with C_1 in a "loose" complex on the surface of the cellulose crystallite. Equally well, of course, there may be other differences between the various insoluble growth substrates that are important determinants of the structure of the active site. These differences could be either at the molecular or at the supermolecular level.

Some correlation of the results with crystal structure is possible in that mixtures of C_x enzymes induced on a cellulose II crystal form (which would be present in the α-celluloses) and the C_1 induced on the cellulose I crystal form (cotton) showed much lower capacities for degrading cotton than did a mixture of C_1 and C_x enzymes which had both been induced on cotton. Avicel, too, presumably would also be in the cellulose II form since it had been prepared from wood α-cellulose. Of all the C_x enzymes tested, that induced on Avicel showed the lowest capacity for degrading cotton when it was acting in synergism with the C_1 induced on cotton.

Whatever the reason for the variation in the synergistic activity observed between C_1 and the various C_x enzymes, it would appear that the answer must lie in the C_x fraction and the manner in which the various components interact with C_1. For this reason, we have looked at the C_x system to *T. koningii* in some detail.

Multiplicity of C_x *Components in* T. koningii *Cellulase*

As mentioned earlier, C_x enzymes exist in multiple forms in most cellulase systems. As a rule they vary in the degree of "randomness" of their attack on CM-cellulose, and they may be distinguished by plotting

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the change in fluidity in the presence of each component against the corresponding increase in reducing power (*44, 48*). A component showing the steepest slope is considered to be the most random.

Five endoglucanases have been isolated from *S. pulverulentum* cellulase (*23*), four from *T. viride* "Onozuka" cellulase (*37*), and three from *T. viride* "Meicelase" (*31*), but there are many other reports of the fractionation of multiple C_x components. *P. funiculosum* cellulase contains three and possibly four endoglucanases (Figure 7), while we have reported *T. koningii* cellulase to contain five (*17*) and recently six (*46*).

Of the six C_x components isolated from *T. koningii* cellulase, only four were major components (C_{x1}, C_{x3a}, C_{x3b}, and C_{x4} in Scheme 4). One of the components (C_{x1}) had a molecular weight very much smaller (13,000) than the other three (C_{x3a} and C_{x3b} have 38,000; C_{x4} has 31,000), but all four were similar in that they could degrade CM-cellulose and H_3PO_4-swollen cellulose. The pattern of attack differed, but there was a definite relationship in the mode of action of each on these substrates. Figure 10 shows that C_{x1} (the low-molecular-weight C_x) was the most random in its attack on CM-cellulose when the change in viscosity was compared with increase in reducing power, while C_{x3b} was the least random. The pattern was the same when the insoluble H_3PO_4-swollen

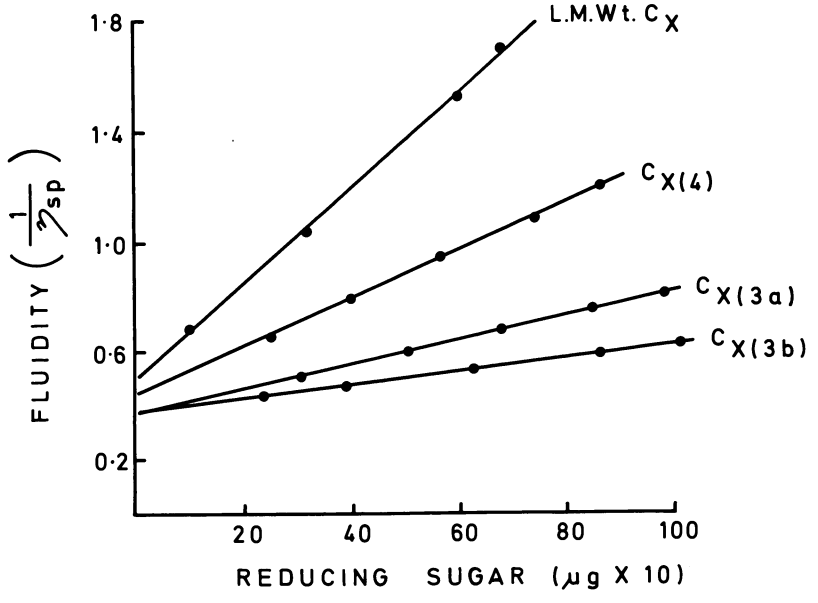

Figure 10. Relationship between fluidity and reducing power during the hydrolysis of CM-cellulose by the C_x *components of* T. koningii *cellulase (46).*

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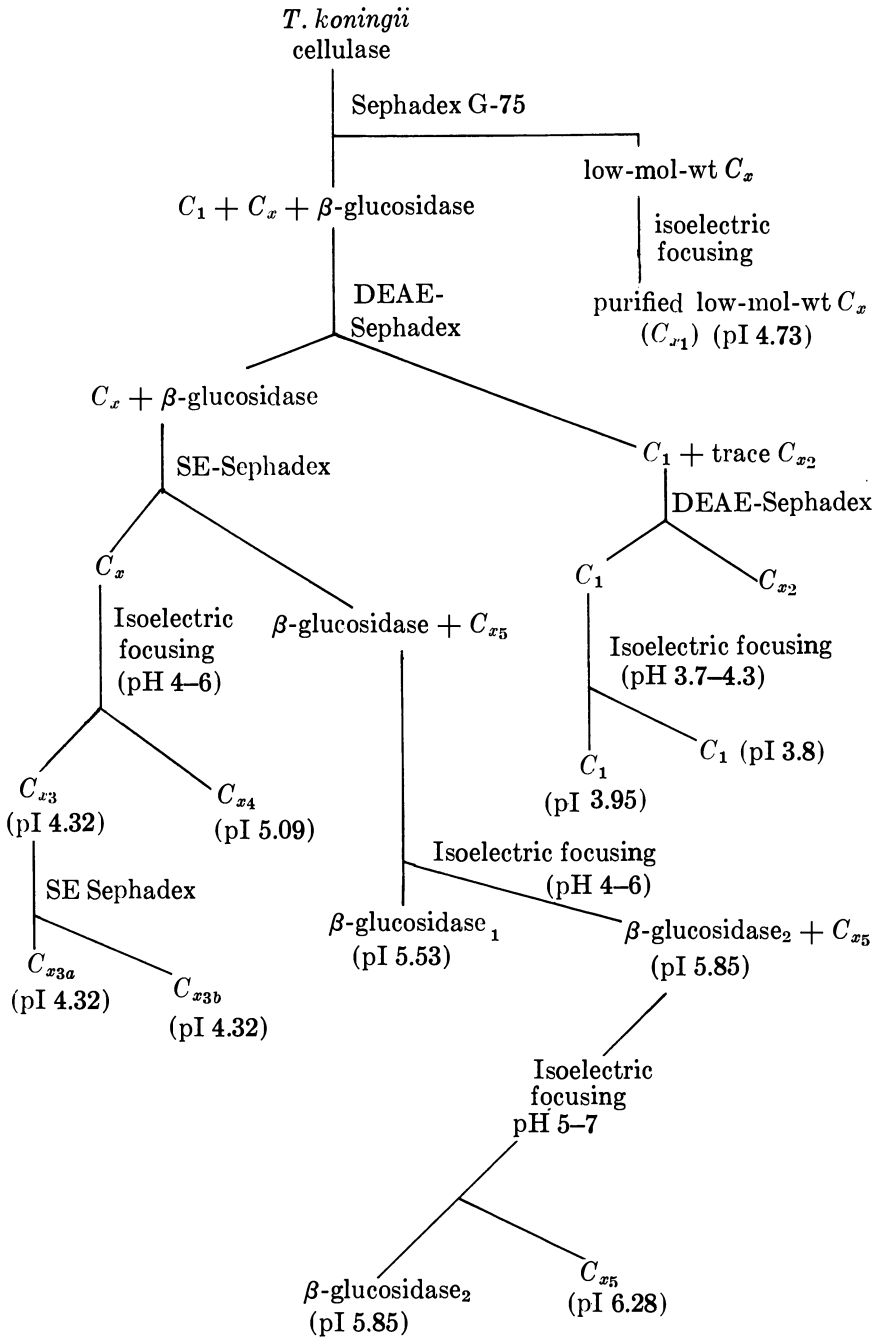
Scheme 4. Fractionation of T. koningii *cellulase*



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cellulose was used as substrate—i.e., the low-molecular-weight C_x component produced the greatest rate of change of degree of polymerization (Figure 11). Clearly, on the basis of these observations, it would appear that the order of increasing randomness of attack is $C_{x3b} < C_{x3a} < C_{x4}$ < low-molecular-weight C_x. If, then, synergistic action between C_1 and C_x enzymes can be explained simply in terms of exoglucanase (C_1) hydrolyzing the new chain ends generated by the endoglucanase (C_x), as has been suggested, and we can assume that there is a relationship between attack on H_3PO_4-swollen cellulose, CM-cellulose, and cotton, then a solution containing C_1 and C_{x1} (the most random) should show the highest capacity for solubilizing cotton fiber. Table X shows, however, that this was not the case. Indeed, the mixture of C_1 and C_{x1} did not show any synergism. Clearly, the problem is more complex than this.

Leatherwood (*49*) interprets his observations made with roll tube cultures of the anaerobic bacterium *Ruminococcus albus* to indicate that a single cellulase complex is formed from enzymes diffusing from two different colony types. He discussed the mechanism in terms of a non-

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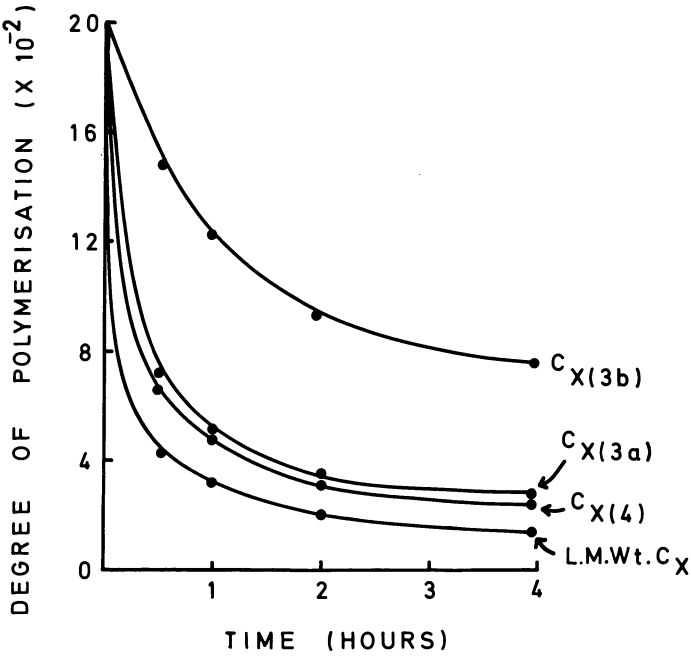

Figure 11. Effect of C_x *enzyme components of* T. koningii *cellulase on the degree of polymerization of* H_3PO_4*-swollen cellulose. The degree of polymerization was determined by viscometry after dissolution in Cadoxen (46).*

Table X. Solubilization of Dewaxed Cotton by Reconstituted Mixtures of the Components of the Cellulase Complex of *T. koningii*[a]

Enzyme	*Solubilization of Cotton (%)*
C_1 (cellobiohydrolase) + C_{x_1}	2
$C_1 + C_{x3a}$	34
$C_1 + C_{x3b}$	2
$C_1 + C_{x_4}$	51
$C_1 + C_{x3a} + C_{x_4}$ + 1β-glucosidase	53
$C_1 + C_{x_1} + C_{x3a} + C_{x3b} + C_{x_4}$	72
Unfractionated complex	71

[a] C_{x_2} and C_{x_5} in Scheme 4 were minor components. All components were recombined in the same proportions in which they were present in 1.0 mL of starting material (20–80% saturated $(NH_4)_2SO_4$ fraction, diluted 50-fold). From Ref. *46*, in part.

hydrolytic affinity factor, i.e., C_1, and a hydrolytic factor, i.e., C_x, forming an active cellulase complex. We would suggest again that the formation of a complete cellulase complex for the enzyme system in *T. koningii* cellulase is a possibility that must be considered. However, since there is no real evidence for the involvement of a nonhydrolytic C_1 component of the type envisaged by Leatherwood, we would suggest that extensive hydrolysis of highly ordered cellulose is accomplished only by those pairs of hydrolytic enzymes, (C_{x3a} and C_1) and (C_{x_4} and C_1), that have formed a loose complex on the surface of the cellulose crystallite.

In essence, then, we consider the enzymatic hydrolysis of cellulose to involve sequential action, where the C_x enzymes initiate the attack and the new chain ends that are generated are then hydrolyzed by the endwise acting C_1 components. However, where attack is on the crystalline areas, we now suggest that the second stage must follow the first instantly to prevent reformation of the glucosidic linkage between two glucose residues held rigidly in position by intermolecular hydrogen bonds.

To account for the fact that C_1 apparently has a much greater affinity for crystalline cellulose than C_x, we suggest that while C_x action causes the first cleavage of the glucosidic linkage, C_1 is actually already in position awaiting the approach of C_x. In other words, C_1 is an affinity factor, but it is different from that envisaged by Leatherwood in that it is also involved in the hydrolytic process.

A "loose-complex" hypothesis is highly speculative, but it is one that could fit the facts that have been established. It is compatible with the fact that the relative proportions of C_x to C_1 are important for maximum rate of solubilization (*16*). It could also explain the variation in the synergism between C_1 of one fungus and C_x of another, or the variation

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in synergism between C_1 induced on one carbon source with C_x induced on another. In some respects, it is even compatible with Reese's hypothesis, for it seems reasonable to assume that the adsorption of a charged molecule such as C_1 on the surface of the cellulose crystallite as a preliminary to C_x–C_1 complex formation would result in the localized disruption of hydrogen bonds.

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11

The Mode of Enzymatic Degradation of Cellulose Based on the Properties of Cellulase Components

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From Driselase, a commercial product from Irpex lacteus, *a cellulase component was fractionated and purified. The enzyme produced exclusively cellobiose (G_2) from cellulosic substrates such as Avicel, carboxymethyl cellulose (CMC), cellotetraose (G_4), and cellohexaose (G_6), but a mixture of glucose (G_1), cellobiose, and cellotriose (G_3) from cellopentaose (G_5). In the hydrolysis of CMC, it showed a time-course curve typical of exocellulase. Its synergistic action with an endocellulase of higher randomness was very strong and it occurred in the hydrolysis of CMC as well as of Avicel. It mutarotated the products from cellopentaitol in an upward direction as endocellulases did, indicating that the β-glucose configuration was retained. Based on the properties of this exocellulase and of several endocellulases already reported, the in vivo degradation of native cellulose by this fungus was discussed.*

In previous work, we obtained several cellulase components from culture filtrates of *Irpex lacteus* (*Polyporus tulipiferae*) or from Driselase, a commercial enzyme preparation of this fungus; they behaved practically as a single protein (*1, 2, 3*). They were different in randomness of the hydrolysis of carboxymethyl cellulose (CMC), expressed as the ratio

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between the decrease in the viscosity of substrate and the increase in the simultaneous production of reducing power. The hydrolysis by F-1 was most random, that by S-1 was least random, and that by F-2 was medium.

On the other hand, the presence of several endocellulase components of different randomness also was demonstrated in Cellulase–Onozuka, a commercial enzyme product from *Trichoderma viride,* by the studies of our group (*1, 4, 5, 6, 7*) and others (*8, 9*). In addition, we obtained an exocellulase component of Avicelase type from Cellulase–Onozuka (*10*). This *Trichoderma* exocellulase showed a strong synergistic effect on the hydrolysis of not only Avicel but also CMC in association with endocellulase components of higher randomness. This cellulase, therefore, seemed to play an important role in the degradation of cellulose in vivo. In addition, an exocellulase component of glucosidase type was obtained from Cellulase–Onozuka (*7*), and the cellulase seemed to play an important role in the hydrolysis of cellooligosaccharides into glucose in vivo, because it split them more easily than the ordinary β-glucosidase.

With this background, we then tried to obtain the possible exocellulase component from *I. lacteus* using Driselase as the starting material, and we succeeded in isolating it. Its enzymatic properties were also precisely investigated and the possible role of this cellulase component in vivo was discussed with reference to the properties of other cellulase components already published. This report deals with these results, but most of the data for F-1 and S-1 were cited from previous work (*2, 3*).

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Experimental and Results

Fractionation and Purification of E_x-1 Cellulase Component from Driselase. Driselase powder (50 g) was extracted with several aliquots of water and the precipitate formed upon salting out with ammonium sulfate (on a saturation between 20% and 80%) was fractionated on a DEAE–Sephadex A-50 column. Each fraction was tested for β-glucosidase, xylanase, CMCase, Avicelase activities, and protein content. The elution patterns are shown in Figures 1 and 2.

The E-3 peak was high in Avicelase activity and in protein content as compared with CMCase activity. This peak was further fractionated on a Bio-gel P-100 column; five protein peaks (E-3-1 to E-3-5) were obtained, of which E-3-2 peak was highest among them in Avicelase activity and protein content. The elution patterns are shown in Figure 3, and the time course of hydrolysis of CMC by these cellulase fractions measured by a decrease in the viscosity is shown in Figure 4. Randomness of them is in the order of E-3-5 < E-3-2 < E-3-1 ≦ E-3-4 ≦ E-3-3. The E-3-2 fraction was subjected to further purification on a CM–Sephadex C-50 column because E-3-5 was very low in the Avicelase activity.

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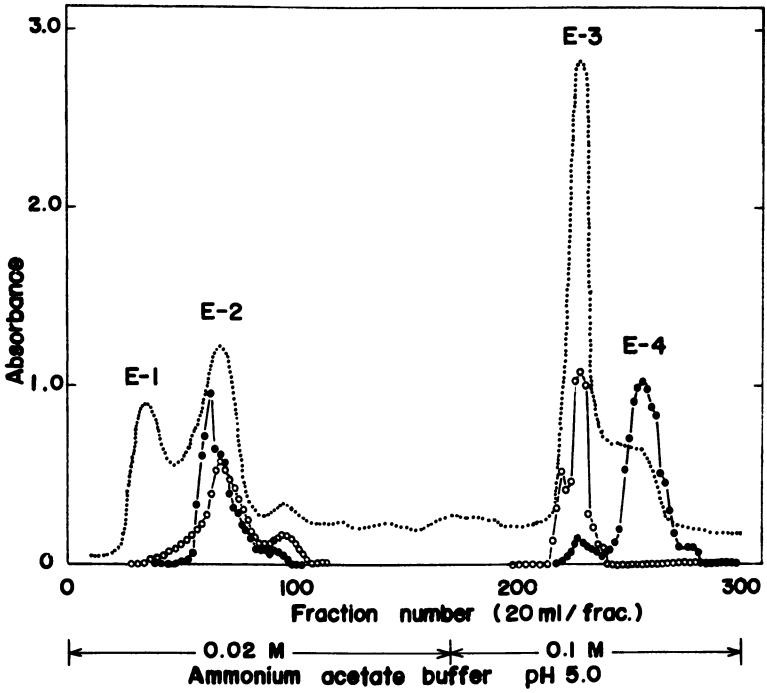

Figure 1. Elution patterns of the ammonium sulfate-precipitated enzyme preparation from a DEAE–Sephadex A-50 column. (●) CMC-saccharifying activity (5-min incubation) of eluates diluted 60-fold, (○) Avicel-saccharifying activity (1-hr incubation), (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 5.0 × 50 cm; flow rate: 20 mL/8 min; one fraction: 20 mL.

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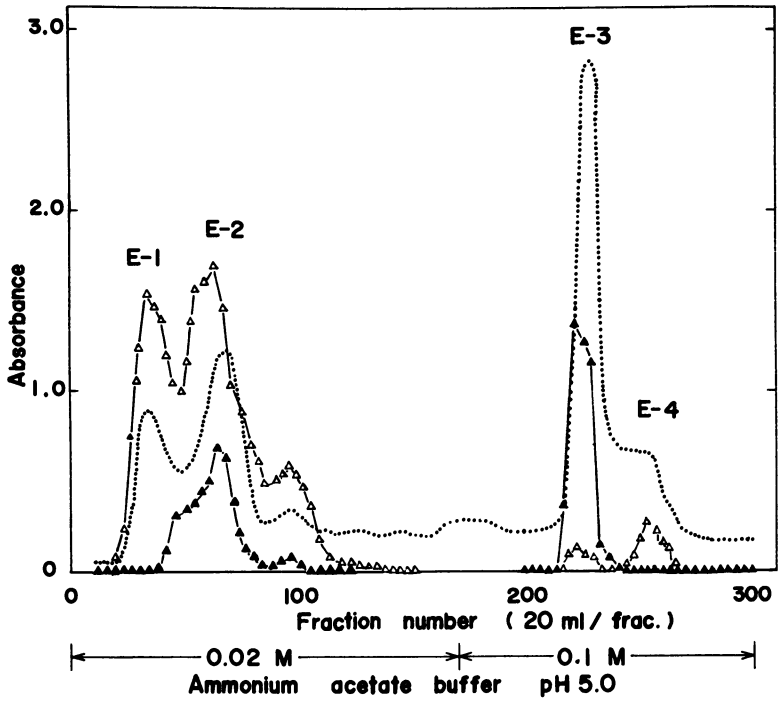

Figure 2. Elution patterns of the ammonium sulfate-precipitated enzyme preparation from a DEAE–Sephadex A-50 column. (▲) β-glucosidase activity (3-hr incubation) of eluates diluted 60-fold, (△) xylan-saccharifying activity (3-hr incubation) of eluates diluted 60-fold, (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 5.0 × 50 cm; flow rate: 20 mL/8 min; one fraction: 20 mL.

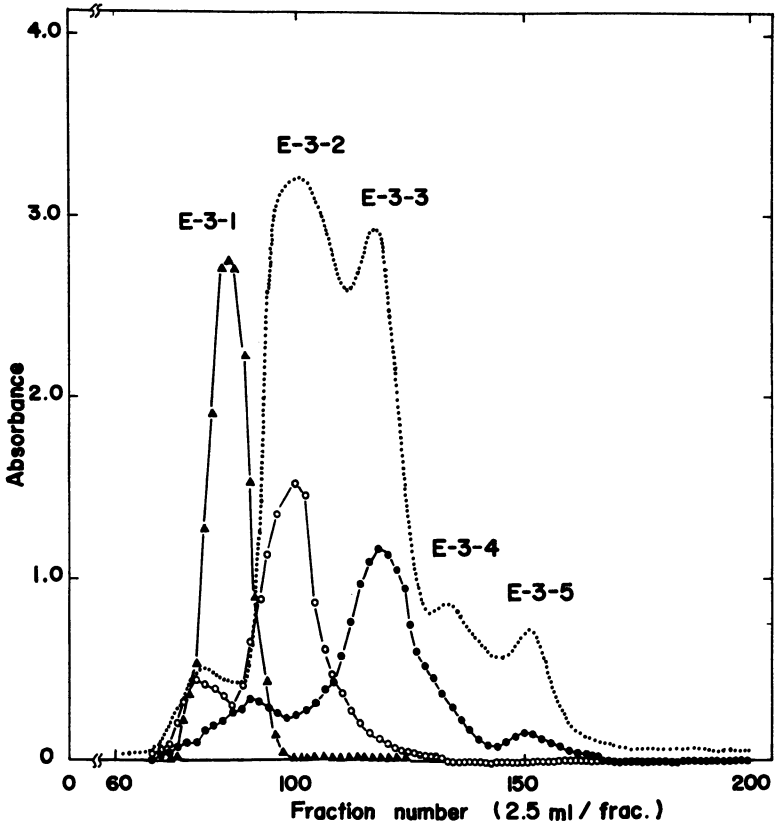

Figure 3. Elution patterns of E-3 on a Bio-gel P-100 column. (●) CMC-saccharifying activity (5-min incubation) of eluates diluted 20-fold, (○) Avicel-saccharifying activity (30-min incubation), (▲) β-glucosidase activity (15-min incubation), (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 2.7 × 133 cm; flow rate: 5.0 mL/hr; one fraction: 2.5 mL.

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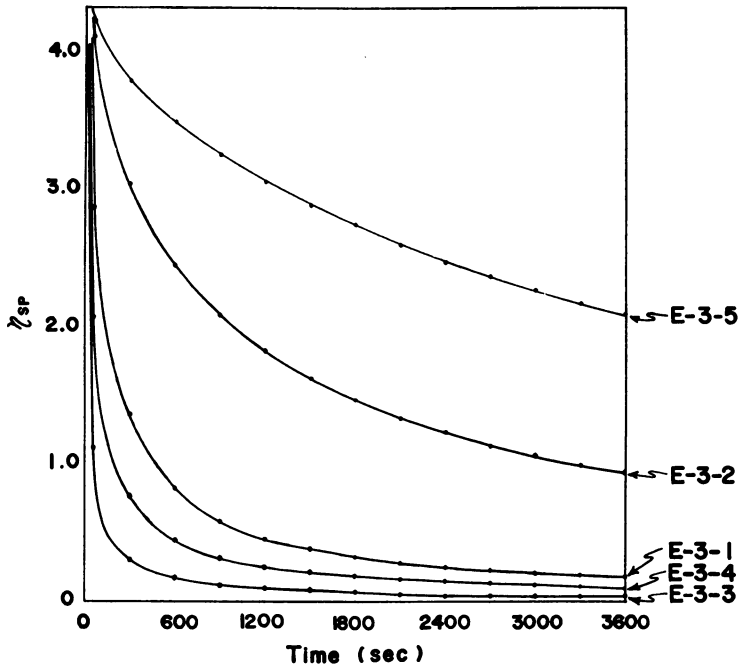

Figure 4. Decrease in specific viscosity during the hydrolysis of CMC solution by E-3-1, E-3-2, E-3-3, E-3-4, and E-3-5. Reaction mixture consists of 3.0 mL 1% CMC, 7.0 mL 0.1M sodium acetate buffer, pH 4.0, and 2.0 mL enzyme solution containing an amount of protein equivalent to an optical density of 0.05 at 280 nm. The reaction mixture was carried out at 30°C in an Ostwald viscometer.

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As shown in Figure 5, the E-3-2-2 peak seemed to be a cellulase fraction of Avicelase type while the E-3-2-3 peak appeared to be a cellulase fraction of CMCase type. The E-3-2-2 peak was then fractionated on a DEAE–Sephadex A-50 column and two peaks were obtained: a cellulase fraction apparently a CMCase type (E-3-2-2-1) and another cellulase fraction (E-3-2-2-2) seeming to be of an Avicelase type (Figure 6). E-3-2-2-2 was eluted from a Bio-gel P-100 column as a single peak, as shown in Figure 7. This cellulase component migrated as a single band on SDS–polyacrylamide disc electrophoresis (Figure 8) and it was named E_x-1.

Overall procedure of fractionation and purification of E_x-1 is shown in Figure 9, and these steps and yields are presented in Table I.

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Table I. Purification Steps of E_x-1 and Their Yield

Purification Step	*Specific Activity*[a] *(units/mg)*		*Yield*[d] *(%)*
	CMC[b]	*Avicel*[c]	
Starting solution	0.200	0.0008	100 (as dry matter)
Ammonium sulfate precipitation (0.2–0.8 saturation)	0.283	0.0018	35.0
1st. DEAE–Sephadex A-50 column chromatography (E-3 component)	0.433	0.0032	12.4
1st. Bio-gel P-100 gel filtration (E-3-2 component)	0.124	0.0052	3.0
CM–Sephadex C-50 column chromatography (E-3-2-2 component)	0.070	0.0056	0.46
2nd. DEAE–Sephadex A-50 column chromatography (E-3-2-2-2 component)	0.001	0.0438	0.17
2nd. Bio-gel P-100 gel filtration (E_x-1 component)	0.001	0.0571	0.08

[a] Specific activities are defined as activity units/mg of enzyme protein.
[b] One unit is the CMC-saccharifying activity that produces reducing power equivalent to 1.0 μmol glucose per min.
[c] One unit is the Avicel-saccharifying activity that produces reducing power equivalent to 1.0 μmol glucose per min.
[d] The yield at each purification step is expressed in terms of wt % of the dry matter of the starting solution.

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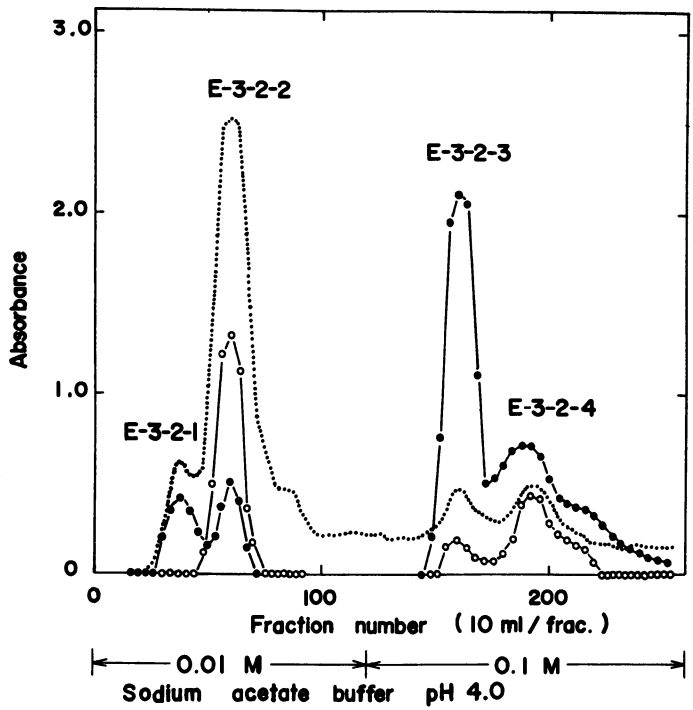

Figure 5. Elution patterns of E-3-2 on a CM–Sephadex A-50 column. (●) CMC-saccharifying activity (30-min incubation) of eluates diluted 10-fold, (○) Avicel-saccharifying activity (30-min incubation), (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 3.0 × 30 cm; flow rate: 20 mL/hr; one fraction: 10 mL.

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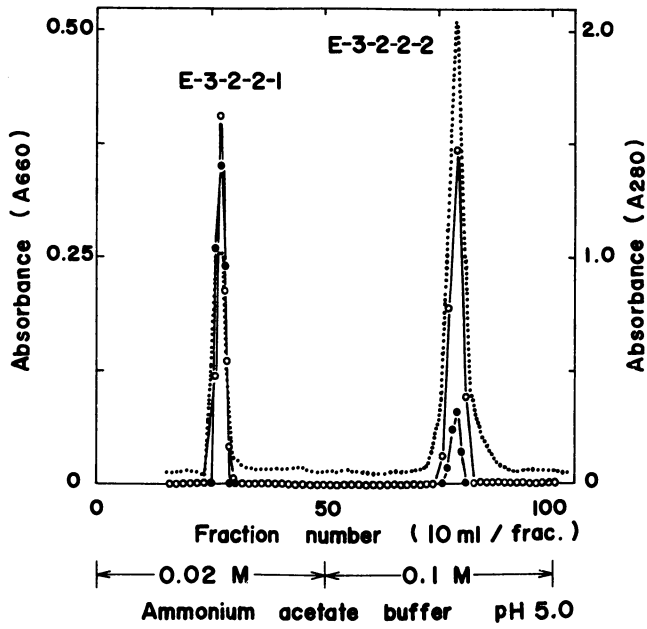

Figure 6. Elution patterns of E-3-2-2 on a DEAE–Sephadex A-50 column. (●) CMC-saccharifying activity (10-min incubation) of eluates diluted 10-fold, (○) Avicel-saccharifying activity (30-min incubation), (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 3.0 × 30 cm; flow rate: 26 mL/hr; one fraction: 10 mL.

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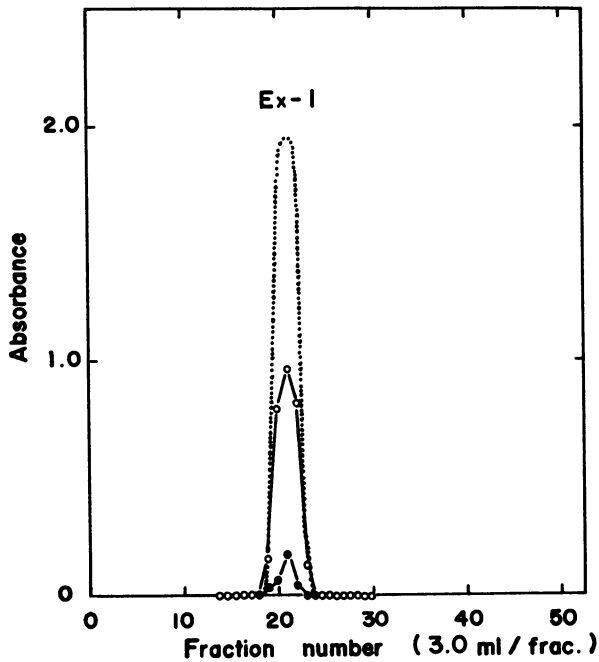

Figure 7. Elution patterns of E-3-2-2-2 on a Bio-gel P-100 column. (●) CMC-saccharifying activity (30-min incubation), (○) Avicel-saccharifying activity (30-min incubation), (· · ·) protein concentration measured in terms of the absorbance at 280 nm; column: 1.2 × 140 cm; flow rate: 3.0 mL/hr; one fraction: 3.0 mL.

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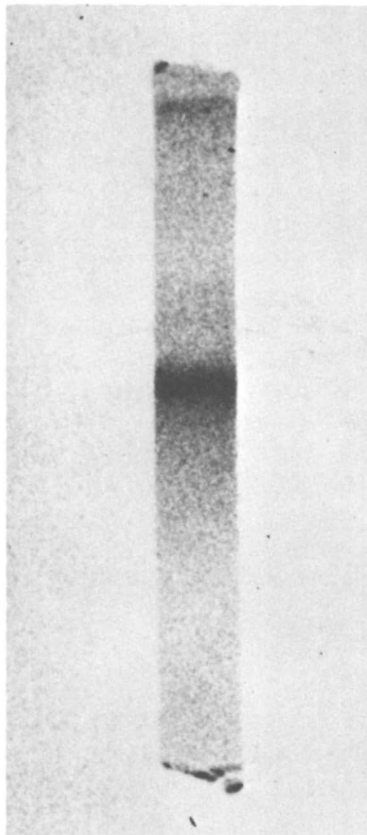

*Figure 8. Polyacrylamide-gel disc electrophoresis of E_x-1. Electrophoresis of the purified enzyme was carried out by the method of Weber and Osborn, using 10% acrylamide, pH 7.0, containing 0.1*M *sodium phosphate and 0.1*M *sodiumdodecyl sulfate.*

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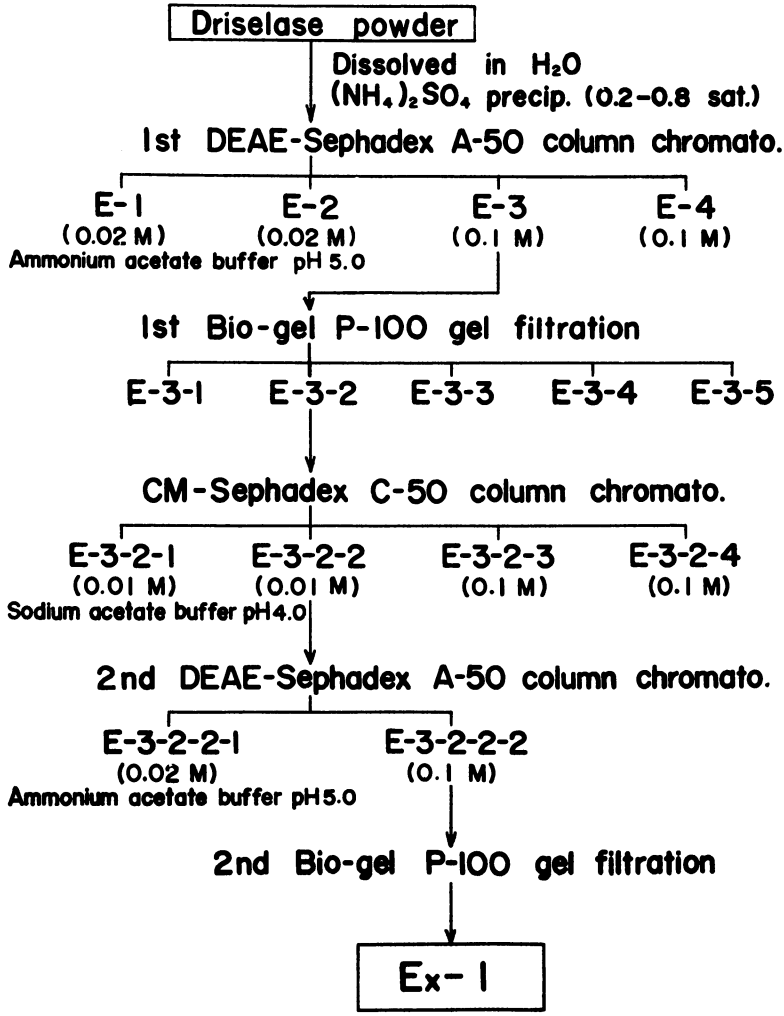

Figure 9. Overall procedure of fractionation and purification of E_x-1

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Table II. Temperature and pH Optima and Stabilities

Component	*pH Stability*	*Optimum pH*	*Temperature Stability*	*Optimum Temperature (°C)*
E_x-1	3.5–6.0	5.0	100°C for 10 min 95% inactivation	50
S-1	4.0–5.0	4.0	80°C for 10 min 100% inactivation	50
F-1	4.0–5.0	5.0	80°C for 10 min 100% inactivation	40

Properties of E_x-1 Cellulase Component. pH AND TEMPERATURE OPTIMA AND STABILITIES. E_x-1 was practically stable in the pH range of 3.5–6.0, while S-1 (an endocellulase of lower randomness) and F-1 (an endocellulase of higher randomness) were stable at pH's between 4.0 and 5.0. The optimum pH of E_x-1 was at 5.0, similar to that of F-1, while that of S-1 was at 4.0. The activity of E_x-1 was optimum at 50°C similar to S-1, while that of F-1 was 40°C. E_x-1 retained 5% of its optimum activity after 10 min at 100°C, whereas F-1 and S-1 were totally inactivated after 10 min at 80°C (Table II).

MOLECULAR WEIGHTS, AMINO ACID COMPOSITION, AND CARBOHYDRATE CONTENT. The molecular weight of E_x-1 was estimated by gel filtration to be 65,000 and it contained 2.35% carbohydrate as glucose. Those of S-1 and F-1 were 56,000 and 35,000, respectively and their carbohydrate contents were 12.20% and 10.92% as glucose, respectively (Table III).

Amino acid composition of E_x-1 is shown in Table IV together with those of S-1 and F-1. The amino acid composition of E_x-1 has a pattern similar to those of S-1 and F-1 except for minor differences in a few amino acid contents such as proline, glycine, isoleucine, and glutamic acid.

Table III. Molecular Weight, Carbohydrate Content, and the Ratio of Avicelase to CMCase Activity

Component	*Molecular Weight*	*Carbohydrate Content as Glucose (%)*	*Avicelase:CMCase*[a]
E_x-1	65,000	2.35	9.56
S-1	56,000	12.20	3.00
F-1	35,000	10.92	0.18

[a] Avicelase and CMCase activities were measured for 1-hr and 30-min incubations, respectively.

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Table IV. Amino Acid Composition of Various Cellulase Components from *I. lacteus*[a]

Amino Acid	E_x-*1*	*F-1*	*F-2*	*S-1*
Lys	3.2	3.8	2.9	2.8
His	1.0	2.0	2.2	1.3
Arg	2.5	2.1	2.0	2.2
Asp	12.5	13.9	13.3	12.6
Thr	9.5	8.2	7.6	8.4
Ser	8.7	9.0	9.6	9.5
Glu	6.3	9.2	9.7	9.3
Pro	3.7	5.8	5.9	7.1
Gly	9.7	7.9	7.4	11.8
Ala	8.1	7.5	7.8	7.8
Cys	1.0	2.5	2.6	2.3
Val	5.0	7.0	7.4	5.2
Ile	2.4	4.5	3.8	3.6
Leu	4.7	6.7	7.4	5.7
Tyr	3.1	4.2	3.9	4.3
Phe	3.4	5.9	6.6	6.1
Trp	3.1	—	—	—
NH_3	13.1	—	—	—
Met	1.2	—	—	—

[a] All data reported as molar ratios.

EVIDENCE FOR E_x-1 TO BE AN EXO-TYPE COMPONENT. The time course of CMC hydrolysis by E_x-1 is shown in Figure 10. The hydrolysis proceeded rapidly at first, but it reached a plateau and seemed to stop after 3 hr. This is characteristic of the hydrolysis by exo-type cellulase, as has been reported for exocellulase of glucosidase type from *T. viride* (*7*) and for another *Trichoderma* exocellulase of Avicelase type (*10*).

Similar hydrolysis patterns were observed for the hydrolysis of CMC of different degrees of substitution, and they were entirely different from the patterns obtained by E_n-1, which was fractionated and purified from the E-4 peak of Figure 1. The purification procedure is not given in this chapter. These time-course patterns are shown in Figures 11 and 12.

As further evidence, we demonstrated by paper chromatography that hydrolysis products from cellooligosaccharides by E_x-1 are G_1 and G_2 from G_3, and G_1, G_2, and G_3 from G_5, but only G_2 from G_4, G_6, CMC, cellodextrine, and insoluble cellulose such as Avicel, swollen cellulose, absorbent cotton, and filter paper (Figures 13 and 14). However, G_3 was formed from G_6 when E_x-1 was incubated with a mixture of G_6 and G_1. There is no indication that G_6 was split by this cellulase into G_3 plus G_3, but rather that G_2 produced from G_6 was transferred immediately to G_1 to form G_3. The results are shown in Figure 15.

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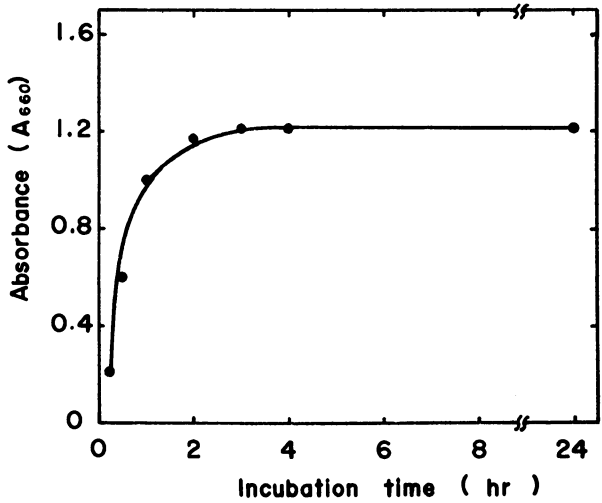

Figure 10. Time course of E_x-1 activity for CMC. Reaction mixture of enzyme and CMC solutions was incubated at 30°C for different reaction times, then examined for reducing sugar production. Final enzyme concentration: 3.0×10^{-2}%.

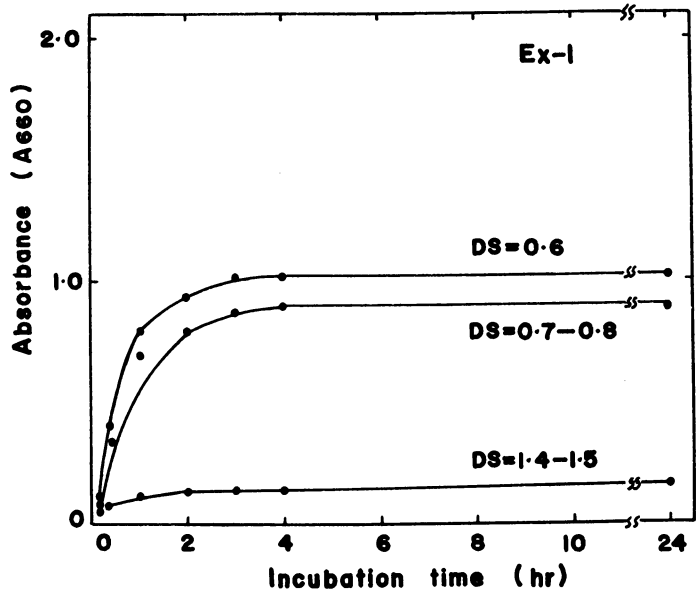

Figure 11. Time course of E_x-1 activity for CMC with different degrees of substitution. Mixtures of enzyme and CMC solutions were incubated at 30°C for different reaction times, then examined for reducing sugar production. Final enzyme concentration: 2.82×10^{-2}%.

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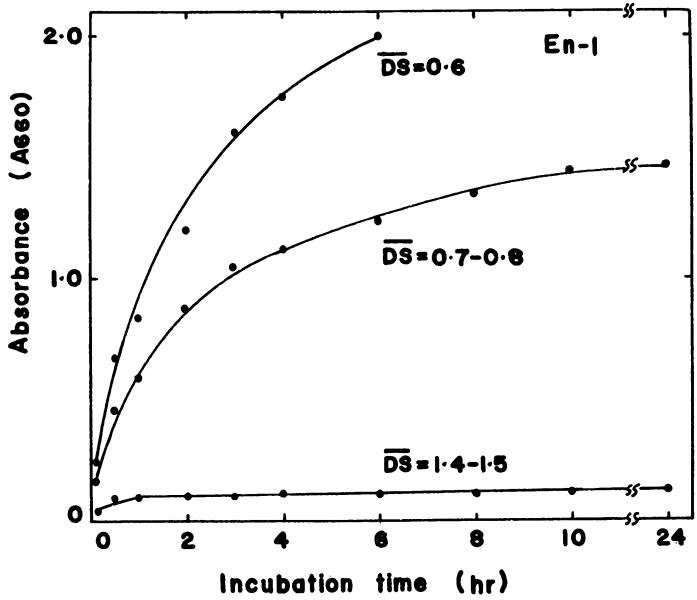

Figure 12. Time course of E_n-1 activity for CMC with different degrees of substitution. Mixtures of enzyme and CMC solutions were incubated at 30°C for different reaction times, then examined for reducing sugar production. Final enzyme concentration: 3.75×10^{-5}%.

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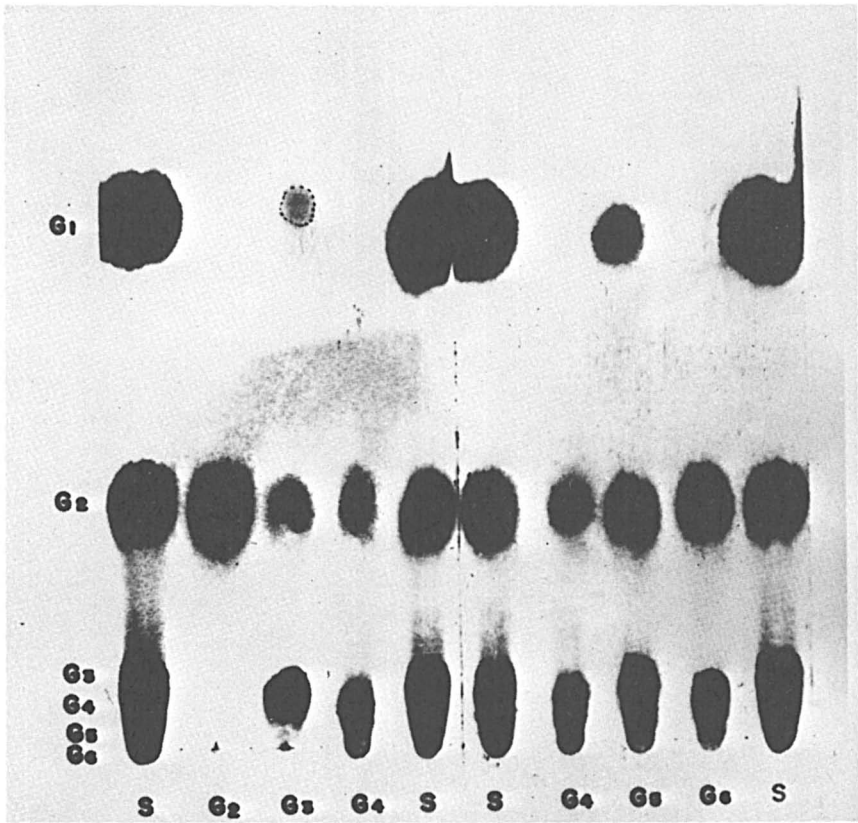

Figure 13. Paper chromatogram of the hydrolysis products from cello-oligosaccharides by E_x-1. Developed by the descending technique for 72 hr at room temperature on Whatman No. 1 paper, using 1-butanol: pyridine:water (6:4:3, v/v) as a solvent; (S) standard, (G_1) glucose, (G_2) cellobiose, (G_3) cellotriose, (G_4) cellotetraose, (G_5) cellopentaose, (G_6) cellohexaose; final enzyme concentration: 3.0×10^{-2}%.

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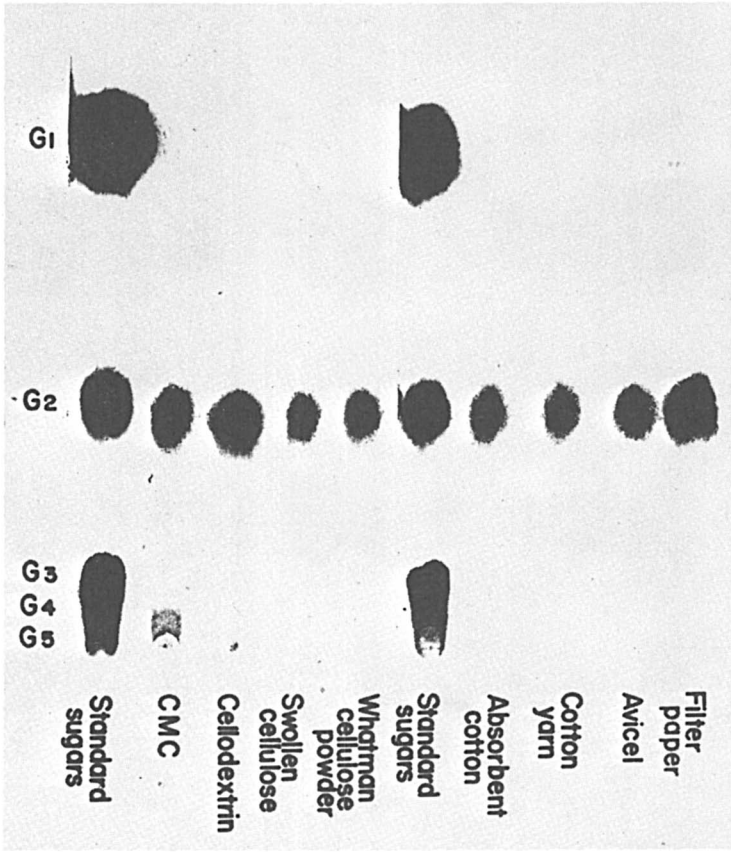

Figure 14. Paper chromatogram of the hydrolysis products from higher cellulose substrates by E_x-1. Developed by the descending technique for 96 hr at room temperature on Whatman No. 1 paper, using 1-butanol: pyridine:water (6:4:3, v/v) as a solvent; (S) standard, (G_1) glucose, (G_2) cellobiose, (G_3) cellotriose, (G_4) cellotetraose, (G_5) cellopentaose, (G_6) cellohexaose; final enzyme concentration: 2.82×10^{-2}%.

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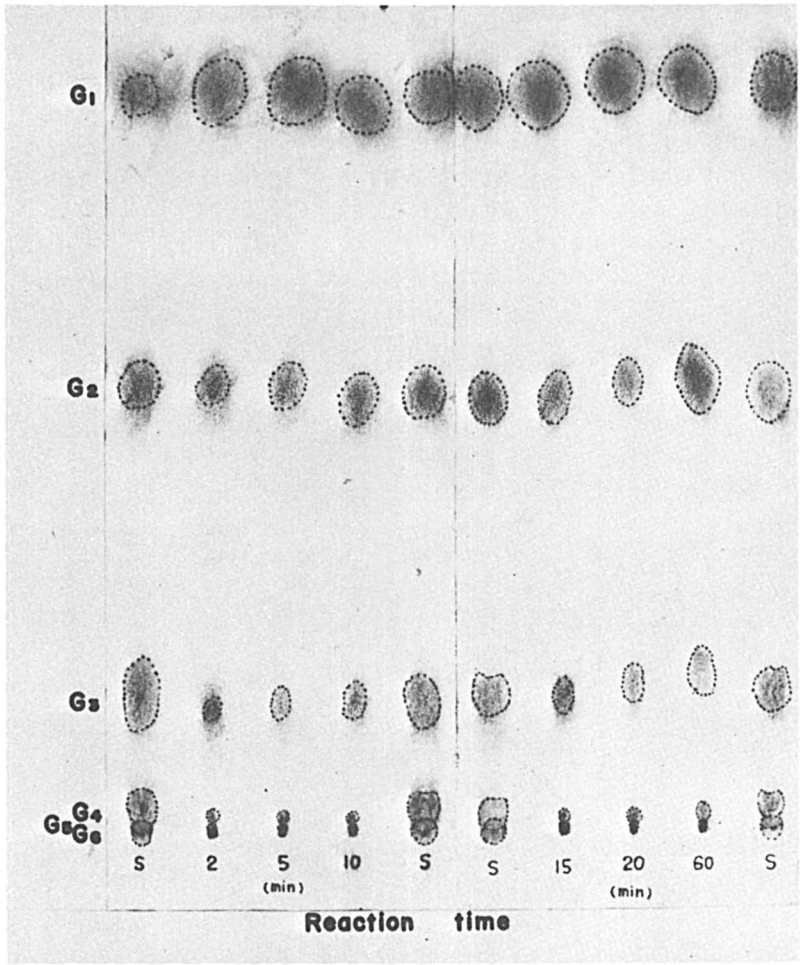

Figure 15. Paper chromatogram of the hydrolysis products from cellohexaose by E_x-1 in the presence of previously added glucose. Developed by the descending technique for 100 hr on Whatman No. 1 paper, using 1-butanol:pyridine:water (6:4:3, v/v) as a solvent; (S) standard, (G_1) glucose, (G_2) cellobiose, (G_3) cellotriose, (G_4) cellotetraose, (G_5) cellopentaose, (G_6) cellohexaose; final enzyme concentration: 6.5×10^{-2}%.

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COMPARISON OF RANDOMNESS OF E_x-1 AND ENDOCELLULASES ON THE HYDROLYSIS OF CMC AND COTTON. That E_x-1 is least random (as compared with S-1 and F-1) on the hydrolysis of CMC and cotton was verified by the observations of the relationships between fluidity of CMC or the decrease in degree of polymerization (DP) of cotton and the simultaneous production of reducing power. These results are shown in Figures 16 and 17. Further, as shown in Figures 18 and 19, the difference in the hydrolysis patterns of both types of cellulase becomes more clear with the comparison between time-course patterns of changes in the viscosity of CMC by both E_x-1 and E_n-1. The latter is a typical endocellulase component as described relative to Figure 12.

SUBSTRATE SPECIFICITY OF E_x-1. It is very noticeable that G_3 and G_4 were attacked only very slowly by E_x-1 as compared with G_5 and G_6, but the hydrolysis of G_5 was much faster than that of G_6. Therefore, the order of the hydrolysis of cellooligosaccharides is $G_5 > G_6 > G_4 > G_3$. Cellobiose was not hydrolyzed by E_x-1 even during a prolonged incubation, but *p*-nitrophenyl β-cellobioside was hydrolyzed at either its holo-

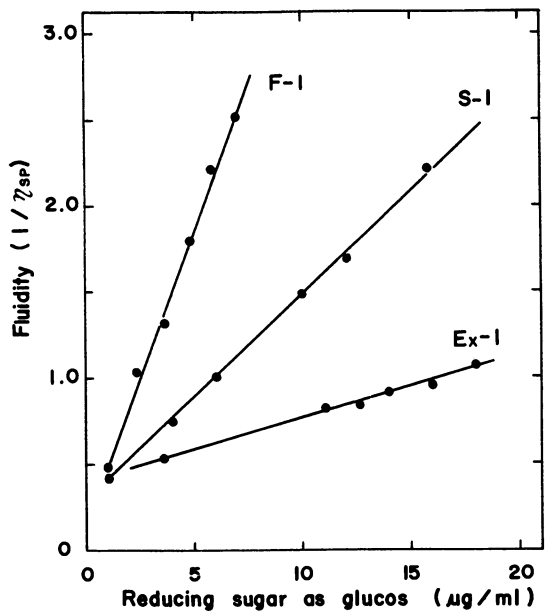

Figure 16. Relationship between increase in fluidity and reducing power during the hydrolysis of CMC by E_x-1, S-1, and F-1. Reaction conditions for viscosity measurement: 3.0 mL 1% CMC, 7.0 mL 0.1M sodium acetate buffer, pH 4.0, and 2.0 mL enzyme solution. The reaction mixture for reducing power measurement was made under standard conditions. Final enzyme concentrations: 3.0 × 10^{-3}%, 8.0 × 10^{-3}%, and 6.3 × 10^{-3}% for E_x-1, S-1, and F-1, respectively.

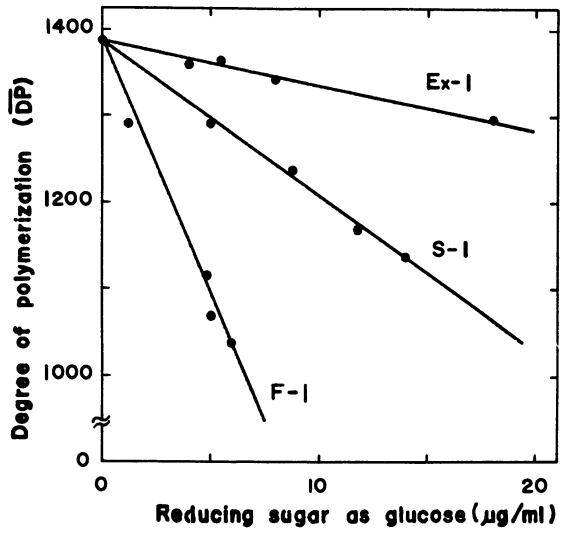

Figure 17. Relationship between the decrease in the average degree of polymerization and the increase in reducing sugar during the degradation of absorbent cotton by E_x-1, S-1, and F-1. Reaction mixture contained 30 mg absorbent cotton and 10 mL 0.1M sodium acetate buffer, pH 4.0, containing enzyme solution. The residual cotton was dissolved in cuprammonium solution and its relative viscosity was measured at 30°C under a nitrogen atmosphere. Final enzyme concentrations: 3.0 × 10^{-2}%, 2.2 × 10^{-2}%, and 3.7 × 10^{-2}% for E_x-1, S-1, and F-1, respectively.

side or aglycon bond. The rate of the hydrolysis of the aglycon bond was, however, slightly larger than that of the holoside bond. The slower attack of E_x-1 toward the cellobioside seemed rather particular as compared with other cellulase components obtained previously from *T. viride* (*7, 11*) and *I. lacteus* (*1, 2, 3*). Thus, K_m values for G_3 and G_4 were difficult to estimate, but those for G_5 and G_6, estimated from Lineweaver–Burk plots, were 0.190m*M* and 0.303m*M*, respectively.

SYNERGISTIC ACTION OF E_x-1 WITH OTHER ENDOCELLULASES ON THE HYDROLYSIS OF CMC AND AVICEL. The synergistic action of E_x-1 with F-1 was investigated in the hydrolysis of both CMC and Avicel. For comparison, we investigated a similar action between S-1 and F-1. The results are shown in Table V.

The synergistic effect caused by a mixture of a typical endocellulase, F-1 (CMCase), and an endocellulase of lower randomness (Avicelase) is slightly smaller than that caused by a mixture of F-1 and E_x-1 (an exocellulase of Avicelase type) in the hydrolysis of both CMC and Avicel. This may be explained by the postulation that this kind of synergistic effect should be caused by the cooperation between cellulase components of extremely different types of hydrolysis. Consequently, the

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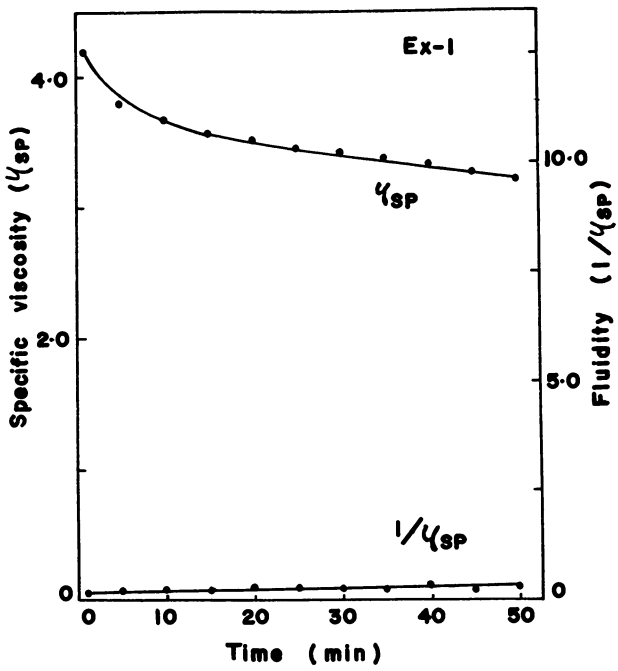

Figure 18. Decrease in specific viscosity during the hydrolysis of CMC solution by E_x-1. Reaction mixture contained 3.0 mL 1% CMC solution, 7.0 mL 0.1M sodium acetate buffer, pH 4.0, and 2.0 mL enzyme solution. The mixture was incubated in an Ostwald viscometer at 30°C; final enzyme concentration: 8.5×10^{-4}%.

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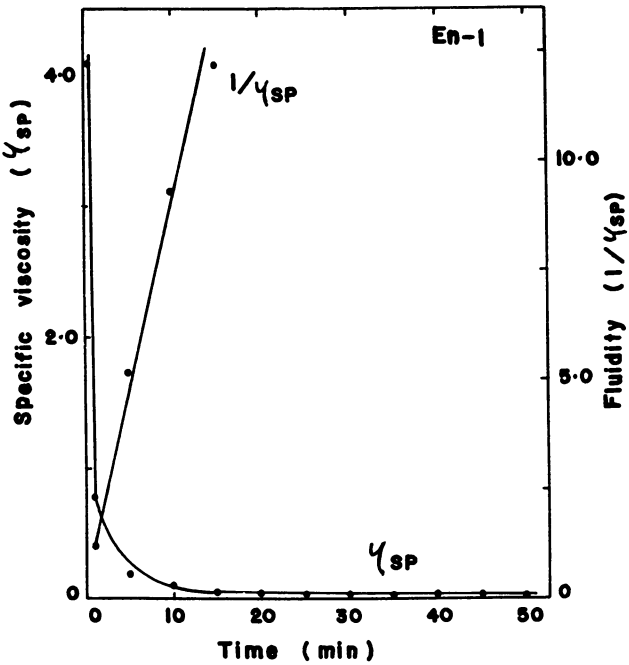

Figure 19. Decrease in specific viscosity during the hydrolysis of CMC solution by E_n-1. Reaction conditions are the same as in Figure 18; final enzyme concentration: 2.2×10^{-4}%.

Table V. Synergistic Action Among E_x-1, S-1, and F-1

Cellulase Component	*Enzyme Concentration (mg/mL of Reaction Mixture)*	*CMC (DS 0.63) (A_{660})*[a]	*Avicel (A_{660})*[a]
E_x-1	0.25	0.038	0.606
S-1	0.20	0.091	0.137
F-1	0.10	0.330	0.082
Sum			
(E_x-1) + (F-1)	0.35	0.368	0.688
(S-1) + (F-1)	0.30	0.421	0.219
Mixture of two cellulases			
(E_x-1 + F-1)	(0.125 + 0.05) (0.175)	0.472	1.713
(S-1 + F-1)	(0.10 + 0.05) (0.15)	0.460	0.381
Ratio			
$\frac{(E_x\text{-}1 + F\text{-}1)}{(E_x\text{-}1) + (F\text{-}1)}$	—	1.3	2.5
$\frac{(S\text{-}1 + F\text{-}1)}{(S\text{-}1) + (F\text{-}1)}$	—	1.1	1.7

[a] Reaction time: 10 min for CMC and 2 hr for Avicel.

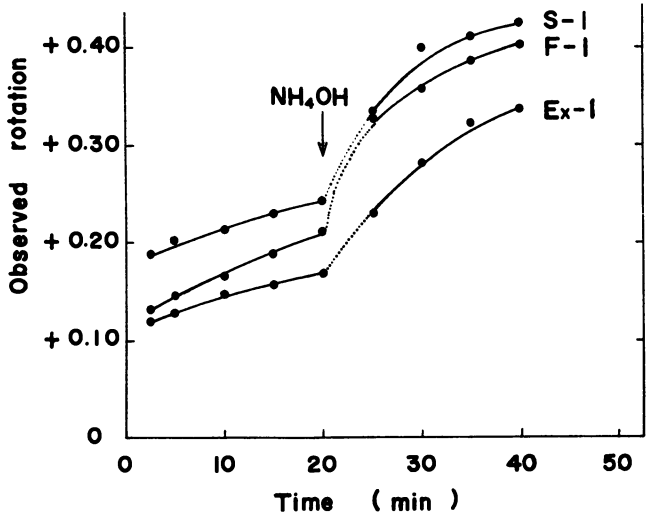

Figure 20. Changes in optical rotation during the hydrolysis of cellopentaitol by E_x-1, S-1, and F-1, and after base-catalyzed mutarotation. A drop of concentrated ammonium hydroxide was added to the digest at the time indicated by an arrow and the optical rotation was followed during 40-min incubation. Final enzyme concentrations: 3.0 × 10^{-2}%, 2.0 × 10^{-3}%, and 1.5 × 10^{-3}% for E_x-1, S-1, and F-1, respectively.

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synergistic action between cellulase mixtures with larger differences of randomness seemed to be higher than that between the components with smaller differences of randomness.

MUTAROTATION OF HYDROLYSIS PRODUCTS BY E_x-1. The mutarotation of hydrolysis products from cellopentaitol by E_x-1 was investigated. For comparison those by S-1 and F-1 were observed. As shown in Figure 20, the mutarotation of hydrolysis products by E_x-1 (exclusively G_2) increases, indicating that products are released in the β-cellobiose configuration. Entirely similar results were observed for the hydrolysis products by S-1 and F-1 (a mixture of G_1, G_2, and reduced G_3 in different proportion for each reaction mixture). Therefore, these cellulase components belong to the same group, as far as the mutarotation pattern is concerned.

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Conclusion

We have obtained at least four cellulase components (including E_x-1) from *I. lacteus* in this work. They were all in a highly purified state and behaved practically as a single protein on ultracentrifugation and/or SDS–disc electrophoresis. Therefore, the difference in randomness of these cellulase components should not be caused by the possible mixtures of endo- and exocellulase components. If this is not the case, the cellulases must be each considered as an independent component having an inherent characteristic property. In fact, S-1 and E_x-1 are from different sources; S-1 originates from the E-2 peak of Figure 1, and E_x-1, is from the E-3 peak of the same figure. Therefore, it does not seem that a trace of F-1 (typical random-type cellulase) still contaminates E_x-1 (typical exo-type cellulase) to form S-1, although this possibility cannot be completely denied. S-1 appears to be an endocellulase, which we have called Avicelase (*2*), and shows lower randomness.

We thus elucidated that three of the four cellulase components are endo- or random-type and the other is exo-type. However, it is difficult to distinguish between the components of least or lowest random-type and those of exo-type. It is rather easy to identify an endo-type cellulase component. In contrast, it is very difficult to determine a cellulase to be exo-type because if the enzyme has a glycosyl-transferring activity the hydrolysis product is not a single sort, which is one of the necessary conditions to be an exo-type. Based on our experiments, measurement of the time course of CMC using a sample of medium substitution degree seems to be the best method of diagnosis to determine a cellulase component to be endo- or exo-type. With some enzymes, direction of mutarotation of reaction products is useful to resolve this problem, as is illustrated by the classic example of the starch hydrolysis by α- and β-amylases. If this is true for our cellulases, the mutarotation of reaction products would be a

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criterion of the hydrolysis type: retention of configuration for endoenzymes, inversion for exoenzymes. To justify this argument, we must make similar investigations on a series of endocellulase components whose randomness of hydrolysis spreads over a wide range, from higher degrees (i.e., acid hydrolysis) to lower ones (i.e., hydrolysis by exoenzymes). However, in a previous study of *T. viride* cellulases we arrived at the conclusion that the mutarotation of hydrolysis products may not be a criterion of the hydrolysis type (*10*). Taking into account this result, the same conclusion must be drawn for the *I. lacteus* cellulase components.

Thus, we can state that the cellulase system of *I. lacteus* consists of various kinds of endocellulases of different randomness and at least one exocellulase of Avicelase type; it is certain that they attack native cellulose synergistically in vivo to produce reducing sugars. The synergistic action must have been performed not only between endocellulases of higher randomness and exocellulases, but also among the endocellulases of different randomness. However, it is not clear at present whether all the cellulase components are formed in vivo under individual genetic control or whether only some of the components are directly genetically controlled and the rest secondarily formed from the gene-controlled components, as we discussed in a previous work (*12*).

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12

The Cellulase System of *Trichoderma*

Relationships Between Purified Extracellular Enzymes from Induced or Cellulose-Grown Cells

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The use of sophorose to stimulate production of the cellulase enzyme system of Trichoderma reesei *QM 9414, has clarified the nature of the individual enzyme components. The multiple forms of cellobiohydrolases, endoglucanases, and β-glucosidase observed among the extracellular enzymes produced by strains of* T. viride *and* T. reesei *have complicated interpretation of the respective catalytic roles of these proteins and their biosynthetic origins. The rapid production of the system in response to 1m*M *sophorose has allowed isolation of the three principal components of the nascent mixture. An endoglucanase and Cellobiohydrolases I (D) and II comprise about 15%, 60% and 25%, respectively, of the cellulase proteins. Cellobiohydrolase I, form D, is distinctly different from the newly described Cellobiohydrolase II. Other proteins, such as β-glucosidase, can account for only about one percent of the total protein. This eliminates the possibility that there is induced with the cellulase system a significant complement of additional enzymes concerned with the degradation of either cellulose or other components of wood.*

Component enzymes of the cellulase system have been purified from several microbial species (*1–13*), among which mutants of the imperfect fungus *Trichoderma* provide the highest levels of extracellular enzyme activity (*14*). From this organism have been purified β-glucosidases (EC 3.2.1.21), endo-1,4-β-D-glucanases (EC 3.2.1.4) and 1,4-β-D-

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glucan cellobiohydrolases (EC 3.2.1.91) (CBH), which together comprise a system capable of rapidly and effectively converting cellulose to glucose. Each of these enzymes is a glycoprotein and catalyzes both hydrolytic and transglycosylation reactions. Although there have been several reported isolations of multiple forms of these enzymes, the structural, functional, and biosynthetic relationships between them remain unclear (*3, 15, 16, 17*). A central goal of cellulase research has been to determine the role of each enzyme (or enzyme form) and to establish the number of components necessary and sufficient for the effective conversion of cellulose to glucose. This report reviews molecular aspects of these enzymes and presents information regarding the catalytic properties and induction of some enzymes elaborated by the strain *Trichoderma reesei* QM 9414.

The sequential and cooperative roles of the enzymes are shown in Figure 1. Endoglucanases, often called cellulases or carboxymethylcellulases, provide nonreducing chain ends from which the exoCBH's cleave cellobiosyl residues. The cellobiose, which is inhibitory to the depolymerizing enzymes, is cleaved to glucose by the β-glucosidase. Since the product of one enzyme may become the substrate for another, enzyme purification is an essential prerequisite to any clear description of the system.

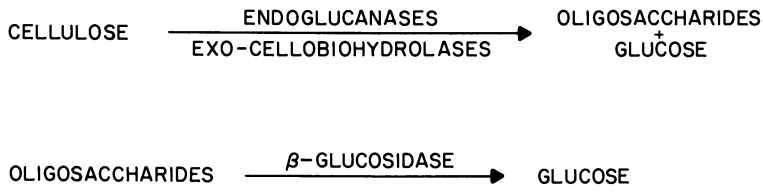

Figure 1. Cooperative action of enzymes of the cellulase system in hydrolytically converting cellulose to glucose

Our overall strategy for purification is shown in Figure 2. When applied to a commercial cellulase preparation, an initial affinity column of microcrystalline cellulose results in separation of the enzymes into two classes. Elution with buffer removes low-affinity components collectively termed the "buffer fraction"; subsequent elution with distilled water removes tightly bound components comprising the "water fraction." The buffer fraction contained a β-glucosidase and two endoglucanases, which were further purified by ion exchange chromatography. Those enzymic components eluted with water included the cellobiohydrolase and two tightly bound endoglucanases, which were purified by ion exchange chromatography and preparative gel electrophoresis.

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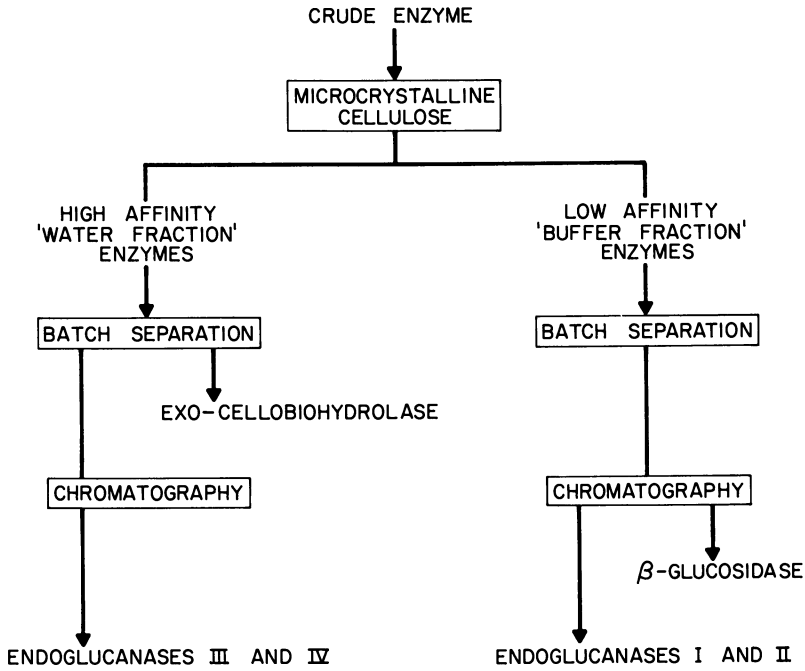

Figure 2. Schematic diagram of the general procedures used in purifying cellulase system components (1, 2, 4)

The categories of substrates which are used for assays of cellulase enzymes are shown in Table I. The use of crystalline, insoluble forms of cellulose as substrates makes assays difficult and has led to such trivial names as "Avicelase" activity. These assays are useful as indications of the capacity of an enzyme system to degrade native cellulose and indicate the presence of CBH enzyme which cannot be assayed in the presence of endoglucanases or β-glucosidase. The susceptibility to enzymatic attack generally increases with the hydration of the polymer chains that accom-

Table I. Cellulase Substrates

Insoluble Polymers	
hydrocellulose	Avicel
α-cellulose	filter paper
swollen cellulose	Walseth cellulose
Soluble Polymers	
derivatized cellulose	carboxymethylcellulose
Oligomers	
cellooligosaccharides	cellopentaose

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panies loss of crystallinity. Seldom are the specific molecular changes caused by enzymic action measured; instead, reducing-sugar production or changes of viscosity or turbidity are reported, leading to nonlinear kinetics. Among the cellulase substrates, only the cellooligosaccharides have specific (i.e., not statistically described) molecular properties. It has been useful to compare the activities on oligomers of enzymes that are involved in depolymerization and saccharification of polymeric substrates. For such studies, oligosaccharides of >99% purity are required to permit evaluation of initial reaction products. A typical separation of soluble cellooligosaccharides is shown in Figure 3. To identify the linkage cleaved in cellooligosaccharides, we employed reduction by sodium borohydride to provide the corresponding alditols. Sugar alcohols subsequently formed by enzymic action will be indicative of origination from the position usually occupied by the reducing end. The entire series of normal and reduced oligosaccharides was separated isocratically by high performance liquid chromatography (HPLC) (*18*).

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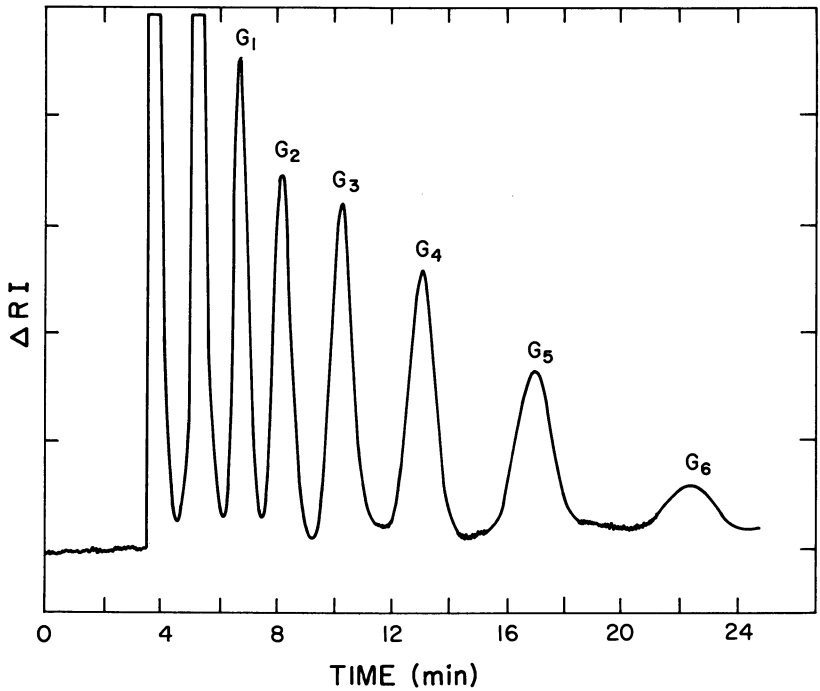

Figure 3. Separation of cellooligosaccharides using the Waters Associates column method (18). The solvent system employed was acetonitrile–water (72:28) at a flow rate of 4.0 mL/min.

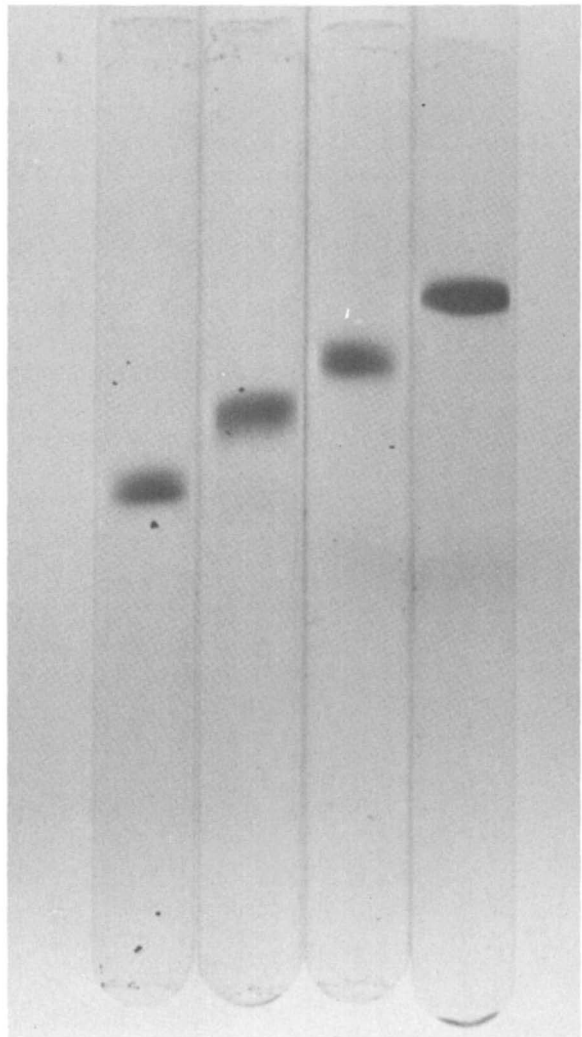

Figure 4. Polyacrylamide-disc gel electrophoretic patterns obtained for forms of CBH I from Trichoderma *(2). The protein samples applied to these gels were, from left to right: 20 μg CBH I (A), 15 μg CBH I (B), 33 μg CBH I (C), and 40 μg CBH I (D). Forms A, B, and C were purified from a commercial* T. viride *cellulase preparation and Form D was purified from a culture of* T. reesei *QM 9123 grown on purified cellulose.*

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1,4-β-D-Glucan Cellobiohydrolases

CBH's are the most abundant components of the *Trichoderma* cellulase system. They are glycoprotein exoglucanases that produce cellobiose. Four forms of CBH previously have been purified (*2*) and characterized. Forms A, B, and C were derived from a commercial *T. viride* cellulase preparation, whereas Form D was produced by *T. reesei* QM 9123 grown in submerged culture. Polyacrylamide disc gel electrophoretic patterns obtained from the four CBH's are shown in Figure 4. These acidic proteins have similar amino acid compositions, molecular weights, ultraviolet absorption spectra, and all cysteine residues linked by disulfide bonds. Some of the properties of these enzymes are summarized in Table II. It was of interest to determine if the small differences in amino acid composition between CBH D from *T. reesei* and the CBH's from *T. viride* were significant. The principal structural differences between the four CBH forms are the respective neutral carbohydrate content and composition (*2*). In each case mannose was found to be the principal neutral sugar component. Analysis of the oligomeric carbohydrate side chains of CBH C, after their removal by β-elimination, revealed that the mannosyl units are linked by $1 \rightarrow 6$ glucosidic bonds, at least some of which have anomeric carbon atoms with α-configuration (*19*). For each CBH C molecule, there are about 17 side chains of one to five monosaccharide units in length, which are linked to the protein by *O*–mannosyl bonds to serine and threonine residues. Immunological studies revealed cross reactivity between all four of these CBH forms and small spurs of "nonidentity" for forms A, B, and D when tested

Table II. Properties of the Forms of

	Cellobiohydrolase I Forms
	A
Molecular weight (by thin-layer gel-filtration chromatography)	53,000 ± 5,000
Neutral carbohydrate content (wt %)	1.4
Glucosamine (wt % as *N*-acetylglucosamine)	0.8
Thermal stability (activity remaining after 30 min at 60°C)	64
Cellobiose produced from swollen cellulose (wt % of soluble products after 30 min incubation at 40°C)	> 90
Specific activity (μmol reducing sugar released from swollen cellulose/min/mg protein)	1.01

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against antiserum to form C. The β-glucosidase and CBH II described in this report do not react with antiserum to CBH C whereas at least one endoglucanase (IV) does. Thus CBH's A, B, C, and D are structurally related forms of CBH I. The activity of the CBH's increases with chain length of oligomeric substrates up to cellotetraose, but remains essentially constant for cellotetraose, cellopentaose and cellohexaose, indicating that this enzyme may have a limited number of subsites. Examination of the frequency of bond cleavage of normal and reduced oligosaccharides by CBH reveals that: (1) there is a lack of absolute specificity for terminal cellobiosyl groups, and (2) there is little tendency to cleave bonds linking the sorbityl residue at the former reducing end of the substrate (*20*).

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Endo-1,4-β-D-glucanases

Of the four glycoprotein endoglucanases purified from *T. viride*, three were obtained in quantities large enough to provide some structural information (*3,4*). Endoglucanases I and II have a low affinity for cellulose, whereas Endoglucanases III and IV bind tightly to a column of microcrystalline cellulose. These enzymes are distinguished from the other types of enzymes in the cellulase system by their ability to reduce the viscosity of CM-cellulose solutions. A summary of some of the properties of the endoglucanases is presented in Table III. Endoglucanase II has the highest specific activity (expressed in terms of change in specific fluidity/min/mg protein). Disc gel electrophoresis of the endoglucanases yields a broad but distinctly different band for each enzyme. Each has a similar pH optimum and is labile above pH 8.0. Endogluca-

Cellobiohydrolase I of *Trichoderma* (*2*)

	Cellobiohydrolase I Forms	
B	*C*	*D*
53,000 ± 5,000	53,000 ± 5,000	53,000 ± 5,000
5.8	10.4	6.7
0.8	0.5	0.5
68	65	65
> 90	> 90	> 90
0.67	0.56	0.53

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Table III. Endoglucanases of *Trichoderma viride* (3, 4)

	Endoglucanase		
	II	*III*	*IV*
Molecular weight	37,000	52,000	49,500
Specific activity (Δ specific fluidity/min/mg protein) substrate: CM-cellulose	1010	60	250
Specific activity (μmol reducing sugar/min/mg protein) substrate:			
CM-cellulose	28	28	9
phosphoric-acid swollen cellulose	9.6	9.9	7.4
Avicel	0.1	0.1	0.1
pH optimum	4.5	4.5	4.5
Stability	labile at pH > 8.0	labile at pH > 8.0	labile at pH > 8.0
Neutral carbohydrate content (wt %)	4.5	15.0	15.2

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nase II has a distinctly lower molecular weight as determined by sedimentation equilibrium ultracentrifugation. The amino acid compositions are similar, particularly those of Endoglucanases II and IV. Since the former exceeds the latter only with respect to apparent leucine and and methionine content there is a real possibility of a precursor/product (IV/II) relationship. The high affinity forms, Endoglucanases III and IV, have the highest neutral-carbohydrate content, about 14–15% by weight as determined by the phenol–sulfuric acid method of Dubois et al. (*21*) or by gas chromatography of derivatives of the neutral sugars (*22*).

A useful comparison of the modes of action among endoglucanases is the plot of decrease in specific fluidity vs. concomitant production of reducing sugar (*3*). Endoglucanases II and IV, like liquefying amylases, effect a large change in specific fluidity relative to Endoglucanase III, which is analogous to "saccharifying" amylases. CBH C, by comparison, has essentially no endoglucanase activity and must be assayed at a concentration ten-fold higher than that of the endoglucanases in order to cause a measurable increase in fluidity.

The endoglucanases exhibit higher maximum velocities on longer cellooligosaccharides, as expected for "endo" action. These enzymes have little or no xylanase or amylase activity but display the highest specific activity (μmol reducing sugar released/min/mg protein) when acting on CM-cellulose. Activity on swollen cellulose is much greater than that on Avicel. The transglycosylation activity of Endoglucanase IV is demon-

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strated by the formation from cellopentaose of hexa-, hepta-, and octasaccharides in addition to the hydrolytic products cellobiose and cellotriose. Since both the shorter and longer oligosaccharide products are substrates for the endoglucanases, the pattern of products from the cleavage of longer oligomers (e.g. cellohexaose) changes with time as the sugars produced initially are hydrolyzed further (*3*). Thus the array of products observed depends on the extent of the reaction. The initial reaction rate may be measured as the decrease in substrate concentration per unit time. To elucidate the specificity of bond cleavage, one must note the array of products formed after only a few percent of substrate has reacted. Endoglucanase III, unlike Endoglucanases II and IV, cleaves or transfers cellobiosyl units from the nonreducing ends of substrates. The use of reduced oligosaccharides to permit identification of the bond cleaved was illustrated by the action of Endoglucanase IV on cellotetraitol and cellopentaitol (*3*).

In order to demonstrate directly the transfer of cellobiosyl units by Endoglucanase III and to confirm the origin of these units as the nonreducing ends of oligomers, cellotetraitol and cellopentaitol were again employed as substrates (*3*). The latter yielded cellotriitol whereas from the former was produced cellobiitol together with cellotriose and cellotriitol without corresponding quantities of cellobiose, glucose, or glucitol. Rechromatography of the cellotetraitol mixture revealed a hexaitol peak which could serve as an intermediate (formed by cellobiosyl transfer) by cleavage to equal amounts of G_3 and G_3H. This would account for the production of these trisaccharides without concomitant production of glucose or glucitol.

To determine the roles of the endoglucanases in the degradation of insoluble cellulose, we incubated each of them with filter paper discs for two days (*3*). As seen in Figure 5, only Endoglucanases III and IV reduced the discs to short fibers. This demonstrates the importance of the high-affinity enzymes that, although less active in degrading CM-cellulose, are effective in degrading pure cellulose.

β-Glucosidase

The β-glucosidase is a glycoprotein which cleaves β-linked glucose oligomers as well as aryl-β-D-glucosides (*1, 20*) and transfers glucosyl units. Since the majority of the glucosidase and cellobiase activities from a commercial *T. viride* cellulase preparation copurified, it was concluded that one enzyme is responsible for these two types of activity. Two minor components having aryl glucosidase activity are removed during the purification process. The amino acid composition of the β-glucosidase reveals that it is less acidic and more hydrophobic than

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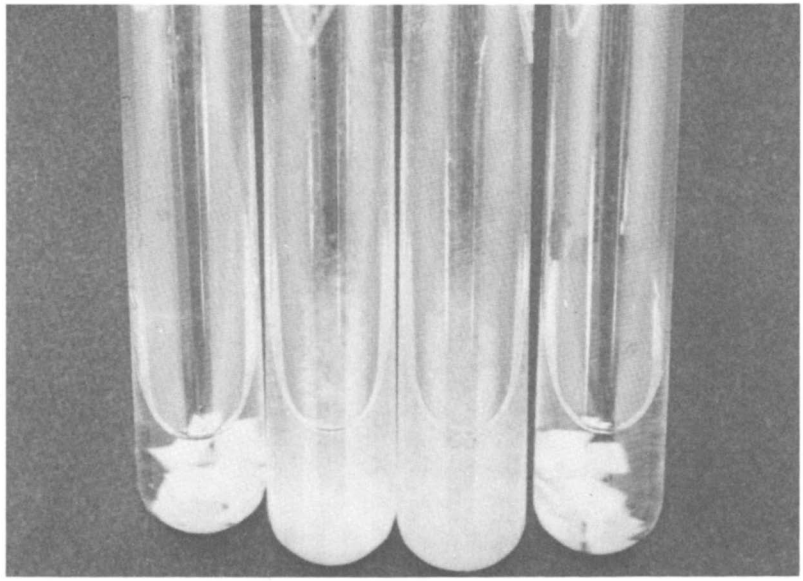

Figure 5. Reaction mixtures demonstrating short-fiber-forming activities of endo-1,4-β-D-glucanases. The reaction mixtures are shown after two weeks incubation at 40°C with 20 mg filter paper (3). Represented from left to right are a control without enzyme, Endoglucanase IV (235 μg/mL), Endoglucanase III (87 μg/mL), and Endoglucanase II (75 μg/mL).

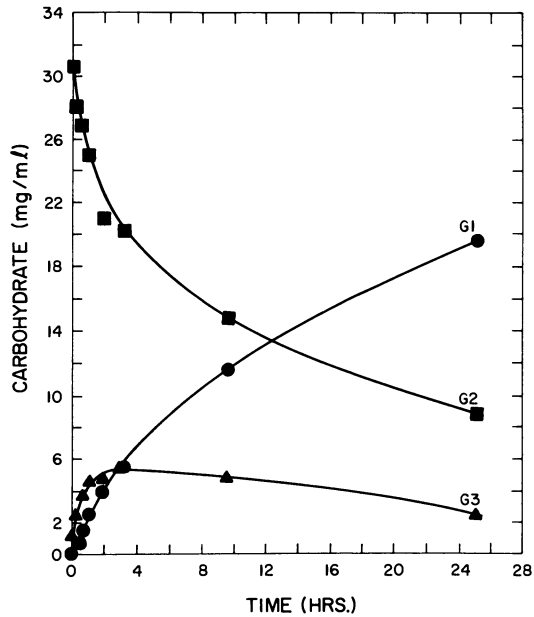

Figure 6. Transglycosylation activity of β-glucosidase. The reaction mixture initially contained 3% cellobiose, 0.0009% β-glucosidase, and 0.05M sodium acetate buffer, pH 5.0. Incubation was carried out at 45°C in a reciprocating shaker. Each sample was boiled for five minutes and analyzed by HPLC (G_1 = glucose, G_2 = cellobiose, G_3 = trisaccharides.)

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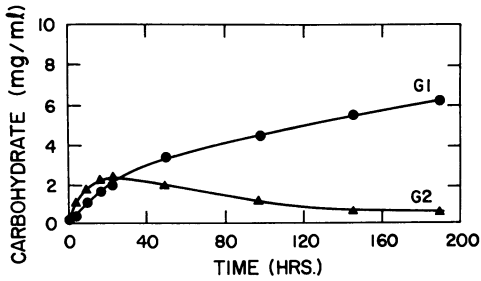

Figure 7. Production of glucose and cellobiose from Avicel. The reaction mixture contained 1% Avicel PH-101, 1% Trichoderma reesei *QM 9123 cellulase protein produced in a pH 3.6 culture medium, and 0.05M sodium acetate buffer, pH 5.0. Incubation conditions and analysis are the same as Figure 6.*

the exo- and endoglucanases. It reacts rapidly with each of the cello-oligosaccharides, reacting most rapidly with cellotriose. It is inhibited by gluconolactone ($K_i = 3.2 \times 10^{-5}M$) and by glucose ($K_i = 1 \times 10^{-3}M$). Substrate inhibition of glucose production is observed at high concentrations of cellobiose or cellotriose due to the transfer of glucosyl units from one substrate molecule to another forming tri- or tetrasaccharide, respectively. One may observe these transfer products with HPLC (Figure 6). The importance of the β-glucosidase for the production of glucose during the action of the cellulase system on cellulose was demonstrated by use of a cellulase enzyme system that had been produced at low pH and that contained little active β-glucosidase. Cellobiose is the predominant product for the first 24–30 hours of incubation (Figure 7). If sufficient pure β-glucosidase is added to restore the normal level of the enzyme to the system, little cellobiose is formed and hydrolysis occurs rapidly and proceeds to completion (Figure 8).

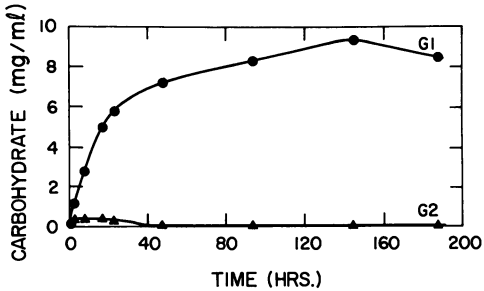

Figure 8. Glucose production after addition of β-glucosidase. Reaction conditions are identical to those for Figure 7, except that 0.001% β-glucosidase was added.

Culture Conditions and the Enzyme System

The problems caused by the substrate cellulose in the studies of these enzymes are manifold. Most of the glucosidic linkages are inaccessible in the interior of the crystalline matrix. Much of the enzyme is bound to the cellulose, making its concentration in the reaction suspension non-uniform. Of the glucosidic bonds joining glucosyl units at the surface of the crystalline cellulose substrate, only half are exposed in equivalent orientations at any given time. Interchain hydrogen bonding interferes with release of oligosaccharide products, which thus may undergo further reaction before being released as soluble products. All of these factors may cause the observed reaction rates and product patterns of the enzymes toward insoluble substrates to differ from that observed with hydrated chains or soluble substrates such as cellooligosaccharides.

It is a common observation (*23, 24*) that the enzymes are not fully recovered from cultures due to binding to residual cellulose in the medium. Some workers (*25*) have attempted to avoid this problem by prolonging the culture period until all of the cellulose has been depleted. This is not only time-consuming but permits a lengthy exposure of these enzymes to the cells, substrates, and other enzymes of the culture incurring the risk of partial degradation of the native enzymes.

In our attempt to identify the normal complement of enzymes of the cellulase system without degradation or loss due to binding, it occurred to us that there was a method at hand for the rapid production of the cellulase system without any exposure of the organism to cellulose or a cellulose derivative. It had been reported previously (*26*) that sophorose (o-β-D-glucopyranosyl-(1 → 2)o-α-D-glucopyranose) is a very potent inducer of cellulase activity in *Trichoderma viride* QM6a. (This organism and strains derived from it are now referred to as *T. reesei.*) Later, an unidentified strain of *Trichoderma* was shown to produce carboxymethyl cellulase activity (determined viscosimetrically) and aryl-β-glucosidase activity in response to sophorose (*27*). Recently, Eriksson's laboratory has reported that although sophorose stimulates the production of endoglucanase activity by *T. viride* QM6a, no free enzyme was found in the incubation medium (*28*).

Methods

Since we had been able to purify the enzymes of the cellulase system of *T. viride,* the purification and identification of the *T. reesei* system was attempted. It was of interest to compare the enzymes produced during growth on cellulose with those produced in response to sophorose. The

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induction technique employed was that of Nisizawa (*27*) and the isolation and purification steps were similar to those reported for enzymes of the *T. viride* cellulase system (*1, 2, 4*).

The organism *Trichoderma reesei* strain QM 9414 (ATCC 26921) was grown from a spore inoculum on a standard medium (*29*) using glucose as the carbon source. The cells were transferred after 48 hr to fresh medium and after a further 24-hr incubation, the mycelia were harvested. The cells were washed twice in 17m*M* potassium phosphate buffer, pH 6.0, incubated in buffer (*27*) with a variety of compounds, and the resulting suspension medium tested for enzyme activity. Three activities, which generally are considered to be part of any complete cellulase system, were measured at intervals over a 24-hr period: (a) β-D-glucosidase activity, measured by the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside, (b) endo-1,4-β-D-glucanase activity (measured viscosimetrically) and (c) Avicelase activity, measured by the production of reducing sugar from Avicel, a microcrystalline cellulose. It should be emphasized that the latter assay does not measure the activity of any individual enzyme, but reflects the activity of the entire cellulase system, of which the principal components are exoCBH's.

Protein concentrations were determined according to the method of Lowry et al. (*30*). Electrophoresis of proteins in polyacrylamide gels was carried out at 4°C, using the discontinuous buffer system No. 1 described by Maurer (*31*) and modified by Emert et al. (*1*). Protein was stained with 0.1% Coomassie Brilliant Blue R250 in a water–acetic acid–methanol (45:10:45) solution. Carbohydrates were stained with the periodic acid–Schiff (PAS) reagent using the method described by Lang (*32*).

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Results

The stimulation of the synthesis of the cellulase system of *T. reesei* QM 9414 by sophorose was established as shown by the results of experiments summarized in Table IV. Other than sophorose, of the glycosides and oligosaccharides tested, only lactose caused even a limited production of the enzymes of the cellulase system. Lactose is not as closely related structurally to sophorose as is, for example, the disaccharide laminaribiose; it is more closely related structurally to cellobiose, which, despite being the major product of cellulose breakdown, does not promote enzyme production under the conditions of this experiment. It was noted that both intra- and extracellular constitutive enzyme levels produced by cells growing on glucose (or by resting cells without inducer, Table IV) are less than 0.5% of the fully induced levels and thus are negligible.

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Table IV. Effect of Several Carbohydrates on the Production of the Enzymes of the Cellulase System by *T. reesei*

Additions[a]	*Activity*[b]			
	Aryl-β-D-glucosidase	*Endo-1,4-β-D-glucanase*	*Avicelase*	*Protein*
	milliunits/mL supernate			*mg/mL supernate*
None	0.8	10	0.4	0.12
Kojibiose	0.8	8	0.4	0.09
Nigerose	1.4	10	0.6	0.12
Maltose	0.5	5	0.5	0.11
Isomaltose	0.5	8	0.6	0.13
Lactose	5.0	2500	9.0	0.25
Sophorose	200	9000	200	0.50
Sophoritol	0.8	10	0.4	0.11
Laminaribiose	0.8	10	0.4	0.14
Cellobiose	0.9	10	0.5	0.12
IPTG[c]	0.8	9	0.5	0.12
Gentiobiose	0.6	10	0.4	0.10
Laminaritriose	0.6	9	0.4	0.22
Gentiotriose	0.6	8	0.5	0.35
Maltotriose	0.7	9	0.4	0.10

[a] Each carbohydrate was supplied at an initial concentration of 1m*M* to a suspension of 2–3 mg/mL mycelium.
[b] Observed after 24-hr incubation. All values are averages of replicates with standard error $\leq \pm 5\%$.
[c] Isopropyl thio-β-D-glucopyranoside.

A very significant increase in enzyme levels in the extracellular medium was also observed on incubation of *T. reesei* mycelia with transglycosylation products formed when *T. reesei* β-glucosidase was allowed to react with cellobiose, at a concentration which favored transfer rather than hydrolysis. The transglycosylation properties of components of the *T. reesei* cellulase system have been demonstrated by other investigators (*33*). These transglycosylation products, trisaccharides containing $\beta(1 \rightarrow 6)$ together with some $\beta(1 \rightarrow 4)$ and a low concentration of $\beta(1 \rightarrow 2)$ glycosidic linkages (*20*), upon incubation with *T. reesei* mycelia gave 250 μg/mL protein and 6000 milliunits/mL endoglucanase activity when used at a concentration of 0.46m*M*. Control values and enzyme levels in the presence of sophorose were the same as those shown in Table IV.

The fact that transglycosylation products of one of the enzymic components of the cellulase system of *T. reesei* can stimulate the production of the system may be of great importance in elucidating the mechanisms of the processes involved in the biosynthesis of these enzymes. Sophorose may be an early transglycosylation product, whose

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formation, by the action of basal levels of enzymes on cellulose, may be a signal for the cells to synthesize more enzyme. Similarly, glucanase or glycosidase induction by disaccharides containing glycosidic linkages other than that of the substrates have been observed for *Streptomyces* $\beta(1 \rightarrow 3)$ glucanase induction by gentiobiose (*34, 35*) and *E. coli* β-galactosidase induction by allolactose (*36*), a transglycosylation product of this enzyme (*37*). The results shown in Table IV, in general, do not point to any structural requirements for an inducer. But since synthesis of the enzyme system is promoted significantly by transglycosylation products formed by one of the components, it is possible that such an inducer may actually exist. Finally, it is reasonable to suppose that the biosynthesis of the *T. reesei* cellulase system is a function of both specific stimulation by an inducer molecule and metabolic regulation of the growth rate of the organism.

The presence in the control incubation mixtures (Table IV) of extracellular protein which was not active cellulase protein led to the consideration that, under those incubation conditions, constitutive enzymes might still be synthesized, or other enzymic activities could be derepressed. Amylase has been reported (*38*) to be a constitutive enzyme in *T. viride* which becomes derepressed when a rapidly metabolizable carbon source is not available. However, further stimulation of amylase synthesis has been shown to occur in the presence of α-linked disaccharides of glucose, especially maltose in the case of *T. reesei* (*38*) and other amylase-producing fungi (*39*). The results which appear in Table V confirm previous studies. Significant amylase activity is present when *T. reesei* mycelia are incubated in phosphate buffer, in the absence of any other compounds. Glucose has been shown to repress amylase production under these conditions. It is also seen (Table V), in agreement with previous studies, that the presence of maltose in the medium resulted in a two-fold stimulation of extracellular amylase activity. Since there is not a corresponding increase in the amount of extracellular protein, it is possible that a very active amylase is produced in very small quantities, or that stimulation of amylase activity by maltose in this case occurs at the expense of other constitutive enzymes. It was also observed during this experiment that carbohydrates structurally related to maltose, such as kojibiose, nigerose, and isomaltose, cause a stimulation of production of *T. reesei* amylase which is comparable to that due to maltose. It appears from the results shown in Table V that the regulation of amylase production by *T. reesei* must differ from the regulation of the production of the enzymes of the cellulase system.

The two compounds which stimulate cellulase production, sophorose and lactose, obviously do not promote amylase production. This is especially evident in the case of sophorose, since the reduction in amylase

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Table V. Effect of Oligosaccharides on Amylase Production by *T. reesei*

Additions[a]	*Amylase*		*Protein*
	milliunits/mL supernate	*units/mg protein*	*mg/mL supernate*
None	146	1.0	0.14
Sophorose	90	0.2	0.48
Lactose	112	0.6	0.20
Kojibose	180	1.9	0.10
Nigerose	247	2.0	0.13
Maltose	260	2.1	0.13
Isomaltose	200	1.7	0.12
Maltotriose	112	1.2	0.10
Laminaritriose	167	1.1	0.15
Gentiotriose	173	0.9	0.20

[a] Experimental conditions are the same as those described for Table IV.

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specific activity is not fully accounted for by the increase in extracellular protein due to cellulase production. It is not unreasonable to conclude that a slight repression of amylase production has occurred under conditions favoring the production of cellulase.

An interesting feature of the experiment described in Table IV is the apparent delay in the increase of aryl-β-glucosidase activity as compared to the glucanase activities. A very small amount of β-glucosidase activity could be detected at six hours of incubation of the fungus with sophorose, whereas at four hours it was already possible to detect endoglucanase and Avicelase activity in the medium. This delay in the appearance of extracellular β-glucosidase activity appears more clearly in a plot of maximum specific enzyme activities vs. incubation time (Figure 9). Whereas, at early times, endoglucanase and Avicelase activity seem to appear at an almost equal rate, as determined by the rate of increase of their respective specific activities in the medium, the rate of synthesis of β-glucosidase appears to be slower. In Figure 9 it can be seen that, whereas Avicelase and endoglucanase reach 50% of their maximum specific activity at six and seven hours, respectively, β-glucosidase activity does not reach half of its maximum level until after 10 hr of incubation, i.e., it appears that the β-glucosidase not only appears later, but is synthesized or released at a slower rate during the early hours of incubation than are the enzymes responsible for the other two activities.

When the organism is grown on cellulose, such a delay in the appearance of β-glucosidase in the medium could be explained in terms of the lack of availability of a substrate for the enzyme at early times: β-glucosi-

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dase need not be synthesized or released, until cellobiose appears in the extracellular medium as a product of the degradation of cellulose by the other enzymes of the system. Thus, cellobiose may serve as a "messenger" for the need to synthesize or release more β-glucosidase. Since β-glucosidase is considered responsible for the production of glucose from cellobiose, it might be possible to determine indirectly if its rate of synthesis at early incubation times during the experiments described is actually lower than that of the other enzymes by studying the products of cellulose degradation by enzyme preparations from several early points of the incubation period. A decreasing proportion of cellobiose (and a correspondingly increasing amount of glucose) in the products of cellulolysis by enzymes produced at later incubation times could be interpreted as signifying that, at early times, the β-glucosidase is not released rapidly enough into the medium to cleave cellobiose produced by the other enzymes (e.g., CBH's). At later times, as the rate of appearance of β-glucosidase activity approaches and eventually becomes equal to that of the other enzymes, the ratio of cellobiose to glucose among the products of cellulose hydrolysis would decrease, until, with enzyme preparations from longer incubation times, it would become virtually zero, leaving glucose as the sole product of hydrolysis. The results of such an experiment are shown in Figure 10. During this experiment, enzymes produced

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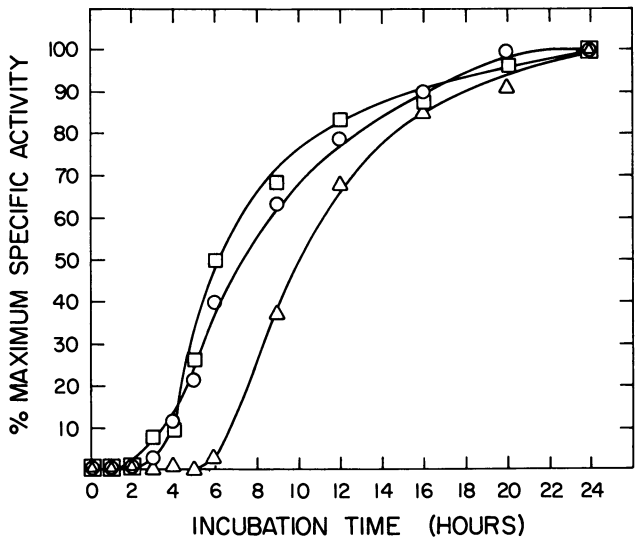

Figure 9. Enzyme production by T. reesei *QM 9414 incubated in the presence of 1mM sophorose. The incubation medium (27) included 17mM potassium phosphate buffer, pH 6.0, at 28°C. The appearance of aryl-β-*D*-glucosidase activity (△) in the extracellular medium is delayed in comparison to endoglucanase activity (○) and Avicelase activity (□).*

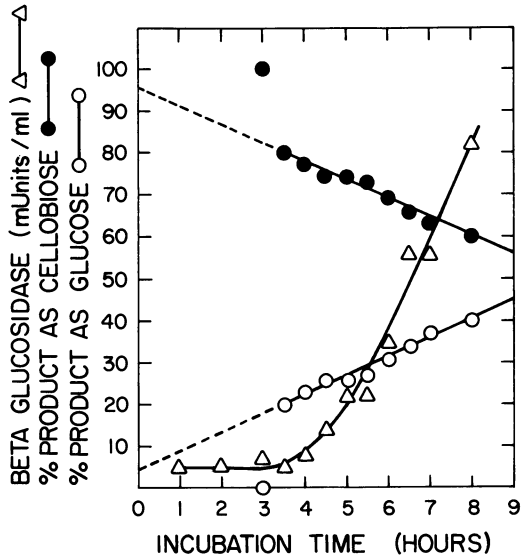

Figure 10. Product distribution from the incubation of enzymes (produced by T. reesei *QM 9414 in response to sophorose) with phosphoric-acid swollen cellulose (PSC). "Incubation time" in this figure indicates the length of incubation of* T. reesei *mycelia in the presence of 1mM sophorose. Enzymes produced at those times were incubated with PSC for 15 hr, and the products analyzed by HPLC (18).*

when mycelia were exposed to sophorose for the indicated lengths of time were incubated with phosphoric-acid-swollen cellulose (PSC), and the products of PSC digestion were analyzed by HPLC. The percent of cellobiose in the products from PSC decreases in a linear fashion and the percent of glucose increases correspondingly. When the substrate is incubated with the enzyme system present at eight hours, the ratio of cellobiose to glucose in the products is approximately 60:40. In this enzyme system, produced during eight hours of incubation, the β-glucosidase activity in the medium totals 82 milliunits/mL supernate, which is approximately one-third of the level of β-glucosidase activity that can be found in the extracellular medium at 24 hr, during an experiment of this type. When a 3-hr enzyme preparation was incubated with PSC under the same conditions, the product, as determined by HPLC, was 100% cellobiose (Figure 10). It is likely that some glucose was also present, but at a level below the detection limits of the technique under the conditions of this experiment.

Loewenberg and Chapman (*40*), working with a different strain of the same organism, found that there was a 6-hr lag before any CM-cellulase (measured viscosimetrically) could be detected in the extracellular medium, whereas Nisizawa et al. (*27*) reported a lag period of

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only two hours. It is important to note that during the present investigation, when cells suspended in 17m*M* potassium phosphate buffer, pH 6.0, were disrupted prior to exposure to sophorose, control levels of enzyme activity were observed in the homogenate. This result indicates that sophorose does not cause release of preexisting, active, cell-bound enzyme, but rather results in de novo synthesis of enzyme or activation of an inactive precursor.

Identification of Enzymes of the Cellulase System

The significance of the enzymes produced by a resting cell suspension in the presence of sophorose depends largely on their relationship to the enzymes produced when the organism is grown on cellulose. It was demonstrated (Figure 11) that the extracellular proteins that are pro-

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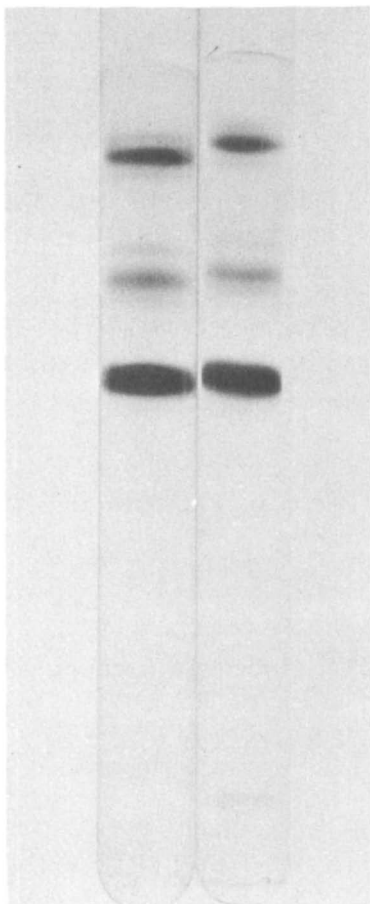

Figure 11. Polyacrylamide disc gel electrophoretic patterns of extracellular proteins produced by T. reesei *QM 9414. The sample applied to the gel on the left was 130 μg extracellular protein from* T. reesei *mycelia grown on 1% Avicel (29), that applied to the gel on the right was 120 μg extracellular protein produced from sophorose-incubated mycelia. The bands shown here were stained for protein with Coomassie Blue and could, in all cases, also be stained for carbohydrate with the periodic acid–Schiff reagent.*

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duced when *T. reesei* is incubated in the presence of sophorose exhibit an electrophoretic pattern identical to that of the proteins found in the extracellular medium of 48-hr cultures grown on cellulose. The principal components of the cellulase system synthesized in submerged culture by the strain of *T. reesei* that has been used throughout this study have been isolated in pure form (*41*). As expected, these components are similar to components previously isolated and characterized from commercial cellulase preparations derived from other strains of *Trichoderma* and apparently identical to enzymes produced in response to sophorose. In order to understand the nature of the biosynthetic processes leading to the formation of the cellulase enzymes, each of the biosynthetic products must be identified.

The cellulase components that are synthesized in the presence of sophorose were investigated by the basic procedures previously described (*1, 2, 4*) for the isolation of cellulolytic components from commercial cellulase preparations. The purification to homogeneity of the proteins that yield the three predominant bands when the crude preparation is subjected to disc gel electrophoresis was accomplished by ion exchange chromatography.

The respective proteins of the lower, middle, and upper bands have been identified by their substrate specificity and product array as CBH I (D), an endoglucanase (EG), and CBH II (*42*). The latter enzyme, to our knowledge, has not previously been described. The CBH I, CBH II, and EG are present in proportion (by weight) of 60 ± 5: 25 ± 5:15 ± 2, respectively, in the cellulase system produced either in response to sophorose or during growth on cellulose. Although we have not associated an electrophoretic band with the β-glucosidase, it is clear that a small amount of it is present. From the specific activity of the purified *T. viride* β-glucosidase (*1*) we can calculate that only about 0.5% of the protein of the cellulase system should be ascribed to β-glucosidase.

The CBH I (D) is identical in composition and activity to the CBH I (D) previously described (*2*) from *T. reesei* QM 9123. The close correspondence of their amino acid contents (Table VI), the nearly identical content of neutral carbohydrate: 6.8% by weight for the CBH I (D) produced in the presence of sophorose and 6.7% for *T. reesei* QM 9123 CBH I (D) grown on cellulose (*2*), and identical electrophoretic properties clearly argue for a common molecular structure for these CBH's I (D). The CBH II is clearly different from all other CBH's in electrophoretic mobility (Figure 12) and amino acid composition (*41*), but is devoid of endoglucanase activity and produces predominantly cellobiose (>90% by weight of soluble products) from cellulose. It has a sedimentation coefficient of 3.71 in comparison to CBH I (D), for which a value of 3.66 was obtained.

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Table VI. Amino Acid Composition of CBH I (D)

Amino Acid	T. reesei *QM 9414* *(mol %)*	T. reesei *QM 9123*[a] *(mol %)*
Lysine	2.7	2.7
Histidine	1.1	1.1
Arginine	1.7	2.1
Aspartic acid	11.8	11.2
Threonine	11.2[b]	11.4
Serine	11.0[b]	11.0
Glutamic acid	9.1	9.1
Proline	6.2	6.6
Glycine	13.0	13.5
Alanine	6.5	6.2
Half cystine	4.0[c]	3.8[c]
Valine	5.2[d]	4.8
Methionine	1.4[c]	1.8[c]
Isoleucine	2.4	2.3
Leucine	5.4	5.3
Tyrosine	4.0	4.8
Phenylalanine	3.4	3.1

[a] Composition of 24-hr hydrolysate (*2*).
[b] Values extrapolated to zero-time hydrolysis to correct for destruction of serine and threonine.
[c] Determined after performic acid oxidation.
[d] Based on 72-hr hydrolysate.

The endoglucanase differs from other enzymes of this class purified from *T. viride*. Although further investigation will be necessary to characterize this enzyme, it is clearly not identical to the low-molecular-weight endoglucanase described by Håkansson et al. (*43*). Since each of the enzymes from the *T. reesei* cellulase system that we have investigated are glycoproteins, perhaps the enzyme described by Håkansson et al. is a degradation product that is formed from some precursor during the culture period. The failure of Eriksson and Hamp (*28*) to observe cell-free endoglucanases from *Trichoderma* exposed to sophorose is at variance with our results. Perhaps endoglucanase release by *T. reesei* differentiates it from the strain of *T. viride* employed by these workers.

Since essentially all of the protein in the cellulose-grown culture or the sophorose-incubation mixture can be accounted for by the three principal proteins and the β-glucosidase, it seems reasonable to conclude that the multiplicity of enzymes seen after several days of culture growth are caused by degradation. This may be attributable either to inherent instability (*3*) or to the presence of hydrolytic enzymes (*16*). There seems to be no evidence for the "C_1" enzyme as a "nonhydrolytic" or "affinity" factor, since all known enzymes of the system are hydrolytic. That the sophorose-stimulated system (with no possible loss of the "C_1"

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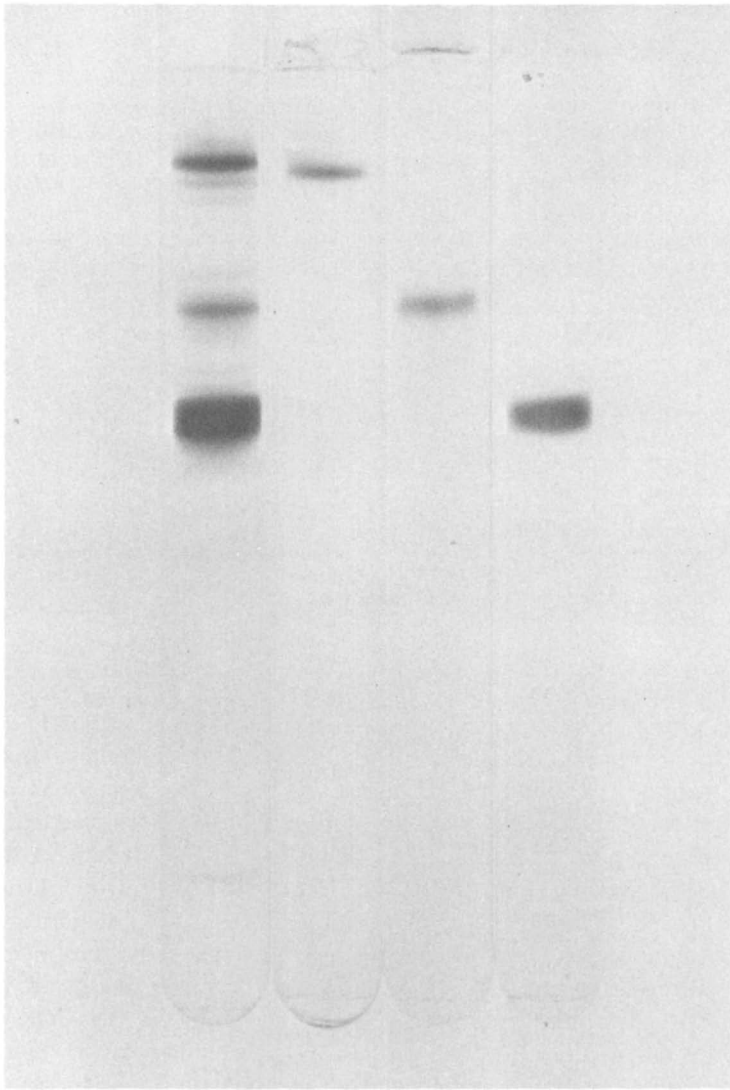

Figure 12. Polyacrylamide disc gel electrophoretic patterns of enzymes purified from the extracellular protein produced by T. reesei *QM 9414 in response to 1mM sophorose. To the gels, from left to right, were applied 175 μg extracellular protein mixture, 45 μg CBH II, 45 μg endoglucanase and 80 μg CBH I (D).*

by binding to cellulose) evidences only hydrolytic enzymes seems a particularly cogent argument for abandoning C_1–C_x nomenclature as a cellulase enzyme descriptor. Recombination of the purified glucanases from *T. reesei* QM 9414 with the β-glucosidase of *T. viride,* in the original proportions of the respective activities, yields a cellulase system which converts Avicel to glucose at exactly the same rate as the native system.

The simplicity of the enzyme system when isolated quickly after synthesis augurs well for productive investigation of the molecular genetic basis of the biosynthesis of these enzymes. Freed of the difficulty of accounting for the synthesis of multiple enzyme forms, investigators should be able to approach with confidence the problem of enzyme synthesis at the genetic level. Although there may be a mechanistic significance to the further conversion of the nascent system to multiple forms, it seems more likely that this process is only a mechanism for the recovery of proteins that have been elaborated unnecessarily into the medium.

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13

Biosynthesis, Purification, and Mode of Action of Cellulases of *Trichoderma reesei*

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Enhanced cellulase producing strains of Trichoderma reesei *QM 9414 were used to study the biosynthesis of cellulases by using soluble (lactose) and insoluble (crystalline cellulose) substrates as the sole carbon source. The major cellulase components were isolated from culture filtrates of* T. reesei *and purified (to homogeneity) by chromatography on ion-exchange resins, by affinity chromatography, and by gel-filtration. These are β-glucosidase, 1,4-β-glucan cellobiohydrolase, and 1,4-β-glucan glucanohydrolase. The latter components were found to form little glucose upon hydrolysis of cellulose. The β-glucosidase had high glucose forming activity upon incubation with cellobiose. Other properties examined with these purified components include molecular weight, specificity of action, and effect of culture media on enzyme activity.*

It is a widely recognized fact that true cellulolytic microorganisms produce three basic cellulase components (*1–5*), and that these enzyme components act in concert to hydrolyze crystalline cellulose to glucose (*6*). Many research laboratories have undertaken the task to purify cellulose components from various cellulolytic microorganisms and to study the mechanisms of cellulose hydrolysis. Much information has accumulated concerning the mode of action of cellulose hydrolysis since Reese et al. first proposed the C_1–C_x concept (*7*). In spite of this, however, conflicting reports still flourish concerning the composition of the "cellulase complex," the multiplicity of cellulase components, the biosynthesis of cellulose, and the mechanisms of cellulose hydrolysis.

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This confusion may be attributable, in part, to factors such as:

(a) the lack of consistancy in the organisms or strain of organisms used for enzyme production, in the cultural conditions used to grow these organisms, in the conditions and substrates used to measure enzyme activity, in the definition of enzyme activity, and in the nomenclature used to identify various cellulase enzyme components;

(b) the variation in techniques used for separation and purification of cellulase components and for verification of homogeneity and purity of the resulting enzyme preparations.

Despite these factors, however, there are some areas of agreement.

The multiplicity of cellulase components has been reported for many cellulolytic microorganisms. For *Trichoderma reesei* (formerly *Trichoderma viride*) at least three endoglucanases (*8, 9*), three isozymes of cellobiohydrolase (*6*), and at least three cellobiases (*10*) have been found in the culture filtrate. Five endoglucanases have been purified for *Sporotrichum pulverulentum* (*11*), while at least eleven endoglucanases have been reported for *Penicillium citrinum* (*12*). While there is agreement that multiple components of the same enzyme may exist, there is disagreement as to the origin of this multiplicity. While it is conceivable that different microorganisms could produce different types of cellulases, it is difficult to understand why the same species of fungus would produce different types of cellulases, i.e. enzymes which are independent entities. One might speculate that it is wasteful for a microorganism, which possesses limited genetic pools, to transcribe and translate so many enzymes for the same function. Alternately, it is conceivable that multiple components may arise from a single enzyme by post-translational modification. Then the question arises as to what causes this modification.

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The Multiplicity of Cellulases

The multiplicity of cellulases is of fundamental interest because of its implications on the basic understanding of cellulose hydrolysis as well as the regulation of cellulase biosynthesis.

It appears that for the much studied *T. reesei* multiplicity is a "fact of life." Some of the physical characteristics of multiple component cellulase enzymes for this microorganism have been elucidated in Brown's laboratory at Virginia Polytechnic Institute. Brown and co-workers purified three cellobiohydrolases from a commercial cellulase preparation. They found these enzymes to be glycoproteins of similar molecular weight with the largest enzyme (cellobiohydrolase "C") predominating (*6, 8*). Shoemaker and Brown (*9*) reported four electrophoretically distinct endoglucanases having molecular weights ranging from 37,200 to 52,000. In addition, Berghem, et al. (*8*) have found two major endoglucanases (one having a high molecular weight and the other a low molecular weight)

In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis; Brown, R., el al.; Advances in Chemistry; American Chemical Society: Washington, DC, 1979.

and several minor ones for *T. reesei.* Okada (*13*) has reported three endoglucanases of differing molecular weight and randomness of action toward hydrolysis of cellulose.

Based on the work done in our laboratory, we believe that multiple enzymes of the same type are derived from the same enzyme and potentially arise from partial proteolysis of such an enzyme (*10*). In previous studies, we have purified three distinct cellobiases from *T. reesei* which are chromatographically distinct yet kinetically similar.

In a similar context, Nakayama et al. (*14*), reported that limit proteolysis of cellulases caused changes in enzyme characteristics and created multiplicity of enzymes. Therefore, it seemed to us that if proteolytic enzymes play an important role in causing multiplicity of cellulases, it should be possible to grow the cultures in such a way that proteolytic enzyme production would be limited. Therefore, we analyzed endoglucanase activity of cultures of various ages after fractionation of the endoglucanase components on DEAE-cellulose chromatography. The results are shown in Figure 1.

There is one sharp, distinct peak of endoglucanase activity from young fermentation broth. In comparison, the ten-day-old culture exhibited additional endoglucanase peaks, while in the fourteen-day-old culture four different endoglucanase activity peaks were observed. The activity peaks of enzyme obtained from older cultures are similar to those obtained from commercial cellulase preparations as shown in Figure 1. Similar results have been observed by Nakayama (*16*).

This data, together with the observation of prolific protease activity in crude commercial cullulase preparations that are probably obtained from older cultures, has led us to speculate that the multiple enzyme peaks in the older cultures could have resulted from protease modification of one parent endoglucanase. This prompted us to discontinue the use of commercial cellulase preparations.

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Proteolysis of Cellulases

The proteolysis of cellulases has been previously investigated. Nakayama et al. (*14*) found that mild proteolysis of endoglucanase from *T. reesei* by a protease prepared from the same fungus resulted in cellulase enzymes which still possessed cellulolytic activity. Earlier, Eriksson and Petterson (*24*) investigated the effect of various proteolytic enzymes on the cellulase activities on *Penicillium notatum.* They found that different proteases affected enzyme activities to different degrees.

It had been reported that *T. reesei* secreted protease into culture media during cellulase production (*21*). We have found that in young culture filtrates of *T. reesei,* the protease activity is very low, but that

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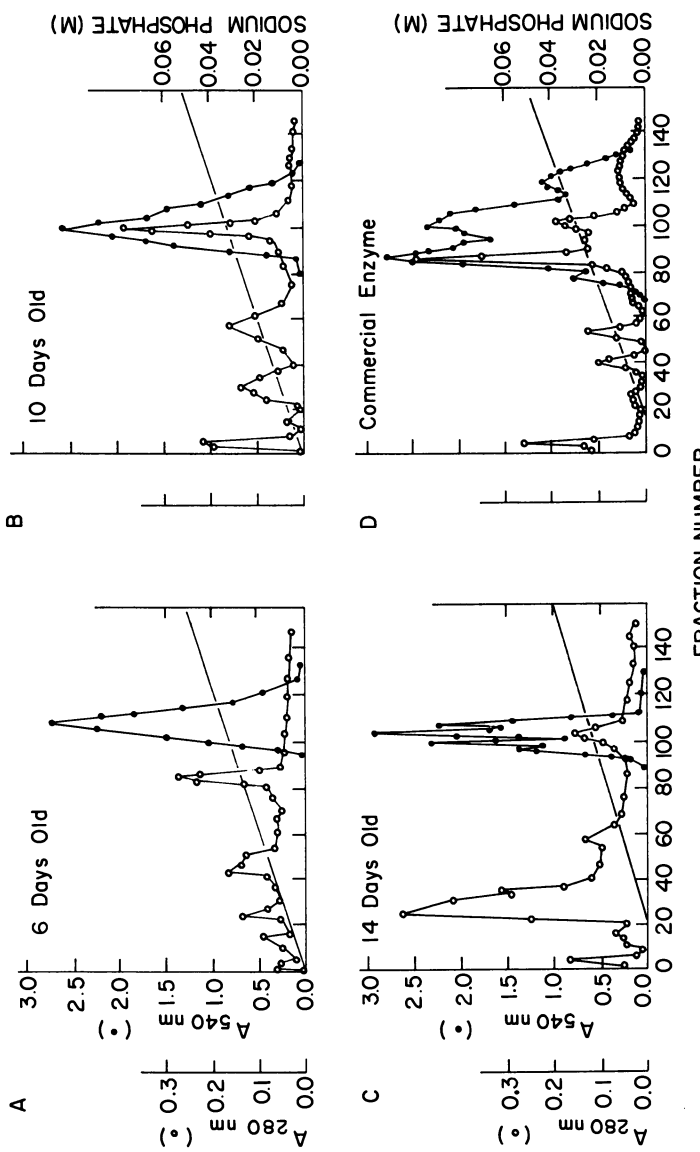

Figure 1. DEAE-cellulose chromatography of endoglucanases from T. reesei *enzymes from (–○–) Relative protein concentration, (–●–) relative activity with respect to CMC. (a) 6-day-old culture, (b) 10-day-old culture, (c) 14-day-old culture, and (d) commercial preparation.*

this activity increases with increasing culture age. Similarly, for crude commercial cellulase preparations, which are harvested from older cultures, we have detected significant amounts of protease activity by Azocoll assay methods (*10*).

It is also instructive to consider some results which have been reported for other enzymes with respect to partial proteolysis. Partial proteolysis of enzymes has been implicated in post-translational modification of both extra- and intracellular enzymes. For example, RNA polymerase (an intracellular enzyme) from spores of *Bacillus subtilis* has been found to be different from RNA polymerase of vegetative cells. The difference is due to the partial proteolysis of the subunits of RNA polymerase from the spore stage. Because of this, the template specificity of the spore enzyme is different from the enzyme of the vegetative cells (*17*). It was later found that this proteolysis could be prevented by careful manipulation of enzyme extracts during enzyme isolation by first removing protease (*18*). Another example is the proteolytic modification of an extracellular enzyme, staphylokinase (*19*). It was found that among the three isozymes of staphylokinase, two enzyme components are derived sequentially from one enzyme by mild trypsin digestion. Thus, a total of three enzymes resulted having different isolectric points and different molecular weights. The two enzymes derived from the digestion were identical to those obtained from the culture filtrate.

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Mode of Action

The modes of action of the three major *Trichoderma* cellulase components: (1) β-glucosidase (i.e., cellobiase), (2) 1,4-β-glucan cellobiohydrolase (i.e., cellobiohydrolase), and (3) 1,4-β-glucan glucanohydrolase (i.e., endoglucanase) are also being intensely studied. These enzymes, which act both individually and together to break down cellulose and cellodextrins, appear to have a complex interdependence which has, to date, been only partially quantified. Many different cellulosic substrates as well as modes of action have been reported for these enzymes (*see* Table I). While cellobiohydrolase produces cellobiose from cellulose through an end-wise cleavage of cellulose chains (*43, 44, 45, 52*), endoglucanase appears to act randomly to hydrolyze cellulose (*8*). Cellobiase hydrolyzes cellobiose and perhaps other cellodextrins as well to give glucose (*10, 46, 47*). The picture is somewhat complicated by the inhibition of cellobiohydrolase by the product cellobiose(*44, 45*). While cellobiase relieves this inhibition by hydrolysis of cellobiose to glucose, cellobiase itself is inhibited by the product glucose (*10, 46, 54*).

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Table I. Summary

General Category	*Other Names*
β-Glucosidase (EC 3.2.1.21)	Cellobiase
1,4-β-Glucan cellobiohydrolase (EC 3.2.1.91)	"C_1" exoglucanase exocellulase avicellase 1,4-β-cellobiosyl-glycanase cellobiohydrolase
1,4-β-Glucano glucanohydrolase (EC 3.2.1.4)	"C_x" CMCase "endocellulase of random type" (*14*) endo-1,4-β-glucanase endoglucanase

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The summary of cellulase mode of action in Table I reviews mainly data reported during the last five years. An excellent comprehensive review article on cellulase mode of action based on information reported prior to 1973 is given by Nisizawa (*55*).

Purification of Cellulase

Previously, both in our laboratory and elsewhere, cellulases subjected to purification procedures were obtained from commercial sources (*5, 6, 8, 9, 10, 13, 39, 46*). Three cellobiases and several endoglucanases and cellobiohydrolases from commercial preparations were purified in our laboratory. While use of protease inhibitors in the fractionation procedures minimized proteolysis during enzyme purification, the existence of enzymes proteolytically modified, presumedly during prolonged fermentation (required for obtaining high titres for commercial production), was a source of confusion, as previously explained. Therefore, we prepared *T. reesei* cellulase harvested from young culture broth. This was used to carry out the enzyme purification procedures described below.

Crude Cellulases. The culture filtrate obtained from four-day-old cultures was concentrated by lyophilization. The protease inhibitors, phenyl methyl sulfonyl fluoride (PMSF, 2.3m*M*) and ethylenediaminetetraacetic acid (EDTA, 0.2m*M*) were added to the culture solution and the salt concentration was adjusted to 0.5*M* with NaCl. After stirring at 4°C for at least 30 min to release cellulases from any enzyme–substrate complex which might have formed, the culture solution was centrifuged

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of Cellulases

Substrates	*Mode of Action*
cellobiose; possible cellotriose (*41*) and cellotetraose (*47*)	Noncompetitive mechanism with product (glucose) acting as an inhibitor (*10, 46*). Apparent substrate inhibition at very high substrate concentration (*46*).
filter paper, Avicel; cellodextrins cellobiose through cellopentose (*43*). bacterial cellulose (*44*). phosphoric acid swollen cellulose (*27*). xylan, *p*-nitrophenyl β-cellobioside (*48*).	Cellobiose producer. A decrystallizing enzyme (*42*) with terminal mode of attack (*2, 43*). Cellobiose is competitive inhibitor (*45, 49*). Glucose is not an inhibitor (*43*). Needed for hydrolysis on highly ordered substrates (*41*).
CMC (*50, 53*) amorphous cellulose (*42*); cellobiose through cellohexaose (*51*) acid swollen cellulose	Characterized by release of free fibers from filter paper. Exact role of this enzyme not yet fully understood (*8*).

to remove mycelial mats. The supernatant, containing crude cellulase, was clarified by additional centrifugation at 12,000 rpm for 15 min. The detailed conditions for this culture growth, harvesting, and processing are given elsewhere (*15*).

Once the crude cellulase solution was obtained, it was concentrated and at the same time separated from most of the salts (left from the fermentation) in the enzyme solution. The steps which accomplished this were: (1) addition of ammonium sulfate (75% saturation) to precipitate protein; (2) recovery of the protein as a pellet by centrifugation at 12,000 rpm for 15 min; (3) redissolution of the protein in 0.1*M* sodium phosphate–0.2m*M* EDTA buffer (pH 6.8); (4) desalting on a 1.5 × 45 cm Sephadex G-25 column; and (5) lyophilization to obtain concentrated enzyme followed by a final ammonium sulfate precipitation to obtain precipitated enzyme.

The concentration procedure in Step (5) was developed by trial and error. Other methods tried were solvent precipitation with acetone and/or alcohol, Amicon ultrafiltration with a PM-10 membrane, lyophilization alone, and ammonium sulfate precipitation alone.

Enzyme Activity Assays. The various enzyme activity assays used in the purification procedures are summarized in Table II.

Overall crude cellulase activity was measured using Remazol brilliant blue acid-swollen cellulose (cellulose–azure, Calbiochem.) (*22*). The assay consists of combining 40 mg cellulose azure, 1.0 mL of 100m*M* sodium citrate buffer (pH 4.8), and 1.0 mL of enzyme solution and

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Table II. Summary of Various Cellulase Activity Assays[a]

Enzyme	*Substrate*	*Volume (mL)*	*Incubation Time (min)*	*Temp (°C)*	*Product Measured*
Crude	cellulose–azure (40 mg)	2	90	50	blue dye
Cellobiase	cellobiose (0.5%)	1	30	50	glucose
Endoglucanase	CMC (0.5%)	0.5	20	50	cellodextrins
Cellobiohydrolase	Avicel (40 mg)	2	30	50	cellobiose

[a] These assays are for quick characterization of activity. Mode of action subsequently verified using pure component cellodextrins or cellulose.

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incubating the mixture at 50°C for 2 hr. After the remaining solids were spun down, the blue dye released into solution as a result of cellulase activity was measured at A_{550nm} with a spectrophotometer. This approach to measuring cellulase activity is very convenient since residual sugars (from the culture filtrate) do not interfere with the measuring and since the activity values obtained are comparable to those obtained with Mandels and Weber's filter paper methods (*23*).

During purification procedures cellobiase activity was monitored by measuring nitrophenol (at A_{420nm}) release for *p*-nitrophenyl-β-D-glucoside (*10*). Kinetic studies and enzyme characterization were carried out using β-D-cellobiose as substrate with the product, glucose, measured with a Beckman Glucose Analyzer (*10*). Assay conditions were pH 4.8 and 50°C.

Enzyme activity towards carboxylmethyl cellulose (CMC) was measured by combining 50 μL enzyme with 0.5 mL of 0.5% CMC in pH 4.8 sodium citrate buffer and incubating at 50°C for 10 min. After this, the reaction was stopped by addition of 1.5 mL dinitrosalicylic acid reagent and the resultant color, which was measured at A_{540nm}, related to the reducing sugar concentration.

Avicel hydrolysis activity was measured by combining 100 μL enzyme with 40 mg Avicel in 2 mL of 50m*M* sodium citrate buffer (pH 4.8) and incubating for 2 hr at 50°C in a Corex tube with constant stirring. The mixture was clarified by centrifugation. Then, 0.5 mL of the clear supernatant was combined with 1.5 mL of dinitrosalicylic acid reagent. The presence of reducing sugars caused color formation upon heating for 3 min in a boiling water bath. Reducing sugar concentration was measured spectrophotometrically at A_{540nm}.

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These methods for enzyme activity determination are easy and convenient for screening enzymes during purification procedures, especially for cellobiase and endoglucanase. Up to 72 of these types of samples can be run at the same time. For cellobiohydrolase it is more difficult, but it is still possible to run up to 24 samples at the same time. In this procedure, the samples in Corex tubes are continuously stirred while kept in a constant-temperature water bath. After incubation the tubes are removed, put on ice, and 5% TCA is added to stop the reaction. The solid substrate is subsequently removed by centrifugation.

Specific hydrolysis products obtained from both cellulose and cellodextrins by cellobiohydrolase and endoglucanases were analyzed by low pressure liquid chromatography using water as the sole eluent (*25, 40*).

Enzyme Purification. Traditional purification techniques reported in the literature include ion exchange resins, gel filtration, and isoelectric focusing (*see* Ref. *26* for a review). Recently, there has been a trend of combining traditional methods with new techniques to perform enzyme purification. Berghem et al. (*8*) used a dipolar adsorbent arginine–ligand affinity resin in combination with other methods to purify endoglucanase from *T. reesei*. Gum and Brown (*27*) and Shoemaker and Brown (*9*) have employed Avicel columns (previously used by Lin et al. (*1*)) to separate various cellulase components. Gong et al. have employed immobilized concanavalin A to separate cellobiase from endoglucanase (*39*).

The methods of purifying enzymes are always tedious and the recovery of enzymes is very low. Recently, we have developed special procedures to purify various cellulase components from young culture broth of *T. reesei*. The procedures used are simple, well-defined, and insure a maximum recovery of three major enzyme components. The purification scheme is summarized in Figure 2. The enzyme cellobiase and low-molecular-weight (LMW) endoglucanase were first eluted from DEAE–Sepharose column by water. These fractions were then subjected to DEAE-cellulose column chromatography. The linear salt elutions resulted in the separation of cellobiase and LMW endoglucanase (*10*). The LMW endoglucanase obtained from DEAE-cellulose chromatography is almost pure, with only a trace of contaminants (Figure 3). Cellobiase can be purified further by subjecting it again to DEAE-cellulose chromatography, with enzyme being eluted out of the column by salt elution at low concentration (*10*). The young culture broth contains only trace amounts of cellobiase. Therefore, further purification of the cellobiase was not attempted although this enzyme was purified previously from a commercial preparation (*10*). The molecular weight and the characteristics of cellobiase from culture broth are the same as those from commercial cellulases.

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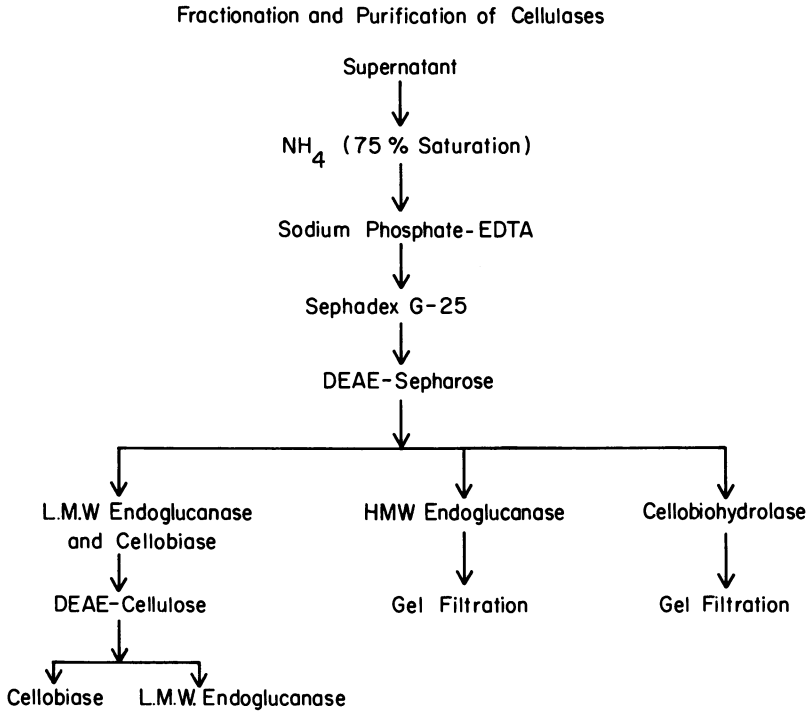

Figure 2. Summary of fractionation and purification scheme for cellulases (15)

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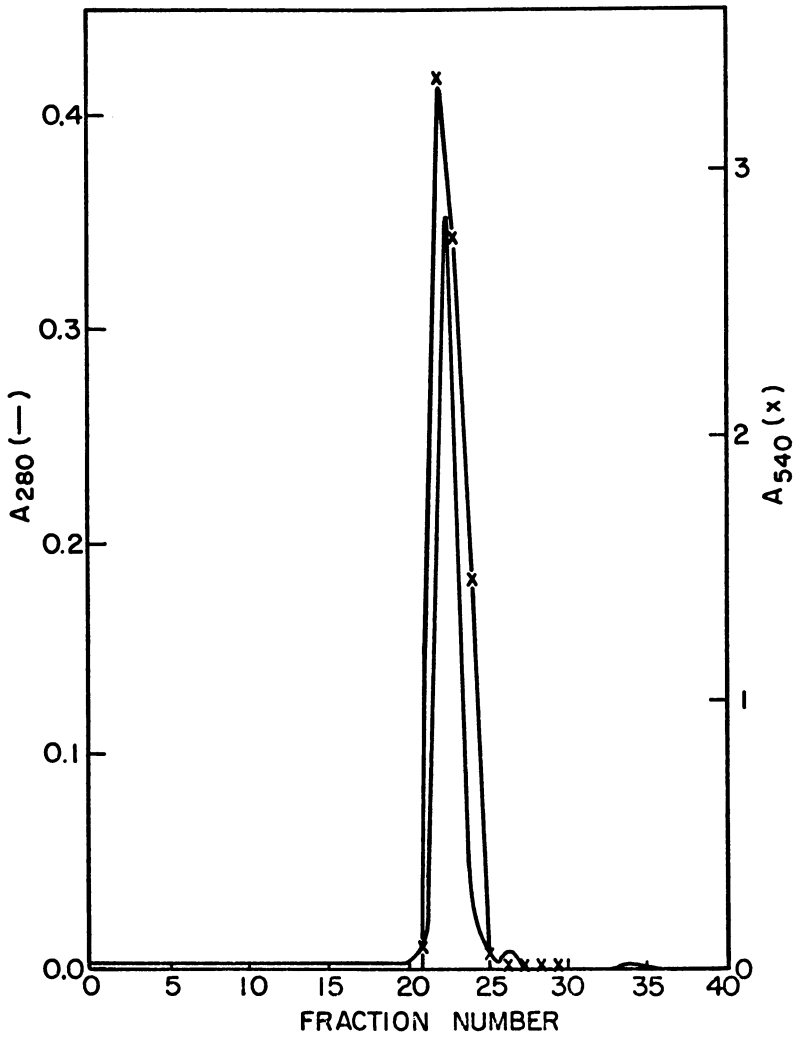

Figure 3. LMW endoglucanase from Sephadex G-75 column chromatography. (—) Protein (measured at $A_{280\,nm}$), (–×–) reducing sugar formed from CMC.

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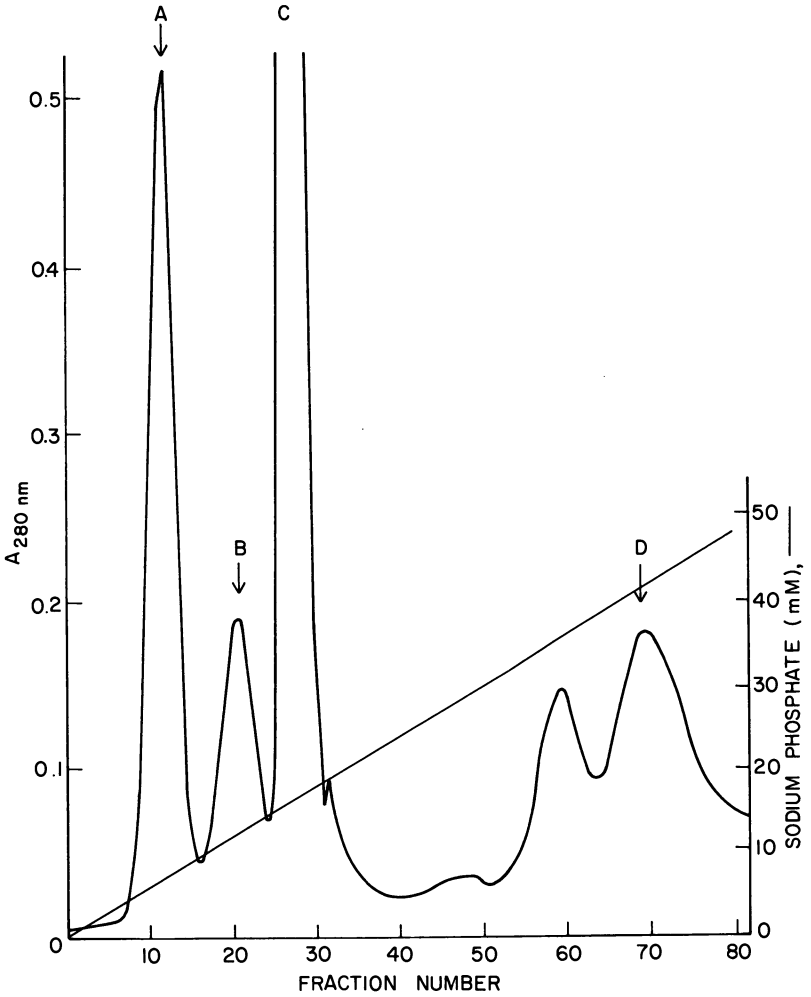

Figure 4. DEAE-sepharose column chromatography of HWM endoglucanase. (A) Peak having major endoglucanase activity and (B), (C), (D) peaks having lower amounts of endoglucanase activity.

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High-molecular-weight (HMW) endoglucanase and cellobiohydrolase are retained on the DEAE-Sepharose after the cellobiase and LMW endoglucanase have been eluted with water. The fractionation of cellobiohydrolase and HMW endoglucanase is accomplished by sequential linear salt gradient elution using sodium phosphate for the HMW endoglucanase (Figure 4) and sodium chloride for cellobiohydrolase (Figure 6).

A total of four endoglucanases was obtained. Three fractions of endoglucanase activity eluted between 0–25m*M* sodium phosphate and one fraction between 25–50m*M* phosphate (Figure 4). The first enzyme peak has the major endoglucanase activity and is homogeneous as was indicated from SDS-gel electrophoresis (*see* Figure 9d). The other three peaks having significant, but lower, endoglucanase activity showed molecular weights that were similar to that of the first peak, as indicated by chromatography of all four fractions on Sephadex G-75 (Figure 5). The molecular weights obtained from the G-75 runs were all about 52,000. On the basis of these data, we concluded that *T. reesei* possesses only one major endoglucanase (Peak A in Figure 4 and Figure 5), while the other endoclucanases (Peaks B, C, D) are post-translational modification products.

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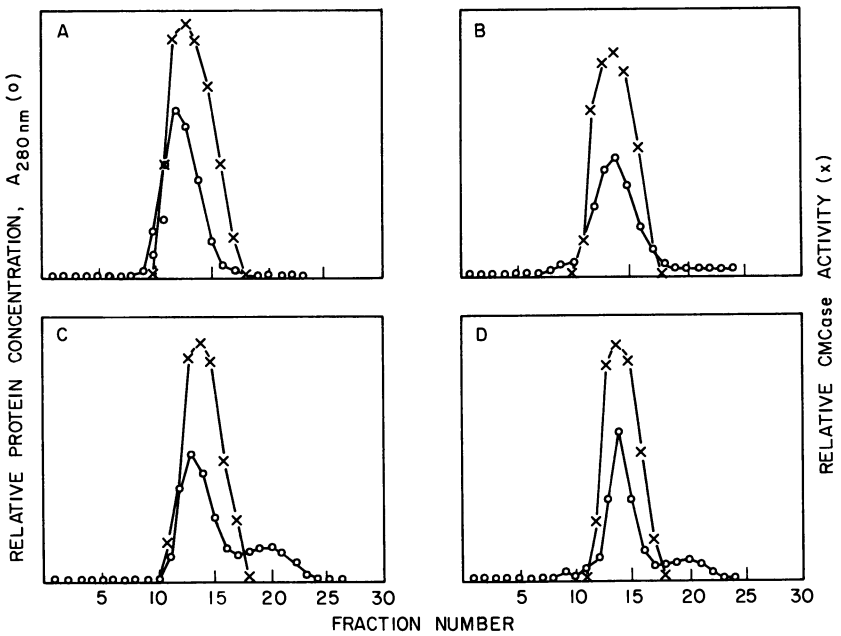

Figure 5. Sephdex G-75 column chromatography of HMW endoglucanase (A), (B), (C), and (D) fractions shown in Figure 4. (–O–) Relative protein concentration, (–×–) relative activity with respect to CMC.

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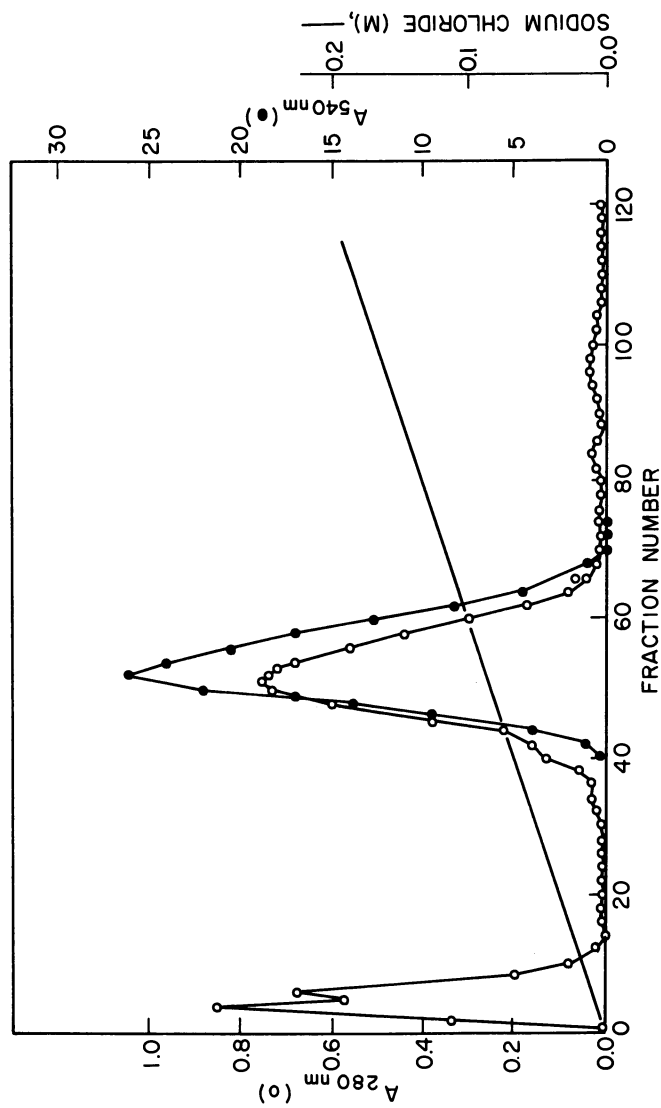

Figure 6. DEAE-sepharose column chromatography of cellobiohydrolase. (–○–) Relative protein concentration, (–●–) relative activity with respect to Avicel.

After the endoglucanase activity had been washed from the column, the cellobiohydrolase fraction was subsequently recovered. In this case however, sodium chloride, rather than sodium phosphate, was used to perform the linear salt gradient (0–200 m*M* NaCl). Only one cellobiohydrolase activity peak was obtained (*see* Figure 6). Even higher salt concentrations (above 200m*M*) failed to wash any other enzyme activity from the column. Thus it is clear that only one enzyme remains after the endoglucanase fractionation step, this single enzyme being cellobiohydrolase. The homogeneity of this enzyme is also verified by the SDS-gel picture shown in Figure 9(g). The homogeneity of the enzyme is also verified by gel filtration on Sephadex G-100 (Figure 7) and also indicated by SDS-gel electrophoresis.

Gel filtration of crude cellulase on Sephadex G-75 gave one cellobiase, a major HMW endoglucanase, a LMW endoglucanase, and one cellobiohydrolase (Figure 8). The purified enzyme and the crude enzyme protein was analyzed by SDS-gel electrophoresis and resulted in the gels shown in Figure 9.

The product specificity of purified cellobiohydrolase and endoglucanase after incubating enzymes with Avicel was analyzed by liquid chromatography (*40*). The chromatograms are Figures 10 and 11.

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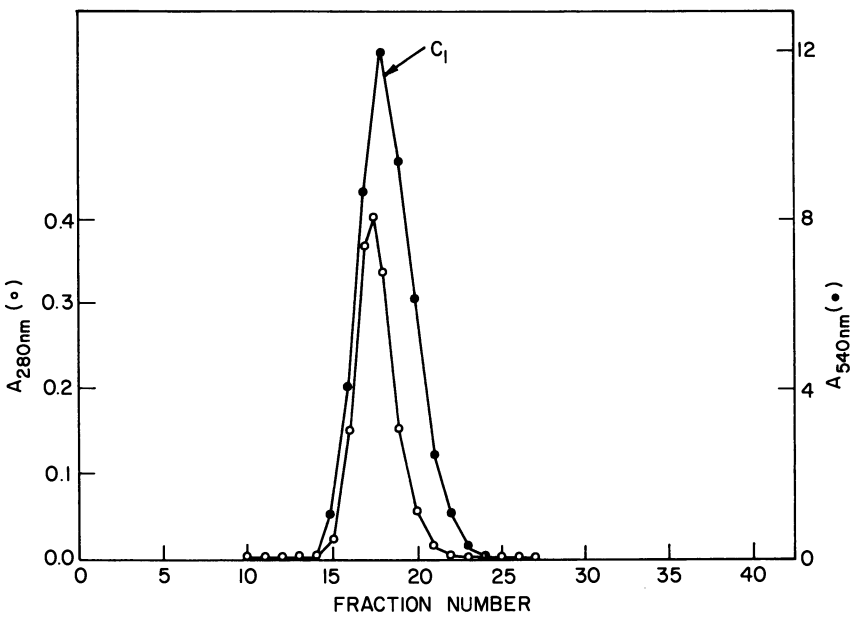

Figure 7. Sephadex G-100 gel filtration of cellobiohydrolase. (–○–) Protein ($A_{280\,nm}$), (–●–) activity with respect to Avicel.

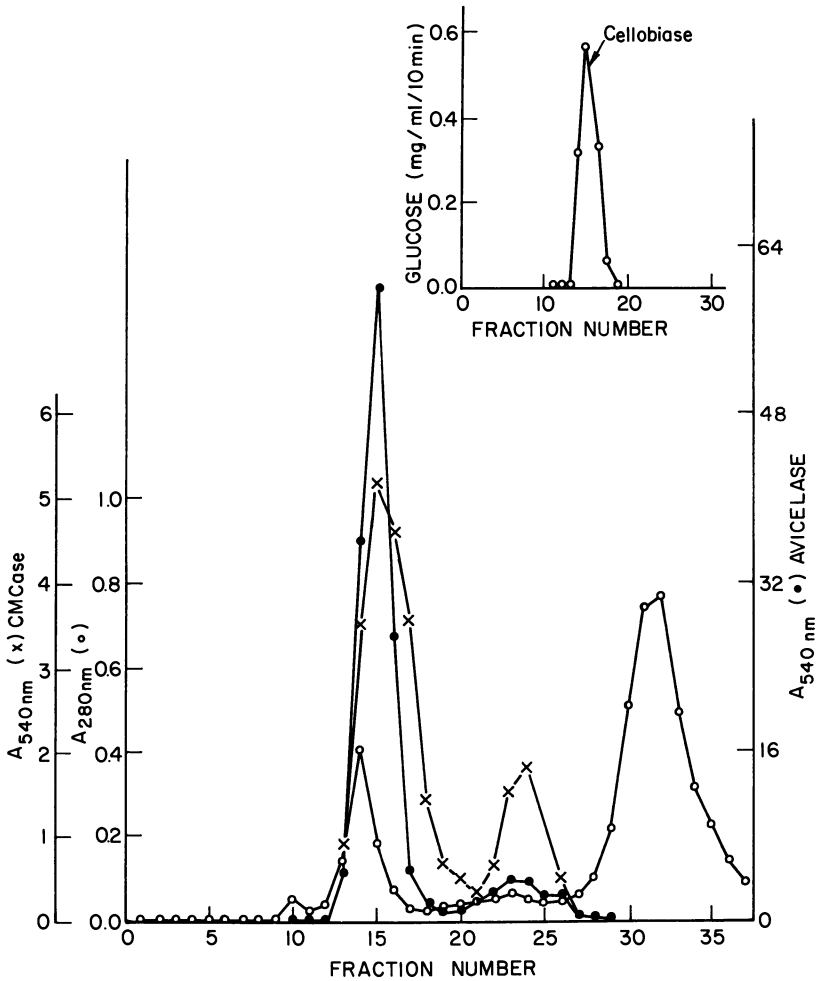

Figure 8. Sephadex G-75 column chromatography of crude cellulases. (–○–) Protein ($A_{280\,nm}$), (–×–) activity with respect to CMC (endoglucanase), (–●–) activity with respect to Avicel (cellobiohydrolase). Inset, *top right: (–○–) cellobiase activity.*

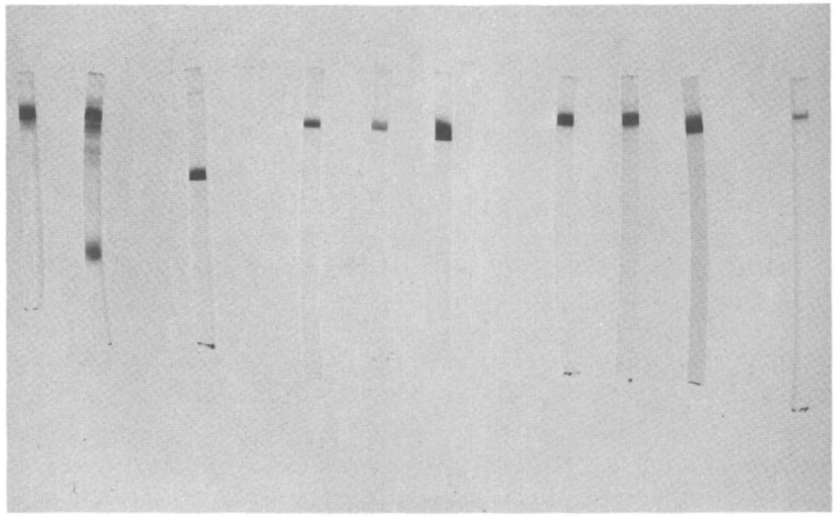

Figure 9. SDS-gel electrophoresis of purified cellulases from T. viride *grown in Avicel and lactose. (a) Crude cellulases (Avicel as growth medium), (b) crude cellulases (lactose as growth medium), (c) purified LMW endoglucanase (Avicel), (d) purified HMW endoglucanase (Avicel), (e) purified HMW endoglucanase (lactose), (f) mixture of (d) and (e), (g) purified cellobiohydrolase (Avicel), (h) purified cellobiohydrolase (lactose), (i) mixture of (g) and (h), (j) purified cellobiase.*

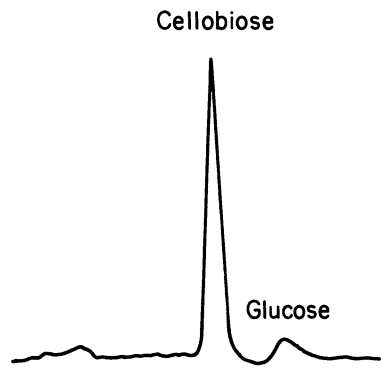

Figure 10. Liquid chromatogram of hydrolysate from action of cellobiohydrolase on Avicel

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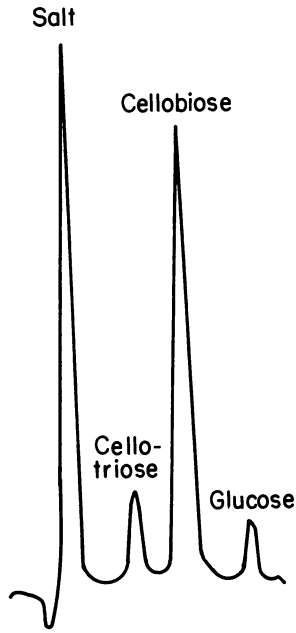

Figure 11. Liquid chromatogram of hydrolysate from action of endoglucanase on Avicel

Results and Discussion

The development of the sequential elution methods makes it possible not only to cleanly fractionate the three cellulase components, but to do the fractionation with very little loss of enzyme. The total recovery of major enzyme components, summarized in Table III, is considerably higher than those reported previously by other researchers (*8, 9*). Table III also gives the molecular weights of the three enzyme components.

Quite surprisingly, cellobiohydrolase is the major cellulase component produced by *T. reesei* and comprised over 35% of the high molecular weight soluble proteins produced by fungus (Table III). Total endoglucanase (G-25 fractions) was less than 8% of total high molecular weight soluble protein and cellobiase was present in only trace amounts. The relatively large amount of cellobiohydrolase in crude cellulase of *T. reesei* is consistent with the results of Berghem et al. (*8*). Cellobiohydrolase also makes up the majority of cellulase enzyme in commercial cellulase preparation (obtained from Enzyme Development Corp., NY, NY).

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Table III. Summary of Cellulase Recovery (*15*)

Fractions	*Total Proteins (mg)*	*% of Proteins Recovered*	*Molecular Weight*[a]
Culture filtrate	1404	100[b]	
After Sephadex G-25 desalting	868	61.8[b]	
Cellobiase	2.6	0.18[c]	76,000
LMW endoglucanase	9.75	0.69[c]	18,000
HMW endoglucanase	28	1.99[c]	52,000
Cellobiohydrolase	312	22.2[c]	71,000

[a] Estimated from SDS–polyacrylamide gel electrophoresis using proteins of known molecular weight as markers.
[b] Based on total soluble protein.
[c] Based on homogeneous protein.

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The cellobiase activity in culture filtrates of *T. reesei* was small relative to that of cellobiohydrolase and endoglucanase. The possibility that cellobiase of *T. reesei* is either an intracellular or membrane-bound enzyme was indicated by experiments in which cellobiose or other carbon sources were used as the substrate for culture growth. While cellobiose can be taken up rapidly by the fungus, very little cellulase activity could be detected in the filtrate (*see* Table IV—cellobiose as carbon source). Furthermore, the appearance of cellobiase did not parallel the appearance of cellulase activity. Increasing culture incubation time did, however, result in increasing cellobiase activity in the filtrate. This data suggested that at least some of the cellobiase present in the filtrate might have been an intracellular cellobiase which was, perhaps, released when some of

Table IV. Growth and Cellulase Production of *T. reesei*

Carbon Source	*Growth*[a] $\left(\frac{\text{Dry weight, gm}}{\text{200 mL culture}}\right)$	*% Cellobiase*[b] *Activity*	*% Cellulase*[b] *Activity*
Cellulose	0.6	100	100
Lactose	0.69	92	81
Glucose	0.63	2	< 1 (trace)
Galactose	0.65	3	< 1 (trace)
Cellobiose	0.67	5	< 1 (trace)
Glycerol	1.03	4	< 1 (trace)
Xylose	0.87	2	< 1 (trace)
Lactose and glycerol	1.01	11	3
Lactose and glucose	0.76	26	21

[a] *T. viride* were grown in 1% carbon source at 28°C for 4 days.
[b] One mL of culture filtrate were incubated with 0.5% substrate at 50°C for 2 hr. Activity from cellulose as carbon source served as the control (100%).

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the cells underwent autolysis. The existance of intracellular and/or membrane-bound cellobiase has been reported for *Sporotrichum pulverulentum* (*28*) and *Neurospore crassa* (*29*). Thus, it would not be unreasonable to suggest that other microorganisms might also have intracellular cellobiase.

Preliminary data showing that cellobiase hydrolyzing activity is found inside the cells, as well as outside, for *T. reesei* is shown in Table V. Cells, when separated from the culture filtrate and broken up by grinding and sonification, showed significant release of cellobiase activity. This phenomenon was observed not only when cellobiose was used as a carbon source during culture growth, but also when other carbon sources such as cellulose, glucose, glycerol, or xylose were employed. Although these results clearly show the existence of an intracellular cellobiase, the question of whether or not the intracellular and extracellular cellobiase is the same must still be answered. These results can also be used to explain why cellobiose is not a good cellulase inducer. Since the cellobiose can be hydrolyzed to glucose and utilized intracellularly by the fungus, the fungus is not forced to produce copious quantities of extracellular enzyme in order to have an assured food source.

While cellobiose is a poor inducer, lactose, the milk sugar, is a good inducer of cellulase biosynthesis. Lactose-induced fungus produces a cellulase complex which is identical to those cellulases induced by cellulose (for SDS gels *see* Figure 9b, e, h, i).

These results, taken together with the data reported previously in this chapter, indicate that *T. reesei* produces one major endoglucanase and one cellobiohydrolase. Cellobiase-hydrolyzing enzymes are probably located intracellularly as well as extracellularly. More work is needed to determine whether or not the intracellular cellobiase is the same as the extracellular cellobiase.

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Table V. Cellobiase Activities[a] of *T. reesei* Homogenate[b]

Carbon Source	*Low-Salt Homogenate*	*High-Salt Homogenate*
Cellulose	2.12	0.66
Cellobiose	1.13	0.77
Glucose	0.72	0.56
Glycerol	1.12	2.94
Xylose	2.38	0.54

[a] mg glucose produced per mL of reaction mixture after incubation at 50°C for 3 hr.

[b] Mycelia from 200 mL of culture were disrupted by grinding and sonification. Soluble proteins were extracted by 50m*M* sodium acetate buffer, pH 5.8. After buffer extraction, the remaining solids were further extracted by high-salt buffer (500m*M* NaCl in 50m*M* sodium acetate, pH 5.8).

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Induction and Biosynthesis of Cellulases

A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cellulolytic enzymes when cellulose is present in the growth media (*20, 23, 28, 30*). However, unlike some of the microorganisms that produce an incomplete cellulase system, *T. reesei,* a true cellulolytic fungus, produces an array of cellulase enzymes, i.e., the "cellulase complex," which is able to hydrolyze cellulose to glucose (*23*).

T. reesei, a saprophytic fungus, is capable of utilizing a variety of carbohydrates. Yet, only a few carbohydrates induce cellulase production. Inducers include cellulose, cellulose derivatives, lactose, and sophorose (*31, 32*). Mandels and Reese (*31, 32*) studied the inducibility of various sugars and found that sophorose is an excellent cellulase inducer in *T. reesei* while having little effect in other fungi or bacteria. On further examination they found that trace amounts of sophorose present in glucose caused the apparent ability of glucose to be a cellulase inducer in *T. reesei.*

Cellobiose, a dimer of β-1,4-linked glucose, is reported to be a cellulase inducer in *T. reesei* as well as in several other fungi (*20, 28, 33, 34*). But whether cellobiose is a true inducer is questionable since Reese et al. (*35*) reported that cellobiose could induce as well as inhibit cellulase biosynthesis. The same is also true for glucose. Whether glucose or cellobiose is an inducer or inhibitor depends on the concentration of sugars in the environment.

Cellulose is a universal inducer of cellulase biosynthesis. Since cellulose is insoluble, the microorganisms are unable to utilize it unless it has been hydrolyzed first to glucose or soluble oligomers of cellulose. But if cellulose cannot be utilized directly without first being solubilized, the question arises as to how it gets into the cells to act as an inducer. There appear to be several possible explanations.

One possible explanation is that the mere contact of the cell surface on the insoluble inducer is sufficient to trigger cellulase production. Another explanation is that cellololytic microorganisms have trace quantities of constitutive cellulases which are continuously released. A third possibility is that the cells are able to synthesize cellulases under starvation conditions.

The first possibility mentioned above, that of induction by cell/inducer contact, may be plausible in the case of cellulose, but it does not appear to account for induction by soluble sugars such as sophorose or lactose. If the cell possesses certain sites on the surface which recognize specific sugar linkages, then why is sophorose not an inducer in other cellulolytic migroorganisms when it is an excellent inducer in *T. reesei*? And why are other uncommon sugars, which possess linkages similar to that of cellulose and sophorose, not inducers (*32*)?

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The explanation of *T. reesei* having constitutive cellulases seems more likely. In the case where cellololytic microorganisms synthesize limited amounts of cellulase, "induction" would be merely an enhancement of cellulase production. Some evidence that *T. reesei* might produce cellulases constitutively was obtained through some preliminary experiments performed in our laboratory. The results are summarized in Table VI. When the culture was shifted into media having no carbon source (i.e., starvation conditions), trace quantities of cellulase activity could still be detected. Even when the microorganism was shifted to a media having the protein synthesis inhibitor, cycloheximide, cellulase was still released into the media. This indicates that the cellulase released is independent of new protein synthesis. These trends were evident for cultures initially grown on either glucose or on lactose and then shifted as indicated in Table VI.

These limited results are not sufficient to explain fully the notion that *T. reesei* could produce cellulase constitutively (in the absence of inducer) but it does produce the cellulase and release it into the media,

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Table VI. Growth and Cellulase Biosynthesis After Media Shifting[a]

Carbon Source 1	*Carbon Source 2*	*Culture Dry Weight (g/200 mL media)*	*Relative Cellulase Activity (%)*
Glucose (control)	no shift	0.62	5
Glucose	glucose	1.03	4
Glucose	lactose	1.03	77
Glucose	lactose with cycloheximide (20 mg/mL)	0.59	3
Glucose	starvation	0.61	4
Lactose (control)	no shift	0.58	100
Lactose	glucose	1.02	16
Lactose	cellobiose	1.01	24
Lactose	lactose	1.06	86
Lactose	starvation	0.56	31
Lactose	starvation with cycloheximide (20 mg/mL)	0.57	26

[a] Cells were first grown in the media with carbon sources as indicated in the Carbon Source 1 column. After 4 days incubation, the mycelia were collected, washed and transferred into the fresh media with the carbon sources as indicated in the Carbon Source 2 column and incubated for an additional 2 days.

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even in the absence of a media carbon source. These results also explain why *T. reesei* could utilize the insoluble substrate, cellulose. This leads us to believe that *T. reesei* or other cellulolytic microorganisms are able to synthesize trace amounts of cellulase which are independent of carbon source present in the media. The reason why these cellulases might be difficult to detect in the media without the inducer is because of their very low activity in the media.

Regulation of Cellulase Biosynthesis

T. reesei is a useful experimental organism for studying regulation of extracellular protein biosynthesis. When grown in a medium in which an exogeneous inducer serves as the major or sole carbon source, *T. reesei*, synthesizes and secretes a cellololytic enzyme into the medium. Similarly, the extracellular cellulase is produced upon limitation of the carbon source and limitation of the utilization of the carbon source (*31*). Presently, there appears to be little data in the literature concerning regulation of cellulase biosynthesis.

Preliminary experiments done in our laboratory showed that antibodies specific for cellobiohydrolase failed to cross react with either purified cellobiase, purified endoglucanase, or crude endoglucanase. These results, together with data reported in the literature, which show that endoglucanase and cellobiohydrolase have different physical structures, indicate that the three cellulases could be transcribed and translated by different genomes. In this context, then, the question arises as to whether cellulase production is regulated by a common regulatory circuit or by different circuits.

The work of Nevalainen and Palva (*36*) may give some clues to the answer for this question. They reported the isolation of a *T. reesei* mutant which was unable to hydrolyze cellulose. The reported results indicated that this single-step reversible mutation led to loss of cellobiohydrolase, endoglucanase, xylanase, and mannase biosynthesis. However, the activity of the aryl-β-glucosidase enzyme was not affected. Thus, these data suggest that both cellulases are regulated by a common regulatory circuit while the β-glucosidase could be regulated independently of the other two enzymes.

Montenecourt and Eveleigh (*37*), using a special agar screening technique, have also isolated a cellulase enhanced mutant (NG-14). They suggest, however, that the cellobiohydrolase and the endoglucanase biosynthesis are regulated by different controls. They base this assumption on data for the relative ratios of cellulases obtained from this mutant needed to hydrolyze different substrates.

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More experiments are needed before any conclusions can be made. It appears that the isolation and study of a mutant which synthesizes only one cellulolytic enzyme component is needed if headway is to be made on determining the nature of regulatory control of cellulase biosynthesis.

Mode of Action

The mode of action of cellulases from *T. reesei* appears to be adapted to the peculiarities of the cellulose substrate. Cellulose, unlike starch or other carbohydrate polymers, is a linear polymer of β-1,4-linked glucose. When cellulose molecules are formed by biosynthesis, about 2% of the total β-linkages are somehow bent (*38*). Consequently, a cellulose molecule of typically a degree of polymerization (DP) of 7,000 will form a "plate" of folded ribbons that allows a high degree of intramolecular cross-links (*38*). A number of such "plates" on top of one another forms a higher structural unit, with each unit approximating a square. The result is a highly crystalline structure. Under hydrolysis attack by a dilute acid, the bent β-links are the first bonds attacked. This leaves straight-glucose-polymeric chains with high levels of interchain cross-links bundled-together, i.e., microcrystalline cellulose (*38*). The microcrystalline structure protects the cellulose from enzymatic hydrolysis.

To hydrolyze crystalline cellulose efficiently by enzymatic means, the inaccessibility of crystalline structures must be overcome. *T. reesei* and some other "true cellulolytic" microorganisms produce a cellulase complex that is capable of efficiently hydrolyzing crystalline cellulose. One explanation of this capability was first proposed by Mandels and Reese (*7*). In this model, two factors, C_1 and C_x worked together to disrupt and hydrolyze cellulose. C_1 first disrupted the crystalline structure of the cellulose while C_x attacked the available sites formed by C_1. In other words, C_1 and C_x exhibit synergism in hydrolyzing cellulose. Since then, the combined action of cellobiohydrolase ("C_1") and endoglucanase ("C_x") has been identified as the source of the apparent synergism (*6, 26, 55*).

The cellobiohydrolase and endoglucanase obtained and purified from *T. reesei* show different modes of action when incubated separately with microcrystalline cellulose (Avicel) at pH 4.8 and 50°C. Cellobiohydrolase gives cellobiose with a small amount of glucose formation (*see* Figure 10). Endoglucanase action on Avicel gives cellotriose, cellobiose, and glucose (*see* Figure 11). The amount of glucose formed is again relatively small.

When endoglucanase (C_x in Table VII), cellobiohydrolase (C_1), and cellobiase are combined and incubated with Avicel, a synergistic effect is observed (*see* Table VII). Cellobiase and endoglucanase have

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Table VII. Hydrolysis of Avicel by Purified Cellulase Components, Alone and in Combination

Enzyme[a]	*Activity (Glucose Equivalent) (μg/mL)*	*% Activity*
Cellobiase (5μg/mL)	0	0
C_x (16μg/mL)	< 1	0
C_1 (500μg/mL)	150	43.9
C_x + cellobiase	< 1	0
C_1 + cellobiase	252	73.7
$C_1 + C_x$	317	92.7
$C_1 + C_x$ + cellobiase	342	100

[a] The terms C_1 and C_x were used to refer to enzyme cellobiohydrolase and endoglucanase, respectively.

very little effect individually while cellobiohydrolase shows significant activity. The most interesting result is that a combination of cellobiohydrolase and endoglucanase, when incubated together with Avicel, gives double the conversion of that when the cellobiohydrolase is used alone. This is especially noteworthy when it is considered that endoglucanase, by itself, has very little activity. The limited hydrolysis to soluble products by endoglucanase is probably due, in part, to the limited accessibility of crystalline cellulose to the enzyme. Even though this enzyme might form many cellulose fragments, these fragments would not go into solution easily because of: (1) the hydrogen bonding between the cellulose fragments and the remaining cellulose structure; and (2) the low solubility of the higher-molecular-weight cellodextrins in water. Thus, it

Table VIII. Pretreatment of Avicel by Endoglucanase[a]

Enzyme[b]	*Activity (Glucose Equivalent) (μg/mL)*
C_x alone (16μg/mL)	< 1
C_1 alone (500μg/mL)	136
C_x (16μg/mL) then C_1	183
C_x (32μg/mL) then C_1	209
C_x (64μg/mL) then C_1	237
C_x (16μg/mL) then C_1 + cellobiase	320
C_x (16μg/mL) then $C_1 + C_x$	305
C_x (16μg/mL) then $C_1 + C_x$ + cellobiase	366

[a] Avicel was first incubated with C_x at 50°C for 30 min; liquids were removed by centrifugation. Pretreated Avicels were then washed with high-salt buffer (0.5*M* NaCl) followed by sodium citrate buffer. The Avicels were then digested by enzymes as indicated.

[b] The amounts of C_1 used were 500 μg/mL; cellobiase, 5 μg/mL; and C_x 16 μg/mL. As in Table VII, C_1 is the abbreviation for cellobiohydrolase and C_x, for endoglucanase.

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appears that endoglucanase has difficulty in hydrolyzing a crystalline substrate. By implication, then, it would seem that cellulose, for which the crystalline structure has been disrupted by some sort of pretreatment, will be more accessible to hydrolysis by endoglucanase.

The action of endoglucanase on Avicel, followed by incubation of the cellulose pretreated with cellobiohydrolase, enhances the apparent activity of the cellobiohydrolase as shown in Table VIII. An explanation for this is that the endoglucanase is able to break up (i.e., nick) the cellulose chains. Therefore, if the crystalline cellulose has been treated first with endoglucanase, reactive sites for cellobiohydrolase attack are created.

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Summary

T. reesei yields one major endoglucanase and one major cellobiohydrolase. The multiplicity of these components is minimal when culture conditions are carefully controlled. The widely reported multiplicity of these components might possibly be due to post-translational modification of one endoglucanase and one cellobiohydrolase. The third major component produced by *T. reesei,* cellobiase, is present intracellularly as well as extracellularly. Whether or not these two enzyme fractions are the same enzyme, remains to be determined.

Endoglucanase and cellobiohydrolase enzyme fractions, obtained from DEAE-Sepharose column chromatography of crude enzyme from in-house-grown *T. reesei* culture filtrates, were examined for activity on cellulose. As expected, cellobiohydrolase action resulted in cellobiose formation while cellotriose as well as cellobiose was formed by the action of endoglucanase. In both cases, the formation of glucose was minimal. Examination of the combined activity of these components on celluloses, showed a certain degree of synergism does exist.

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14

Selective Screening Methods for the Isolation of High Yielding Cellulase Mutants of *Trichoderma reesei*

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Screening methods for the selection of high-yielding cellulase mutants of Trichoderma reesei *have been devised that specifically detect hyperproducing, catabolite repression-resistant, and end-product inhibition-resistant strains. Individual selection techniques employing specific substrates and end-product inhibitors allow for visual screening of a single enzyme in the cellulase complex: endo-β 1–4 glucanase and β-glucosidase (cellobiase) as well as the synergistic action of all the enzymes of the cellulase complex. By use of these methods, mutant strains have been isolated that hyperproduce cellulase and are resistant to catabolite repression by glucose or glycerol.*

Industrial scale enzymatic hydrolysis of cellulose, the world's most abundant renewable carbon source, has been envisioned as a route toward the conversion of cellulose to fuels, food, and chemicals. Enzymatic hydrolysis offers certain advantages in such a process, as enzymes are reusable, energy sparing, nonpolluting, and promote high conversion efficiencies without undesirable side products. To date, the overall conversion of cellulose to glucose employing cellulase enzymes has been hampered by the low yields and associated high cost of the microbial enzymes used. Our specific emphasis has been to increase enzyme production by *Trichoderma reesei,* since this organism is considered the best source of all of the enzymes in the cellulase complex (*1*). There are three primary enzymes thought to act synergistically to degrade crystalline cellulose in *Trichoderma*: 1,4-β-D-glucan 4-glucanohydrolase (EC

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3.2.1.4) (endoglucanase), 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) (cellobiohydrolase), and β-glucosidase (EC 3.2.1.21) (cellobiase). The current concept of the interrelationship of these enzymes envisions an initial attack by the endoglucanases to yield oligosaccharides followed by the release of cellobiose from their nonreducing termini by the action of the cellobiohydrolase. Glucose is subsequently produced from cellobiose through the action of β-glucosidases (*2*). Multiple forms of each of these enzymes have been isolated which show distinctly different biochemical properties and in some cases exhibit different modes of action (*3, 4, 5, 6*).

Mechanisms Controlling Enzyme Synthesis and Activity

The mechanisms controlling the synthesis and activity of each of the cellulase complex of enzymes are summarized in Table I.

Table I. Biochemical Mechanisms Controlling Cellulase Synthesis and Activity in *Trichoderma reesei*

Enzyme	*End-Product Inhibitor*	*Catabolite Repressor*	*Inducer*
Cellobiohydrolase	cellobiose	glucose or metabolites of glucose	cellulose, sophorose
Endoglucanase	cellobiose		
Cellobiase	glucose		cellobiose

Catabolite Repression

The early observations on glucose and catabolite repression in microorganisms were reviewed as long ago as 1943 (*7*). The specific term "catabolite repression" was coined by Magasanik (*8*) and is defined as the mechanism by which organisms decrease the rate of synthesis of catabolic enzymes when the catabolites accumulate to an excess in the cell. The biochemistry and genetics of catabolite repression in prokaryotes are now largely understood and are described in several excellent reviews (*9, 10, 11*). It is generally accepted that catabolite repression in *E. coli* is mediated by the cyclic AMP system (*11*). However, far less is known about the biochemistry and genetics of glucose and catabolite repression of enzymes involved in carbon metabolism in eukaryotes. Cellobiohydrolase, endoglucanase, and cellobiase are all subject to catabolite repression in *Trichoderma* (*12, 13, 14*). A number of other enzymes, for example, invertase (*15*), maltase (*16*), and alcohol dehydrogenase (*17*) have also been shown to be sensitive to catabolite repression in fungi.

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End Product Inhibition

In addition to catabolite repression, the cellulase enzymes themselves are subject to end-product inhibition. For example, as glucose accumulates during saccharification, it interacts noncompetively with cellobiase to inhibit further activity of this enzyme (*6*). Similar inhibition of endoglucanases occurs when cellobiose accumulates in a saccharification reactor (*18, 19, 20*).

Cellulase Induction

All naturally occurring fungal strains of *Trichoderma* require an inducer for cellulase synthesis. In the absence of an inducer such as cellulose, cellobiose (*21, 22*), or sophorose (*12, 13, 14, 23*), *Trichoderma* does not make any detectable "cellulase complex" enzymes. The true physiological inducer of cellulase is currently unknown. Insoluble cellulose is presumably not such an inducer since there is no way for the internal cell machinery to sense the presence of this insoluble material. However, a small transglycosylation product such as sophorose, 2-O-β-glucopyranosyl-D-glucose, may well be the natural inducer.

The simultaneous action of these physiological and enzymatic regulatory mechanisms during industrial enzyme production and large scale saccharification represents one of the primary bottlenecks in the economical production of glucose syrups from cellulosic materials. Our objectives have been to devise selective screening techniques which allow for the isolation of mutant strains that can specifically overcome these catabolite repression and end-product inhibition regulatory mechanisms and at the same time hyperproduce one or all of the individual enzymes in the cellulase complex. Such mutants will be useful in reducing the cost of enzyme production as well as contributing knowledge towards a basic understanding of the mechanisms controlling enzyme synthesis, secretion and mode of action at the cellular level.

Previous efforts to increase cellulase production by *Trichoderma* (*24, 25*) have met with limited success primarily due to the lack of selective screening methods. Problem areas have included an inability to isolate the progeny of a single spore because of the fast-spreading nature of the fungus and lack of a visual screening method that would allow selection on agar medium. All survivors after mutation have previously been tested individually in shake flasks for evaluation of enzyme production. Addition of colony-growth restrictors such as oxgall (*26*), rose bengal (*27*), Phosfon D (*28*), and nonionic detergents such as deoxycholate (*29*) and saponin (*30, 31*), as well as other paramorphologic agents (*32, 33*), have largely overcome the first problem.

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Incorporation of such colony restrictors into agar medium allows development of small discrete colonies from individual spores; as many as 50–100 colonies can be screened per petri plate, depending on the colony-restricting substance used. Visual detection of cellulase activity on agar plates in a reasonably short period of time has proved to be more difficult. Plate assays based on clear zone diameters have been reported utilizing cellulose swollen with phosphoric acid as a substrate (*34, 35*). However, these agar diffusion assays have not been coupled with a colony-growth inhibitor to allow for screening of more than one organism at a time and require 7–14 days for visual detection of clearing. Recently, tests have been described (*36, 37*) which employ cellulose–azure in an agar overlay for detecting fungal cellulase production. Again the test is limited to one organism per tube, which rules out its use in large-scale selective screening of mutants. In screening for mutants that hyperproduce all of the enzymes in the cellulase complex, we have effectively utilized Walseth acid swollen cellulose as a substrate in conjunction with 1.5% oxgall and 500 μg/mL Phosfon D as colony growth inhibitors (*38*). Rapid evaluation of the mutants after colony formation can be achieved after overnight incubation of the plates at 50°C. The oxgall and the Phosfon D act together to retard hyphal spreading and in some way enhance visualization of cellulase activity. Although 50°C is a temperature which is not permissive for growth of *Trichoderma,* it is the optimum temperature for the activity of the cellulase enzymes. Incubation of the screening plates at this temperature allows rapid expression of the enzymatic activities, resulting in distinct clear zones surrounding the potential mutant colonies when acid swollen cellulose is incorporated as a substrate in the agar plates.

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Table II. Screening Methodologies for Isolation of High-Yielding

Enzyme	*Colony Inhibitor*	*Substrate*
Total cellulase complex	1.5% oxgall + Phosfon D	acid swollen cellulose
Endoglucanase	1.5% oxgall + Phosfon D	carboxymethylcellulose
β-Glucosidase	(1) 1.5% oxgall + Phosfon D	esculin
	(2) 1.5% oxgall + Phosfon D	1% cellobiose
	(3) 1.5% oxgall + Phosfon D	any of above

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Selection of Hyperproducing Mutants for Each of the Enzymes in the Cellulase Complex

The screening methodologies used in the selection of high yielding cellulase mutants are summarized in Table II. Mutants with enhanced production of all the enzymes in the cellulase complex can be isolated utilizing oxgall, Phosfon D, and acid swollen cellulose as described above. Sequential increased production can be detected by increasing the cellulose concentration, the agar thickness, and ultimately selection for colonies with enhanced ratios of clear zone diameter to colony diameter.

Selection of hyperproducing endoglucanase mutants can be achieved by substituting a modified cellulose such as carboxymethylcellulose (CMC) for the acid swollen cellulose and retaining the oxgall and Phosfon D as colony growth inhibitors. CMC is not attacked by cellobiohydrolase or cellobiase but is subject to random splitting by the endoglucanase to yield oligomers of glucose. After growth and colony formation, the colonies are replicated and then plates flooded with cetylpyridinium chloride (other quaternary ammonium compounds may be substituted (*39*)). These quaternary ammonium compounds react with the long chain polysaccharides to form a dense white precipitate. Where the organism has secreted large quantities of endoglucanases, the CMC will have been digested and clear zones will be formed. Again sequential enzyme increases may be visualized by raising the CMC concentration and selecting colonies with increased ratios of clear zone diameter to colony diameter.

Cellobiase or β-glucosidase mutants can be detected by a number of plate assay techniques: (1) esculin–ferric ammonium citrate, (2) cello-

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and Catabolite Repression-Resistant Cellulase Mutants

Catabolite Repressor	*Visualization*
5% glycerol	clear zones after incubation at 50°C
5% glycerol or 5% glucose	clear zones after flooding with quaternary ammonium compounds
5% glucose	black rings in the presence of ferric ammonium citrate
0.2% 2-deoxyglucose	large versus pinpoint colonies
5% glycerol	overlay with Glucostat + cellobiose, 50°C, 30 min, red spots

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biose–2-deoxyglucose, or (3) cellobiose–glucose oxidase. In each case oxgall and Phosfon D are incorporated as colony inhibitors. The first method, esculin–ferric ammonium citrate, is a modification of a method previously described by Eberhart (*40*) and employs 0.1% esculin (6,7-dihydrocoumarin-6-glucoside) as a substrate. In the presence of aryl-glucosidase, esculin is split into glucose and esculetin. The esculetin reacts with the ferric ammonium citrate (0.05%), which is present in the agar, to form a black precipitate. In the second method, cellobiose (1%) and 2-deoxyglucose (0.2–0.5%) are incorporated in the agar. Those organisms which are unable to make cellobiase are forced to utilize the 2-deoxyglucose and after minimal growth die because of the toxicity of the antimetabolite. Those mutants capable of synthesizing cellobiase are able to "ignore" the 2-deoxyglucose and grow on the cellobiose. The size of the colony should be directly proportional to the amount of cellobiase and thus mutants are distinguished from wild type on the basis of colony size. The third method for detecting cellobiase activity on plates uses a modification of a method previously described for the selection of high-yielding yeast-invertase mutants (*15*). The surviving colonies are overlayed with a piece of filter paper, which is sprayed with a mixture of cellobiose and Glucostat reagent (Worthington Biochemicals, Freehold, NJ). After incubation at 50°C for 30 min, those colonies with cellobiase activity will yield red spots on the filter paper resulting from the reaction of the glucose, derived from cellobiose, with the Glucostat reagent. It should be noted in the above procedures that two independent enzymes have been considered, cellobiase with optimal activity towards cellobiose and aryl-β-glucosidase with optimal activity towards aryl glucosides. As the substrate specificity of each enzyme is not absolute, the above screening methods do not distinguish between each type of enzyme. Differentiation can only be accomplished through enzyme purification and characterization.

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Isolation of Constitutive, Catabolite Repression, and End-Product Inhibition-Resistant Mutants

Constitutive mutants may be isolated by plating the survivors from mutagen treatment on agar-salts medium containing only colony growth inhibiting substances and a carbon source (e.g., glucose or sucrose) that is not an inducing substrate. Following growth, the plates are overlayed with the specific selective-screening medium described in Table II that will allow visual detection of the desired enzymatic activity. The plates are generally incubated at 50°C overnight. This temperature is not permissive for growth and presumably will not allow de novo protein synthesis. An inhibitor of protein synthesis (e.g., actidione) can be

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incorporated into the overlay if preferred. The plates are subsequently screened for mutant colonies which have made the desired enzyme constitutively. Mutants that no longer require insoluble cellulosic material as an inducer during cellulase production should allow for less sophisticated fermentor design and could be used for continuous enzyme production, as opposed to the present batch production system.

Catabolite repression-resistant mutants can be isolated by adding high concentrations (5% or more) of a catabolite repressor such as glucose or glycerol in combination with the specific cellulosic substrate. All those organisms that remain sensitive to carbon catabolite repression will preferentially grow on the repressor and not synthesize any of the cellulase complex enzymes. Those colonies that are resistant to catabolite repression will secrete the particular cellulase-complex enzyme in spite of the repressor and the activity of the enzyme can be visualized in the appropriate screening agar. The antimetabolite 2-deoxyglucose is particularly useful in selecting catabolite repression-resistant mutants since at concentrations above 0.5% it can be used as a catabolite repressor as well as an antimetabolite. By incorporating 2-deoxyglucose into any of the previously described selective-screening methodologies, it is possible to enrich for the desired phenotype. Catabolite repression-resistant mutants will be useful in lowering the cost of cellulase by allowing substitution of inexpensive crude medium components during enzyme production stage. It may be possible, by utilizing such mutants, to digest cellulosic materials directly to glucose syrups, eliminating the need for two-stage enzyme production and saccharification.

Mutants resistant to end-product inhibition may be obtained by adding high concentrations of the end product to the selective-screening system. For example, high concentrations of glucose will noncompetitively inhibit cellobiase activity (*6*). Mutants screened on any of the three selective media for cellobiase detection that retain this activity in the presence of high concentrations of glucose should show increased resistance to end-product inhibition of the enzymatic activity. Enzymatic saccharifications to date have not achieved 100% conversion of cellulose to soluble sugars (*41*) primarily because of end-product inhibition of the enzymes. Enzymes which are resistant to such inhibition, perhaps used in an immobilized mode, could vastly change the economics of enzymatic hydrolysis of cellulose.

Illustrative Mutants of T. reesei *Isolated Utilizing These Selective Screening Techniques*

Utilizing these screening techniques roughly 800,000 colonies have been screened and approximately 100 mutants have been isolated. Complete characterization of these mutants is a slow and tedious process.

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Rut-NG14 and Rut-C30 have been most fully investigated to date. The genealogy of these mutants is shown in Figure 1.

Rut-NG14 was isolated employing the acid swollen cellulose, oxgall, Phosfon D screening method in the presence of 5% glycerol as a catabolite repressor (*42*). This mutant under fermentor conditions synthesizes approximately 20 times the filter paper activity (a measure of the synergistic activity of all the cellulase complex enzymes) when compared with the wild type QM6a in shake flask. Endoglucanase and cellobiase activities are both increased in this mutant (*42*). Although Rut-NG14 was isolated in the presence of a catabolite repressor (5% glycerol) it is only partially resistant to catabolite repression. Significant amounts of endoglucanase and filter paper degrading activity can be detected, but the yield is not comparable to that under nonrepressed conditions. We insert here a note of caution. Rut-NG14 is representative of a group of mutants whose physiological characteristics in submerged culture do not parallel those on agar plates. In spite of the lack of complete catabolite resistance, Rut-NG14 is an extremely useful mutant. Figure 2 shows the production of cellulase under controlled fermentor conditions by Rut-

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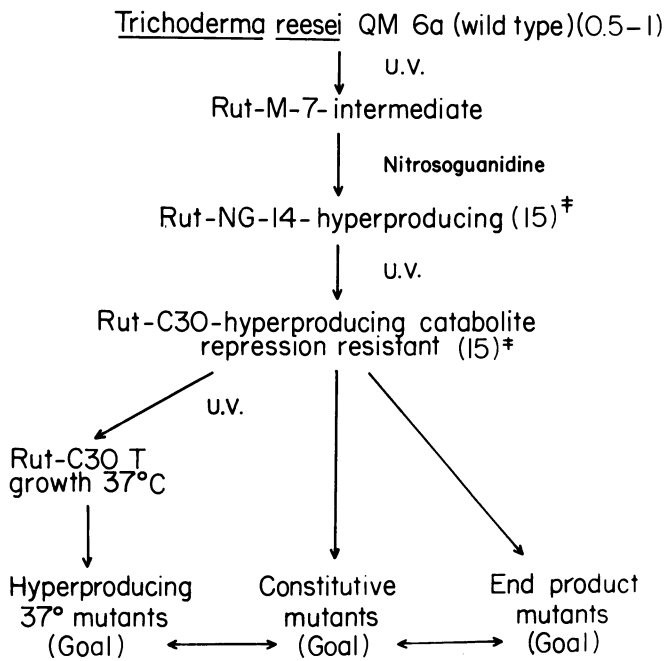

Figure 1. Genealogy of high-yielding cellulase mutants. Yield of cellulase in FP units/mL (42) under controlled fermentor conditions (=) and shake flasks (*).

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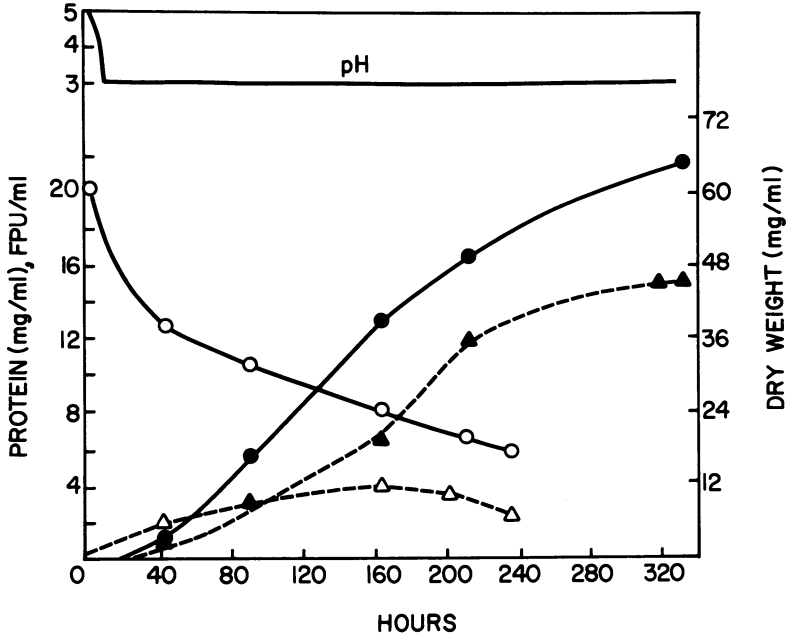

Figure 2. Cellulase synthesis by Rut-NG14 in a 6% cellulose fermentation. (Data from M. Mandels, U.S. Army Laboratories, Natick, Mass.) (○—○) Residual dry weight, (▲—▲) FP units/mL (42), (●—●) soluble protein, (△—△) mycelial protein. Substrate: 6% two-roll milled cotton; Mandels' (42) basic salts medium without urea; inoculum 20% v/v of a 3-day-old shake flask mycelium; pH ≥ 3, continuous addition of NH_4OH.

NG14. This mutant is capable of elaborating 15 FP units/mL with a productivity of 45 units/L/hr. The final concentration of soluble extracellular protein is 21.2 mg/mL. Samples of this enzyme preparation were sent to G. Pettersson at the University of Uppsala, Sweden, for quantification of each of the enzymes in the cellulase complex using purified antibodies to the individual enzymes. The quantitative antigen–antibody reaction showed that 600 mg/g of this enzyme preparation was one enzyme, cellobiohydrolase. This represents a yield of 13 g/L of cellobiohydrolase, which is a 100-fold increase over the amount of cellobiohydrolase obtained with strain QM 9414 (130 mg/L) the best previously existing cellulase mutant (G. Pettersson, personal communication).

Since Rut-NG14 was only partially resistant to catabolite repression but was high yielding for cellobiohydrolase, we used Rut-NG14 as an initial mutagenized strain and after subsequent mutagenesis and selection on cellobiose-2-deoxyglucose medium, isolated Rut-C30. Figure 3 shows cellulase production in shake flasks by Rut-C30 in the presence and absence of a catabolite repressor (5% glycerol). For comparative pur-

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poses, *T. reesei* QM 9414, an organism which is sensitive to catabolite repression, has been run in parallel. Rut-C30 makes almost as much filter paper degrading activity in the presence of 5% glycerol as it does during growth on cellulose alone. If the carbon to nitrogen ratios in such an experiment are adjusted to 10:1 by the addition of 0.6% peptone to the growth medium to match the 6% carbohydrate (5% glycerol and 1% cellulose), no differences in cellulase yield from Rut-C30 can be detected under repressed and nonrepressed conditions. Moreover, the synthesis of cellobiase (Figure 4) and endoglucanase (data not presented) by Rut-C30 are also resistant to catabolite repression by either 5% glycerol or 5% glucose. Rut-C30 has been tested for enzyme production under controlled fermentor conditions and yields similar productivity to Rut-NG14 (15 FP units/mL). Thus both mutants show a 15–20-fold increase in cellulase activity over the wild type strain QM6a (0.5–1.0 FP units/mL) in shake flasks. Quantitation of the specific amounts of the individual enzymes of Rut-C30 with specific antibodies has not been carried out to date. Our objective in isolating catabolite-repression-resistant

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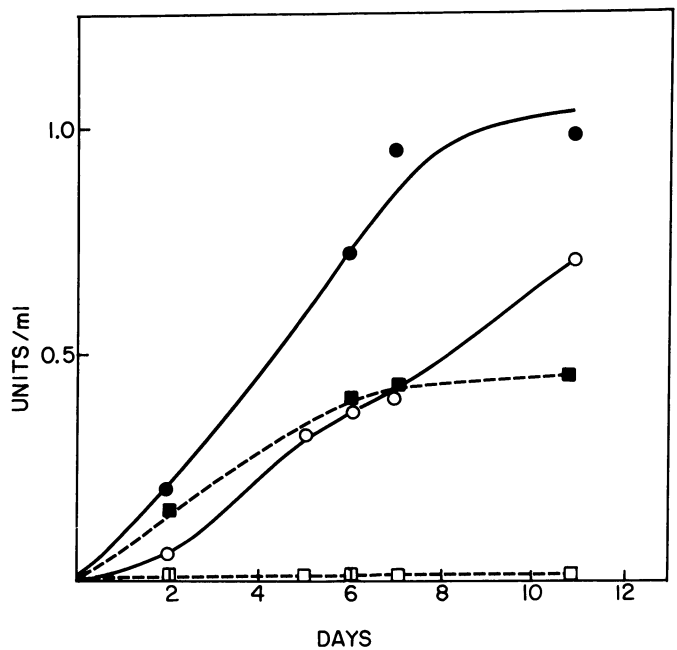

Figure 3. Cellulase production in shake flasks by Rut-C30 and QM 9414 under repressed and nonrepressed conditions. Antibiotic disk as substrate (43). (●—●) Rut-C30, 1% cellulose, (○—○) Rut-C30, 1% cellulose, 5% glycerol, (■—■) QM 9414, 1% cellulose, (□—□) QM 9414, 1% cellulose, 5% glycerol. Vogel's medium (44), 0.1% peptone, 0.2% Tween 80, 1% SW40 Solka floc.

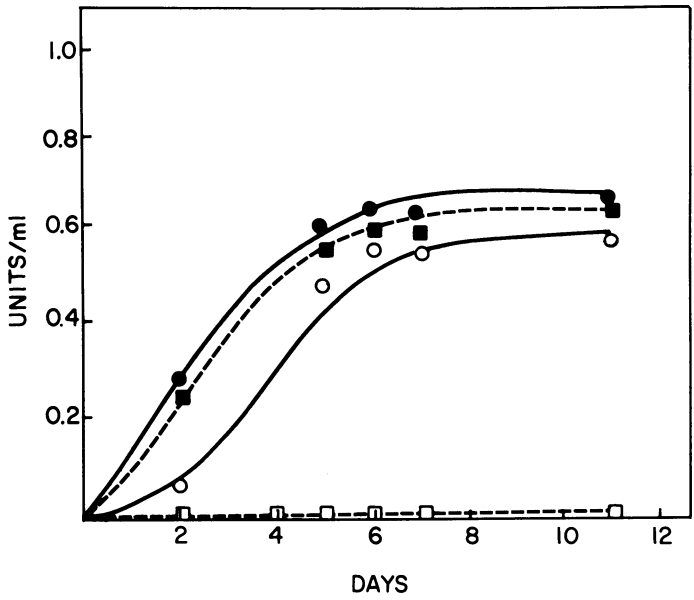

Figure 4. Cellobiase production. Rut-C30 and QM 9414 under repressed and nonrepressed conditions. Growth conditions same as Figure 3. Cellobiase activity was measured using cellobiose as a substrate and quantitating free glucose with Glucostat reagent (Worthington Biochemicals, Freehold, N.J.). (●—●) Rut-C30, 1% cellulose, (○—○) Rut-C30, 1% cellulose, 5% glycerol, (■—■) QM 9414, 1% cellulose, (□—□) QM 9414, 1% cellulose, 5% glycerol.

strains of *T. reesei* was to lower the cost of enzyme production through the use of inexpensive medium components. Rut-C30 has been tested for cellulase production in shake flasks after growth on cellulose and corn steep liquor (an inexpensive nitrogen source). There is no detectable loss in enzyme yield when compared with cellulase production on more costly chemically-defined medium.

Summary

A number of selective-screening methodologies have been devised that have allowed isolation of a series of hyperproducing and catabolite repression-resistant mutants of *T. reesei*. Yields of cellulase of 15 units/mL under controlled fermentor conditions have been achieved with both Rut-NG14 and Rut-C30. Quantitative reaction of Rut-NG14 enzyme preparation with purified antibodies to cellobiohydrolase shows that in this mutant, the cellobiohydrolase is specifically hyperproduced relative to the rest of the enzymes in the cellulase complex. Rut-C30, which was derived from Rut-NG14, shows resistance to catabolite repression for

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filter paper degrading enzymes, endoglucanase, and cellobiase and is capable of producing these enzymes during growth on cellulose and corn steep liquor alone. Future goals are to isolate constitutive mutants and end-product resistant strains as well as continually increase overall cellulase yields. Mutants Rut-NG14 and Rut-C30 are capable of elaborating 2% extracellular protein, the majority of which is cellulase. A yield of 10% extracellular protein as cellulase would greatly improve the economics of cellulose saccharification through enzymatic digestion.

Acknowledgments

We are grateful to Mary Mandels and Raymond Andreotti of the U.S. Army Natick Laboratories for testing the mutants under controlled fermentor conditions (Figure 2); and to Göran Pettersson of the University of Uppsala, Sweden, for the antibody determinations.

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15

Degradation of Delignified Sprucewood by Purified Mannanase, Xylanase, and Cellulases

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Sprucewood holocellulose was treated with an endo-β-1,4-mannanase isolated from Aspergillus niger *and an endo-β-1,4-xylanase, two avicelases, and a cellobiohydrolase C isolated from* Trichoderma viride. *The mannanase hydrolyzed about a quarter of the mannan in 2–3 days without xylan or cellulose degradation. The xylanase hydrolyzed about half the xylan with 10% mannan solubilization. The three cellulases hydrolyzed up to 45% of the cellulose and 20% of the xylan, accompanied by 40–70% solubilization of the mannan. Combined xylanase–mannanase treatment hydrolyzed about half the xylan and mannan. Addition of mannanase to to cellulase-treated samples increased the degradation of the cellulose and mannan. Micromorphological studies of the variously treated specimens revealed a loss of substances in P/S_1, T, and adjacent zones of S_2 of the tracheid wall.*

Fine structural studies on woody cell walls attacked by ectoenzymes of fungi in situ are numerous (cf. *1, 2*). In contrast, investigations on the selective degradation of cell walls by enzymes isolated from fungi are few. Jutte and Wardrop (*3*) attempted the use of crude commercial cellulase preparations to determine the degradation pattern of *Valonia* cellulose and beechwood fibers. Similar use of commercial preparations of enzymes was made by Reis and Roland (*4*) to evaluate the nature of diverse cell walls and to show the distribution of polysaccharides. An endo-β-1,4-xylanase with specific xylanolytic activities was isolated from a commercial cellulase preparation using chromatographic methods and

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acted upon sprucewood holocellulose by Boutelje et al. (*5*) and Boutelje and Hollmark (*6*); they showed the degradation pattern of the tracheid wall using interference microscopic methods. In a similar way, Sinner et al. (*7*) studied the action of endo-β-1,4-xylanases, also isolated from commercial cellulase preparations, on the fine structure of cell walls in beechwood holocellulose; they used transmission electron microscopy to evaluate the fine structural changes of such enzymically hydrolyzed walls. Comptat et al. (*8*) and Ruel et al. (*9*) also conducted studies on the purification of xylanases from commercial enzymes and the action of these on fibres of *Arundo donax* as substrate. Extending their work on xylanases, Sinner et al. (*10*) tested the action of purified xylanases and avicelases on the holocellulose of beechwood; transmission electron microscopy revealed again the fine structural location of xylan and cellulose decomposition.

In the present work, one of the endo-β-1,4-xylanase (E.C. 3.2.1.8), the endo-β-1,4-mannanase (E.C. 3.2.1.78), and the avicelases used in the former experiments with beechwood holocellulose (*10, 11*) were applied to sprucewood holocellulose in order to obtain a better understanding of the individual and combined actions of these enzymes on the complex carbohydrate skeleton of wood. The experiments could be conducted only with a limited number of samples; therefore, the figures given in this chapter have to be considered as preliminary results.

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Experimental

Enzymes. The mannanase was isolated from *Aspergillus niger* (*11*); fraction 4 b was used throughout the experiments (cf. Table 1 in (*11*)). The xylanase 2, the avicelase 1, and the avicelase 2 were isolated from *Trichoderma viride* (*10*). The properties of these enzymes have also been described in the papers cited above. The cellobiohydrolase C was kindly supplied by Dr. R. D. Brown, Jr., and Dr. E. K. Gum, Jr. (Virginia Polytechnic Institute, Blacksburg). The isolation (from *Trichoderma viride*) and the properties of the cellobiohydrolase C are described in their 1974 paper (*12*).

Substrates. Sprucewood particles of 0.07–0.1 mm (*Picea abies* (L.) Karst.) were delignified with sodium chlorite at room temperature (*13*). The following analytical data were obtained on the holocellulose: yield = 75.5% (nonextracted dry wood = 100); moisture content = 10.9% (conditioned holocellulose = 100); KLASON lignin = 3.6% (dry weight = 100). The quantitative analysis of sulfuric acid hydrolysates (hydrolysis according to Saeman et al. (*14*)) by column chromatography (see below) showed the following composition (percentage of total sugars, Figure 1): glucose = 70; mannose = 16.6; xylose = 8.2; galactose = 1.9; arabinose = 1.4; 4-O-methylglucuronic acid = 1.5 (calculated on the basis of the calibration factor of glucose). The calculation of enzymatic degradation of individual cell wall polysaccharides was based on the total sugars in the acid hydrolysate of the holocellulose. For glu-

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cose derived from galactoglucomannan, the ratio mannose:glucose = 3.3:1 was applied (*15*). The galactose found was considered to be derived from the mannan. In this way, the following figures were obtained for sprucewood holocellulose (percentage of total sugars):cellulose: 65; galactoglucomannan: 24; arabino-4-O-methylglucuronoxylan: 11. The preparation and properties of the beechwood (*Fagus sylvatica* L.) holocellulose used in one experiment have been described earlier (*10*) (Figure 1).

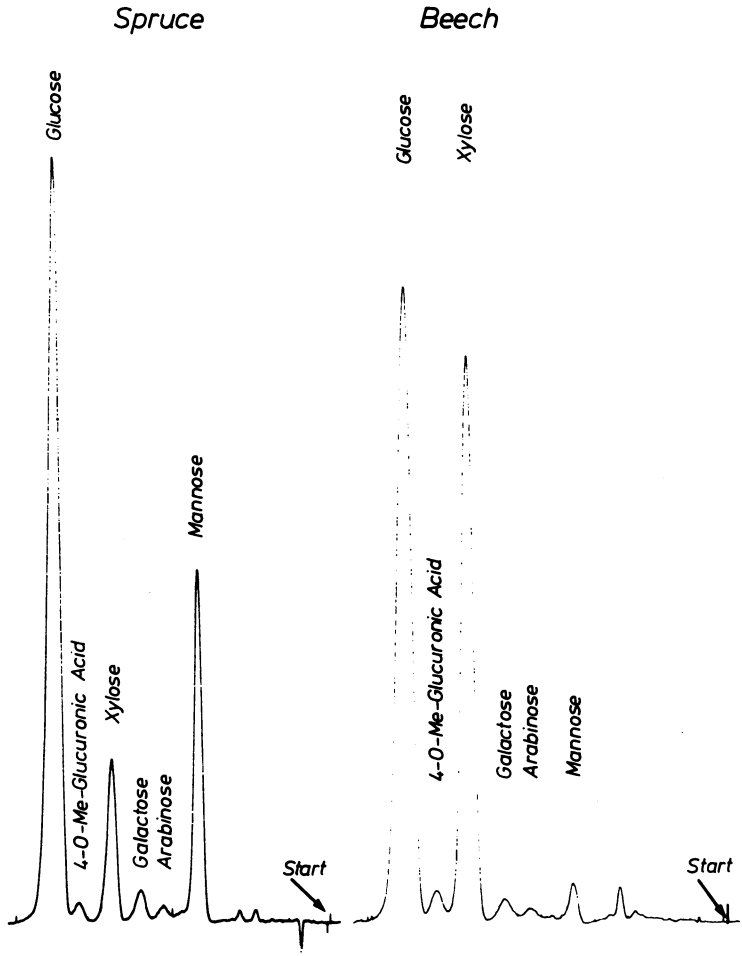

Figure 1. Sulfuric acid hydrolysates of holocelluloses of sprucewood and beechwood. Separation on Durrum DA-X4 (column 0.4 × 30 cm) with 0.49M potassium–borate buffer at pH 9.2 and 60°C (16); detection with 2',2-bicinchoninate (17).

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Enzymatic Degradation. The degradation experiments were carried out as described for beechwood holocellulose (*10*). Buffered suspensions of holocellulose (never dried), equivalent to about a 50-mg dry weight, were treated with mannanase (1.0 mL; equivalent $E_{280\,nm} = 1.4$ and relative activity tested with galactomannan at 37°C = 86 μmol mannose/min), or xylanase 2 (0.2 mL; equivalent $E_{280\,nm} = 2.6$ and relative activity tested with beechwood xylan at 37°C = 268 μmol xylose/min), or avicelase 1 (0.2 mL; equivalent $E_{280\,nm} = 6.6$), or avicelase 2 (0.1 mL; equivalent $E_{280\,nm} = 5.3$), or cellobiohydrolase C (2.08 mg; equivalent $E_{280\,nm} = 4.4$). In a second series, 50-mg holocellulose samples were first treated with xylanase 2, or avicelase 1, or avicelase 2, or cellobiohydrolase C (same quantities as above) and after 48 hr mannanase (1.0 mL) was added. At the end of the experiments, i.e., after 48 or 80 hr, the enzyme–carbohydrate solutions were separated from the remaining solids over a D-4 frit. The solutions were hydrolyzed with sulfuric acid (*14*). The remaining solids were washed on the frit with water and ethanol and stored in ethanol at 4°C for electron microscopy.

Analysis of Degradation Products. The enzymatic degradation of holocellulose was followed by borate complex ion exchange chromatography as described previously (*10, 16*), with a new nonaggressive dye reagent for quantitative detection of sugars in the column eluate (*17*). For this purpose, 5 to 25 μL of the reaction solutions was analyzed at regular intervals and 5 to 10 μL of acid hydrolysates of the separated reaction solutions (see above) was analyzed.

Electron Microscopy. The samples were treated and analyzed as described previously (*10*).

Chemical Results

Mannanase. Sprucewood delignified at room temperature was treated with mannanase as described by Yamazaki (*15*). However, a higher mannanase concentration was used, and the reaction was followed by means of quantitative, column chromatographic analysis of the reducing sugars in the reaction solutions.

The galactoglucomannan of the holocellulose was degraded by the mannanase. The reaction was slow, but conversion showed a steady increase. After 80 hr of incubation, about 25% of the mannan was transformed into water-soluble products (Table I, Column 10). This figure was calculated on the basis of the mannose, glucose, and galactose present in the acid hydrolysate (cf. Experimental).

Mannobiose was predominant throughout the enzymatic reaction; its amount relative to mannose decreased, however, with incubation time (Table II). Besides mannobiose and mannose, glucose was present in the reaction solutions (Figure 2). The amount of glucose relative to the mannosaccharides increased with time: it was about 1:8 after 5 hr of incubation and about 1:4–5 after 23 to 80 hr. No cellobiose and no degradation products from the arabino-4-O-methylglucuronoxylan were found (cf. Figure 2 and Table I, Columns 3 and 4).

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Part of each reaction solution, after 48 and 80 hr of mannanase treatment and after removal of the undegraded material, was hydrolyzed with sulfuric acid. Quantitative sugar chromatography of the hydrolysates showed that the amount of soluble degradation products from the mannan after 48 hr was nearly the same as after 80 hr of incubation with mannanase. The difference between the reducing sugars detected in the reaction solutions before and after acid hydrolysis represented oligomer or polymer mannan fragments (Table I, Columns 2 and 6). They were solubilized by the action of the mannanase but could not be detected by the liquid chromatographic system used. The ratios of mannose:glucose: galactose in the acid hydrolysates after 48 hr and after 80 hr of incubation with mannanase were 1:0.3:0.04. No sugars derived from cellulose or arabino-4-O-methylglucuronoxylan were detected in the acid hydrolysates.

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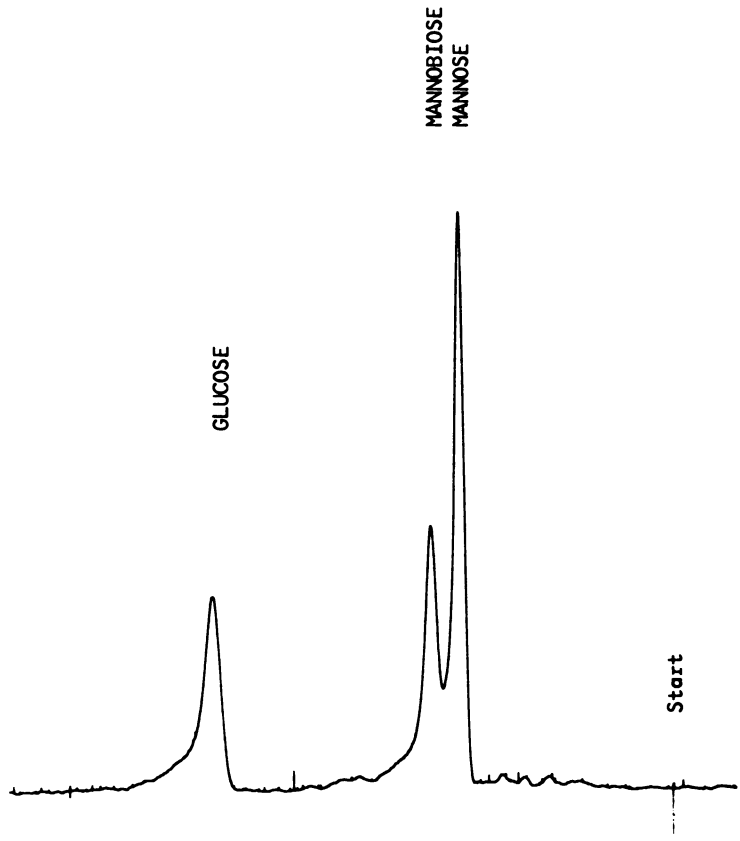

Figure 2. Sugars from sprucewood holocellulose treated with mannanase for 80 hr. (For separation and detection method see Figure 1).

Table I. Degradation of Sprucewood and

Holocellulose (Enzyme Treatment) 1		*Degradation (%)* Reducing Sugars (Dry Sample = 100) *Mannan* 2	*Xylan* 3	*Cellulose* 4	*Total* 5
Spruce–mannanase	(48 hr)	4	0	0	4
	(80 hr)	6	0	0	6
Spruce–xylanase 2	(48 hr)	0	4	0	4
Spruce–avicelase 1	(48 hr)	0	0.6	20	21
Spruce–avicelase 2	(48 hr)	0	0.7	28	29
Spruce–cellobiohydrolase C	(48 hr)	0	0.5	23	24
Beech–cellobiohydrolase C	(48 hr)	0	0.6	9	10

Table II. Weight Ratios of Di- and Monosaccharides Formed

Enzyme Treatment—Ratios of Reaction Products	*Incubation Time (hr)* 5	8	23
Mannanase			
Mannobiose:Mannose	4	3	
Xylobiose:Xylose	—	—	—
Cellobiose:Glucose	—	—	—
Xylanase–Mannanase			
Mannobiose:Mannose	—	—	—
Xylobiose:Xylose	4	2	1
Cellobiose:Glucose	G	G	G
Avicelase 1–Mannanase			
Mannobiose:Mannose	—	—	—
Xylobiose:Xylose	X_2	X_2	X_2
Cellobiose:Glucose	34	22	11
Avicelase 2–Mannanase			
Mannobiose:Mannose	—	—	—
Xylobiose:Xylose	1	2	2
Cellobiose:Glucose	8	6	4
Cellobiohydrolase–Mannanase			
Mannobiose:Mannose	—	—	—
Xylobiose:Xylose	X	X	X
Cellobiose:Glucose	10	10	4

[a] G = glucose only; X = xylose only; M = mannose only; X_2 = xylobiose only; (—) = neither of the two sugars present.

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Beechwood Holocellulose by Individual Enzymes

Degradation (%)						
Total Carbohydrates						
(Dry Sample = 100)				*(Sugars from Each Component in Total Hydrolysate of Dry Sample = 100)*		
Mannan 6	*Xylan* 7	*Cellulose* 8	*Total* 9	*Mannan* 10	*Xylan* 11	*Cellulose* 12
6	0	0	6	24	0	0
6	0	0	6	25	0	0
3	6	0	9	12	55	0
13	2	21	36	55	23	32
17	2	30	49	71	22	46
10	2	17	28	40	15	25
0	3	10	14	0	10	17

by Enzymatic Hydrolysis of Sprucewood Hollocellulose[a]

Incubation Time (hr)				
48	*50*	*54*	*72*	*80*
2 — G	— G	— G	2 — G	2 — G
— 0.8 G	4 0.7 G	3 0.6 G	2 0.5 G	2 0.3 G
— X_2 8	5 4 2	5 4 0.5	4 2 0.1	3 2 0.05
— 2 2	6 4 0.7	5 3 0.3	4 3 0.1	3 3 0.04
— X 2	M X 0.8	3 X 0.3	3 X 0.1	3 X 0.06

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Control samples of sprucewood holocellulose were treated as described before, but without mannanase. The solutions contained almost no reducing sugars. After additional acid hydrolysis, about 1% sugars were found (dry weight of holocellulose = 100). Mannose were predominant; glucose, galactose, arabinose, and xylose were present in smaller amounts.

Xylanase. The xylanase degraded about half the arabino-4-O-methylglucuronoxylan of delignified sprucewood in 48 hr (Table I, Column 11). The main reducing sugars were xylobiose, xylose, arabinose, and oligosaccharides. Xylobiose was predominant throughout the reaction; for the amounts relative to xylose, see Table II. The amounts of arabinose relative to xylose plus xylobiose were in the ratio of 1:3 throughout the reaction, with a slight increasing tendency over the incubation period. Parts of the oligosaccharides probably contained uronic-acid residues. Xylotriose and higher xylooligomers might also be present. Under the separation conditions applied in sugar chromatography, aldo-oligouronic acids were eluted from the column between rhamnose and arabinose, and xylooligomers were superposed on xylobiose. For the areas of the corresponding peaks in the chromatograms, the correction factor of xylobiose was applied to calculate the approximate amount of these oligosaccharides. Therefore, it was to be expected that the degradation rate of xylan calculated from direct sugar analysis of the reaction solution would be inferior to the corresponding value obtained from the acid hydrolysate (Table I, Columns 3 and 7).

The ratios of arabinose:xylose and 4-O-methylglucuronic acid:xylose in the reaction solution after acid hydrolysis were about the same as those of holocellulose before xylanase treatment (cf. Experimental). Oligomer or polymer mannan fragments were present in the reaction solution. They were detected after additional acid hydrolysis, and they represented about 12% of the mannan present in the starting material (Table I, Columns 2 and 6).

The xylanase treatment liberated only minor amounts of glucose; no cellobiose was detected. The higher amounts of glucose found after additional acid hydrolysis probably were derived from the dissolved mannan fragments. No glucose was left when the calculation of the mannan fragments was based on a ratio of mannose:glucose = 3.0–3.3:1, as is generally accepted for sprucewood mannans (*15*).

Avicelases or Cellobiohydrolase C. Treatment of sprucewood holocellulose with the three different cellulose-splitting enzymes gave very similar results. The cellulose was hydrolyzed to about 25–45% in 48 hr (Table I). Cellobiose was the predominant reaction product, but the amount of glucose increased considerably with incubation time (Table II). Acid hydrolysis of the reaction solutions showed that higher-

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molecular-weight, water-soluble degradation products derived from cellulose were present only in minor amounts or even were absent (Table I, Columns 4 and 8).

The arabino-4-O-methylglucuronoxylan was also partly hydrolyzed by the three cellulases. The degradation rates ranged from 15% to 23%. Also, relative to the corresponding values of cellulose degradation, the results obtained for the three cellulases were rather similar (0.5–0.7, Table III, Column 15). The degradation products were mainly oligomer or even higher-molecular-weight xylan fragments, as judged from the results of sugar chromatography before and after acid hydrolysis of the reaction solutions (Table I, Columns 3 and 7). Arabinose was not liberated by the treatments with cellulases. The ratios of arabinose:xylose in the reaction solutions after acid hydrolysis were about the same as in holocellulose before cellulase treatment; the ratios of 4-O-methylglucuronic acid:xylose, however, were lower (1:7 to 1:10, cf. Experimental).

No mannose or mannobiose was detected in the reaction solutions, but high-oligomer or high polymer mannan fragments were present in large amounts. These fragments represented about 40–70% of the initial mannan of the holocellulose. The degradation rates of mannan relative to the corresponding values of cellulose degradation were rather similar for the three cellulases (1.5–1.7; *see* Table III, Column 13) and were higher than in the case of xylan.

Xylanase + Mannanase. The addition of mannanase to a delignified sprucewood sample, which had been incubated with xylanase 2 for 48 hr, effected an increased degradation (Figure 3). Mannan of the holocellulose and the mannan fragments already present in the reaction solution (cf. Mannanase) were hydrolyzed to reducing sugars. Their amounts increased rapidly in the first hours after the mannanase was added, but even at the end of the experiment, the degradation curve for mannan showed a steady increase. At the end of the experiment (80 hr; 48 hr with xylanase alone and 32 hr with xylanase + mannanase), about half the mannan of the starting material was hydrolyzed, mostly to mono- and disaccharides (Table III, Columns 6 and 10).

The main reaction products after the addition of mannanase were mannobiose, mannose, and glucose. Mannobiose was predominant; its amount relative to mannose decreased with incubation time, as in the case of sprucewood treated solely with mannanase (Table II). The ratio of mannose:glucose:galactose in the acid hydrolysate of the reaction solution (80 hr) was 1:02:0.04.

The xylan retained in the tissue after the xylanase treatment (first 48 hr) was left almost unaffected by the simultaneous action of xylanase and mannanase. Only xylobiose and higher-molecular xylan fragments, which were already present in the reaction solution (cf. Xylanase), were

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Table III. Degradation of Sprucewood

Enzyme Treatment 1		Degradation (%): Reducing Sugars (Dry Sample = 100) Mannan 2	Xylan 3	Cellulose 4	Total 5	Total Carbohydrates (Dry Sample = 100) Mannan 6	Xylan 7
Xylanase–Mannanase							
Before adding mannanase	(48 hr)	0	4	0	4	3	6
End of experiment	(48 hr)	12	6	0	18	14	6
Avicelase 1–Mannanase							
Before adding mannanase	(48 hr)	0	0.6	20	21	13	3
End of experiment	(48 hr)	12	1	40	56	23	4
Avicelase 2–Mannanase							
Before adding mannanase	(48 hr)	0	0.7	28	29	17	2
End of experiment	(80 hr)	12	1	43	59	23	3
Cellobiohydrolase–Mannanase							
Before adding mannanase	(48 hr)	0	0.5	23	24	10	2
End of experiment	(80 hr)	10	0.6	33	45	18	3

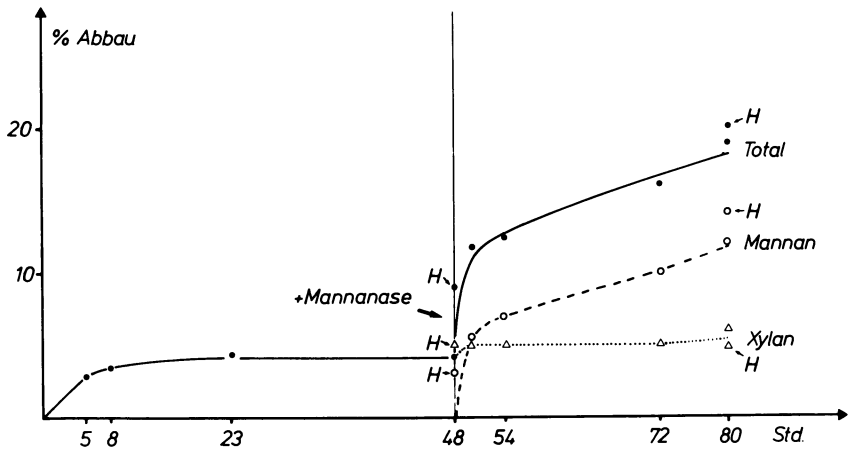

Figure 3. Decomposition of sprucewood holocellulose by xylanase 2 + mannanase, monitored by quantitative sugar chromatography. Ordinate: degradation in percent of dry holocellulose based on the amount of reducing sugars in the reaction solution. (●——●) total degradation products, (▲– · – · –▲) degradation products from cellulose, (○ – – – ○) degradation products from mannan, (△ · · · △) degradation products from xylan.

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Holocellulose by Two Enzymes

Degradation (%)

Total Carbohydrates							
(Dry Sample = 100)		*(Sugars from Each Component in Total Hydrolysate of Dry Sample = 100)*					
Cellulose 8	*Total* 9	*Mannan* 10	*Xylan* 11	*Cellulose* 12	*Mannan/ Cellulose* 13	*Xylan/ Mannan* 14	*Xylan/ Cellulose* 15
0	9	12	55	0	—	4.5	—
0	20	57	57	0	—	1.0	—
21	35	55	23	33	1.7	0.4	0.7
40	67	97	35	62	1.6	0.4	0.6
30	49	71	22	46	1.5	0.3	0.5
43	69	96	31	66	1.5	0.3	0.5
17	28	40	15	25	1.5	0.4	0.6
32	53	77	24	50	1.5	0.3	0.5

split into monosaccharides and low-molecular-weight sugars (Table III, Columns 3 and 7; Table II). At the end of the experiment, xylan and mannan degradation had reached about the same value (57%; *see* Table III).

Cellobiose or other cellulose fragments were not found in the reaction solution, neither after the xylanase nor after the additional action of mannanase. The glucose present probably was derived from the mannan (*see* above and Mannanase).

Avicelases or Cellobiohydrolase C + Mannanase. The action of mannanase on delignified sprucewood holocelluloses that were treated with avicelase 1, avicelase 2, or cellobiohydrolase C for 48 hr gave similar degradation patterns. The rate of total degradation increased rapidly when the mannanase was added; the rate was most pronounced in the case of avicelase 1 (Figure 4).

Mannan fragments that were dissolved by the action of the cellulase and most of the mannan left in the tissue were hydrolyzed when mannanase was added. At the end of the experiment (80 hr; 48 hr with only one of the three cellulases and 32 hr with cellulase + mannanase), about 75–95% of the mannan present in the starting material was hydrolyzed (Table III. Column 10). The calculation of these values was based on the mannose and galactose present in the hydrolysates and on the ratio mannose:glucose = 3.3:1 and was related to the amount of sugars in the total hydrolysate of the starting material (cf. Experimental).

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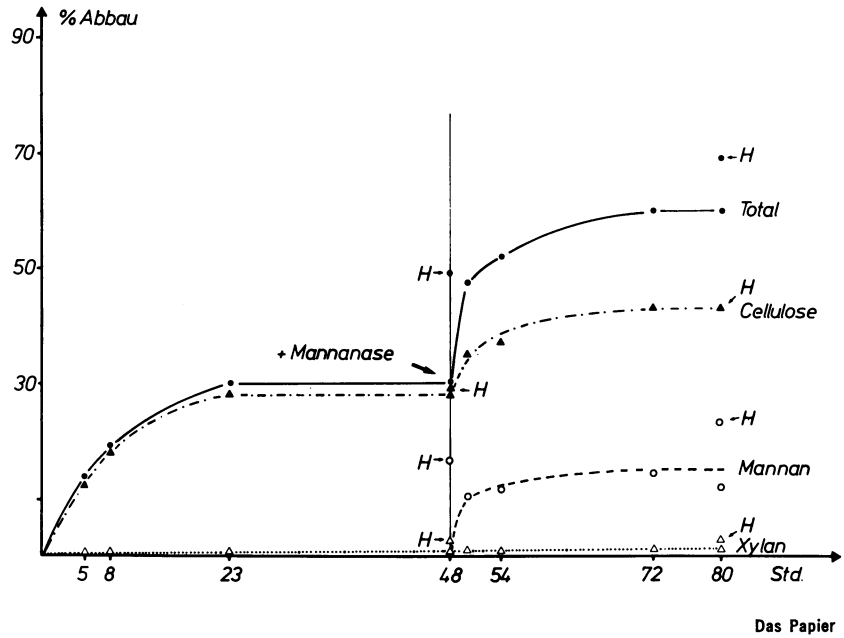

Figure 4. Decomposition of sprucewood holocellulose by avicelase 1 + mannanase, monitored by quantitative sugar chromatography. (For symbols see Figure 3).

Addition of mannanase resulted in high amounts of mannobiose and mannose. The amount of mannobiose relative to mannose was somewhat higher than in the case of the combined xylanase–mannanase treatment (Table II). The ratios of mannose:galactose in the acid hydrolysates of the reaction solutions at the end of the experiment were 1:0.05 for the two avicelases and 1:0.03 for the cellobiohydrolase C.

The presence of mannanase revived the cellulolytic action. The degradation rate increased, although not as rapidly as at the beginning of the experiment, when the holocellulose was solely incubated with one of the three cellulases. The final cellulose degradation amounted to about 25–45%. As in the case of single enzyme treatments, the avicelases gave higher cellulose degradations than the cellobiohydrolase C (Table III, Column 12).

The ratio of cellobiose:glucose decreased rapidly when the mannanase was added. At the end of the experiments, the amount of glucose exceeded about twentyfold that of cellobiose.

Only minor amounts of additional reducing sugars from the xylan decomposition were detected in the reaction solutions. Acid hydrolysis showed, however, that higher-molecular xylan fragments were liberated by the combined cellulase-mannanase treatment. At the end of the experi-

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ment, about 15–30% of the arabino-4-O-methylglucuronoxylan of sprucewood holocellulose was removed by the enzyme treatments.

The xylan degradation rates relative to those of mannan ranged from 0.3 to 0.4 at the end of the experiment (80 hr). The same values were obtained before the additional action of mannanase on the samples treated with one of the three cellulases for 48 hr (Table III, Column 14). The corresponding values relative to the cellulose degradation rates ranged from 0.5 to 0.7 for the samples treated with cellulase only (48 hr) and after combined cellulase–mannase action (Table III, Column 15).

Ultrastructural Observations

Control Sample. The holocellulose material of sprucewood evinces a rather homogeneous contrast across the width of the secondary wall after treatment with potassium permanganate; the middle lamella/primary wall region is, in general, more electron opaque than the other wall areas (Figure 5). In certain cases, a somewhat higher contrast of the tertiary wall region can be observed.

Mannanase. The general appearance of the mannanase treated holocellulose material evokes the impression of a rather widespread attack. In principle, the mode of degradation effects a high degree of decrustation both of S_1 and tertiary wall regions (Figure 6), contributing to an exposure of fibrillar elements. In some cases, the tertiary wall region evinces a more intensive attack than the S_1, as shown in Figure 7. A larger

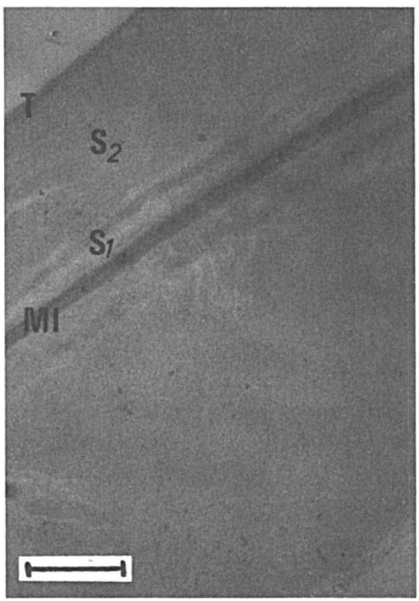

Figure 5. Holocellulose of sprucewood tracheid. Control sample treated with $KMnO_4$, showing higher contrast in the middle lamella and homogeneous contrast in the rest of the wall. Scale = 1 μm.

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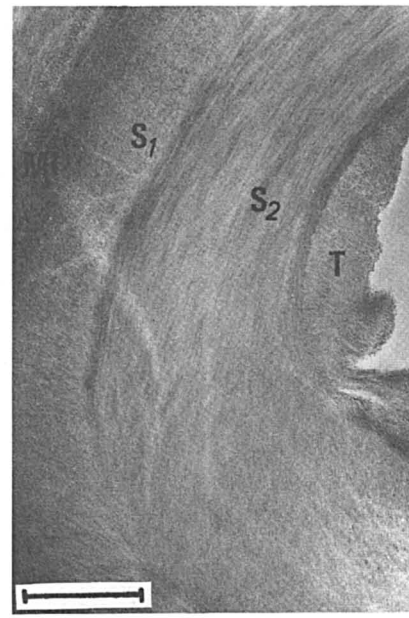

Figure 6. Slightly biased section of a tracheid wall after mannanase treatment. Decrustation in S_1 and T. Scale = 1 μm.

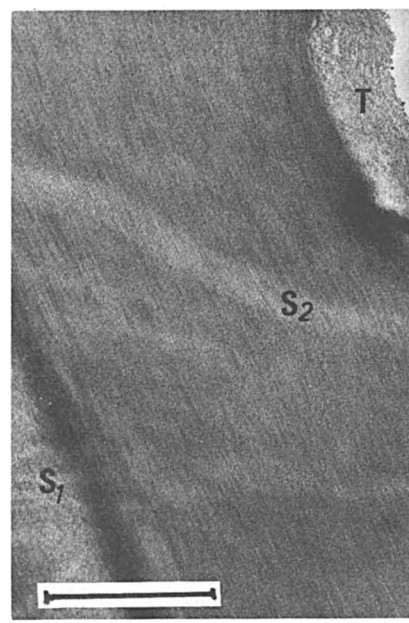

Figure 7. Tracheid wall after mannanase treatment with fibrillar texture of S_2; note also loosened texture in S_1 and T. Scale = 1 μm.

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portion of S_2 becomes clearly exposed, exhibiting a fibrillar texture (Figure 7). The cell corners become affected—in particular, in the peripheral regions of the holocellulose particles, whereby a slight dissolution of substances occurs.

In the ray parenchyma cells, the protective layer is revealed as a transparent zone with a slight loss of wall contrast. On the other hand, the S_1 appears to be affected too, although to a lesser extent.

Xylanase. The sprucewood holocellulose treated with xylanase shows, in general, a mild attack especially in the wall region at the lumen-wall boundary. A progressive dissolution of substances can also be detected from S_1 into the adjacent S_2 as well as from the lumen side into the secondary wall (Figure 8). This decrustation appears to be not very extensive in total, inasmuch as the affected wall areas are proportionally few. Compared with the loss of opacity in the lumen-wall boundary, the S_1 is not affected to such a great extent. The corners of the tracheids exhibit a high transparency, obviously due to removal of substances, as compared to the remainder of the middle lamella region.

The ray parenchyma cells also become affected in the same manner, even though the degree of substance loss is much lower.

Avicelases or Cellobiohydrolase C. In holocellulose treated with an avicelase or with cellobiohydrolase C, the degradation of the tracheid wall is very intense. This is mostly concentrated in the S_1 and adjacent zones of S_2 as well as in the tertiary wall region (Figure 9). In the middle part

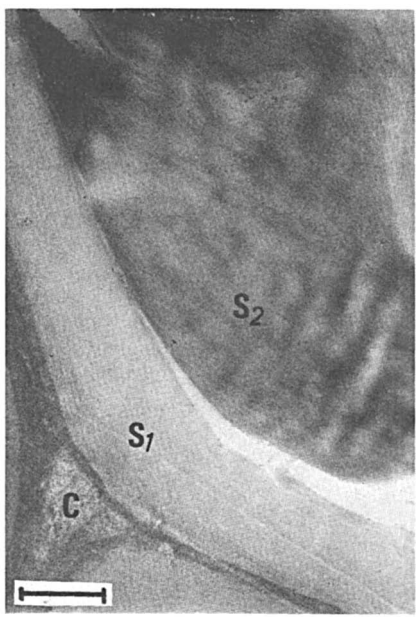

Figure 8. Xylanase-treated latewood tracheid wall with dissolution of cell corner (C) and contrast-poor loosened texture in S_1. Scale = 1 μm.

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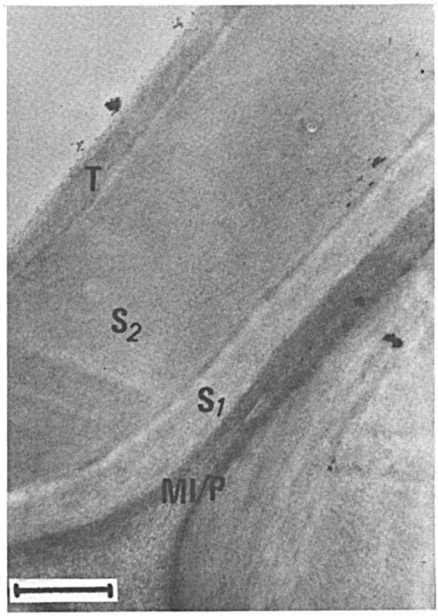

Figure 9. Avicelase-1-treated earlywood tracheid with degradation especially in S_1 and partially in T. Scale = 1 μm.

of S_2, the degradation process shows itself in the exposure of a highly fibrillar texture as well as in the hydrolysis of wall material (Figure 10). The cell corners become only very little affected, especially in the cellobiohydrolase-treated samples. In peripheral regions of the holocellulose particles, the degradation of the S_2 wall region is very extensive, perhaps due to the facility with which the enzymes penetrate.

The ray parenchyma cells behave in a manner similar to the tracheids, with a dissolution of fibrillar material from the S_1 as well as from the wall region near the lumen.

Xylanase + Mannanase. The combined action of xylanase and mannanase leads to a degradation of wall material, which resembles the mode of attack met with in the individual enzyme treatments; only the degree of dissolution is rather more intense in the combined treatment. Of special interest is the decrustation of the cell corners, which appear to be attacked on a large scale (Figure 11). The S_1 and tertiary wall zones also undergo a decomposition process (Figure 11).

The ray parenchyma cells evince an equal degree of attack of both the S_1 and tertiary wall regions, especially in the vicinity of pit connections between contiguous cells. Along with the other wall material, the protective layer also shows a dearth of contrastable substances.

Avicelase or Cellobiohydrolase + Mannanase. The treatment of spruce holocellulose first with avicelase or cellobiohydrolase and subsequently with mannanase effected an overall intensive degradation of

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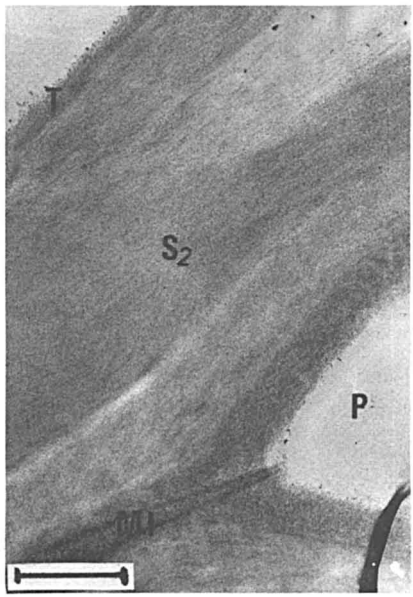

Figure 10. Avicelase-treated earlywood tracheid wall evincing partial dissolution of wall substance in S_2 near a pit chamber. Scale = 1 μm.

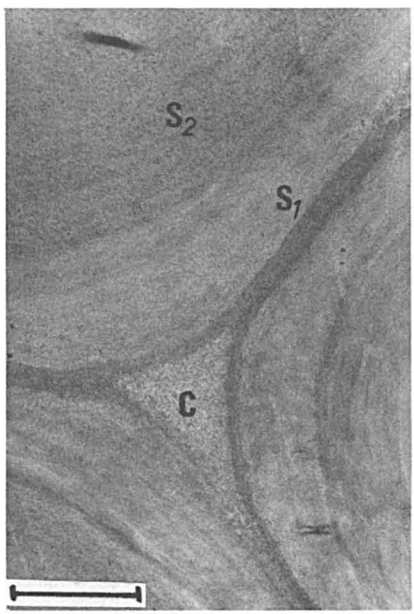

Figure 11. Xylanase + mannanase treatment. Decrustation of S_1 layer and cell corner (C) in an earlywood tracheid. Scale = 1 μm.

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tracheid and parenchyma walls. This degradation comprises both cell corners and secondary wall layers of the tracheids. The S_2 layer becomes very strongly hydrolyzed, leaving remnants of fibrillar structures of 300–400-nm diameters. The defibrillation is characteristically present in the S_2, although the S_1 also shows signs of evident attack (Figure 12). Such a mode of wall dissolution leads in some cases to a separation of the wall layers between S_1 and S_2, partly as a result of the mechanical forces exerted during preparation of the material for electron microscopy. Partially, there is a complete lysis of the tertiary wall zone, with a loosening of the texture in S_1. The S_2 becomes occasionally degraded in a lamellar fashion (Figure 13), with a differential exposition of fibrillar and granular material. The degraded wall layers exhibit remnants of cellulose fibrils with darkly contrasted areas, partially showing the same orientation as the fibrillar components.

The ray parenchyma walls are more intensely attacked than the tracheid walls. The whole secondary wall exhibits a high degree of decomposition. Again, it is the wall area around the pits that is preferably attacked (Figure 14). In the parenchyma walls there occurs also a partial degradation, in a layered manner, of highly contrastable and transparent zones, apparently as a result of enzyme attack both from the lumen and from the middle lamella regions; this mode of degradation results in a checked pattern.

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Figure 12. Cellobiohydrolase + mannanase treatment. Extensive degradation of tracheid wall material in all the layers. Scale = 1 μm.

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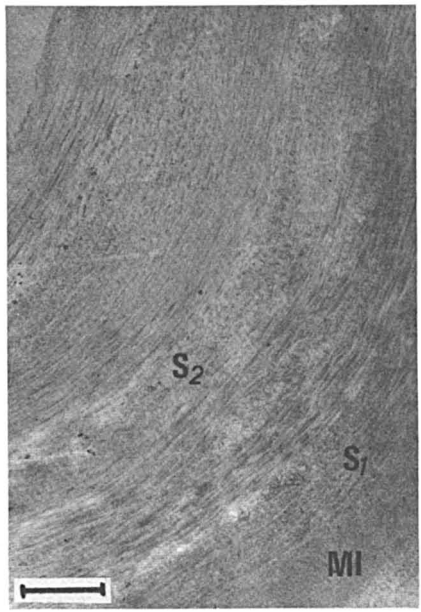

Figure 13. Avicelase 1 + mannanase treatment. Degradation of S_2 in an earlywood tracheid showing lamellar aggregation pattern. Scale = 1 μm.

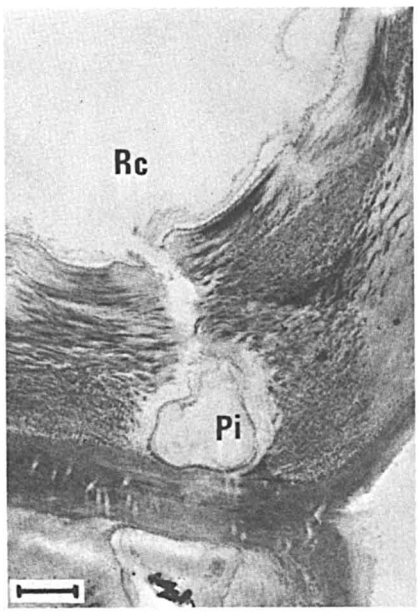

Figure 14. Avicelase 1 + mannanase treatment. Ray parenchyma (Rc) with extensive degradation of wall material near a pit pair (Pi). Scale = 1 μm.

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Discussion

The properties of the enzymes used in this study have been described in former publications (*10, 11, 15*). Important for the following interpretation are their hydrolytic specificities. The xylanase did not hydrolyze either isolated mannans or celluloses—or only to a very small extent (*10*). The same is true for the mannanase with respect to xylans and celluloses (*11, 15*). The avicelases, which were not purified to the same extent as the xylanase and mannanase, did not hydrolyze mannans, but they degraded xylans besides crystalline cellulose (*10*). Also, the highly purified cellobiohydrolase C (*12*) degraded xylan to some extent (Dr. E. K. Gum, Jr., personal communication).

The chemical analysis has shown that the mannanase isolated by Yamazaki (*15*) from a commercial enzyme preparation of *Aspergillus niger* can degrade galactoglucomannan in sprucewood holocellulose to a certain degree. About a quarter of the mannan was dissolved in about two days; the following two days of mannanase incubation brought about predominantly further hydrolysis of the mannan fragments removed in the first days.

Mannan dissolved away by the buffer (control specimen) was low. Based on the mannan content of the sprucewood holocellulose, less than about 4% mannan or mannan fragments were solubilized; they constituted high oligomers and/or polymers. In preliminary experiments of Yamazaki (*15*), the same mannanase was used after an additional purification by gel filtration but applied at a much lower concentration (about ⅛). The amount of low-molecular-weight reaction products was then less than 0.5% (holocellulose dry weight = 100), which was in the range of mannan or high-molecular-weight mannan fragments found in solutions of control specimens. Based on these and on similar results of Boutelje and Hollmark (*6*), it was concluded that the mannanase degraded the mannan in sprucewood holocellulose only to a small extent or not at all (*10*).

The experiments were repeated under similar conditions with the same and another mannanase fraction of Yamazaki's, with sprucewood holocellulose prepared in a slightly different way (*19*). After three weeks' incubation, about 19% of the mannan was dissolved away, and the loci of hemicellulose removal were revealed by electron microscopy. In these experiments, the bulk of hemicelluloses was obviously removed by the buffer solution. In addition to mannose, glucose and galactose derived from mannan, xylose and glucose were found in the reaction solutions in appreciable amounts.

In the present investigation, about eight times more mannanase per milligram of holocellulose was used with shorter incubation periods (up

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to 3.5 days). It became obvious that the mannanase is capable of removing a certain portion of the mannan in sprucewood after mild delignification and that its action is largely selective for mannan. Besides mannose, glucose, and galactose derived from mannan, no other sugars were detected in appreciable amounts. Yamazaki (*15*) has demonstrated that his mannanase yields glucose from softwood mannans. The amount of glucose relative to the mannosaccharides found in the present investigations increased with mannanase incubation time, but it was always markedly inferior to the ratio of mannose to glucose of 3.3:1, which on an average is generally accepted for sprucewood mannans (*15, 20, 21*). In addition, subsequent to acid hydrolysis of the final reaction solutions, the ratio of mannose:glucose was about 3:1. Therefore, it is very likely that the total amount of glucose found was derived from mannan and that the cellulose was left unaffected. The portion of xylan in sprucewood holocellulose is low (about 11%), and the degradation rates attained did not exceed 6% of the starting material. Therefore, it cannot be excluded that some xylan was dissolved away during the mannanase treatment without being detected by the chemical analyses used.

The discrepancy between the mannan removal finally achieved with Yamazaki's mannanase and the failure reported by Boutelje and Hollmark (*6*) for a mannanse isolated from another commercial enzyme product could be due to several factors. It might be simply a question of mannanase concentration; it could also be caused by a different catalytic action of the two mannanases. On the other hand, there is a marked difference concerning the substrates. The sprucewood holocellulose used by Boutelje and Hollmark (*6*) was prepared with sodium chlorite at 70°C. It contained only about 10% mannose, 5% xylose, 0.3% arabinose, and traces of galactose. In the present study, delignification was performed at room temperature, leaving much more hemicelluloses in the tissue (see Experimental). Therefore, it might be that the portion of mannan removed by the mannanase in the present experiments had already been chemically removed during the delignification of the samples treated with mannanase by Boutelje and Hollmark.

The xylanase hydrolyzed about half the xylan in the sprucewood holocellulose. This is in the range of the xylan degradation obtained in former studies with delignified beechwood (*7, 10, 22*). Boutelje et al. (*5*) reported only 20–30% xylan hydrolysis of sprucewood holocellulose by a xylanase, even after repeated treatments. They used the same holocellulose and a xylanase isolated from the same commercial enzyme preparation as were used in the mannanase treatment referred to above. The holocellulose contained only a low xylan portion and very little arabinose; this could be the reason for the inferior degradation rate, and hence it is not surprising that no free arabinose was detected, in contrast to what

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occurred in the present experiments. Moreover, oligosaccharides were not recorded by the method used by Boutelje and coworkers (*5, 6*). In the present study, oligosaccharides consisting of xylose, glucuronic acid, and/or arabinose were shown to be present in appreciable amounts. In addition, high oligosaccharides and/or polysaccharides derived from mannan were detected, representing about 12% of the mannan in the starting material. It is interesting to note that the xylanase treatment—along with 50% xylan hydrolysis—removed about half as much mannan as did the mannanase treatment. The dissolution of mannan could be due to traces of mannan-decomposing activities; it could also be that mannan molecules or mannan fragments are dissolved away along with the hydrolytic removal of xylan. The degree of polymerization of the solubilized mannan-carbohydrates was not determined; hence it could not be decided whether the xylanase treatment resulted in a specific hydrolysis of xylan in delignified sprucewood (*5*) but accompanied by mannan dissolution.

The cellulose was either not degraded by the xylanase treatment or degraded only to a small extent. This is in line with former investigations on holocelluloses of sprucewood (*5*) and beechwood (*7, 10, 22*).

The three cellulases decomposed about 25–45% of the cellulose accompanied by solubilization of about 40–70% of the mannan and, by partial hydrolysis, of about 20% of the xylan present in the untreated sprucewood holocellulose. Based on the degradation products (cf. Table III, Columns 13–15, and Table II), the catalytic actions of the three cellulases—all isolated from *Trichoderma viride*—are similar or identical. The lower absolute degradation values obtained with cellobiohydrolase C might merely be a result of enzyme concentration.

The degradation rates of cellulose obtained with the individual cellulases were, in each case, higher than the corresponding values reached under the same conditions with beechwood holocellulose (*10*). The same tendency had already been found in preliminary tests with crude cellulase preparations (*13*; cf. *23*).

The cellobiohydrolase C was highly purified and proved to be an electrophoretically homogeneous protein (*12*). Nevertheless, like the avicelases (*10*), it possessed, besides the cellulolytic, a xylan-degrading activity. The hydrolytic activity for a xylan isolated from beechwood holocellulose (*18*) was about 10% of the cellulolytic action observed on swollen cellulose (Dr. E. K. Gum, Jr., personal communication). The enzyme tests and the degradation products of sprucewood and beechwood holocellulose indicate that all three cellulases can hydrolyze both cellulose and xylans (arabino-4-O-methylglucuronoxylan and 4-O-methylglucuronoxylan). It might even be that the catalytic activities for these two different substrates are localized in one protein molecule—i.e., the enzyme would have an active center for β-1,4-glucans and for β-1,4-xylans.

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The catalytic action of the two avicelases and the cellobiohydrolase C seems at least to be different from those of the xylanases isolated from *Trichoderma viride*: i.e., the degradation products have a higher degree of polymerization even after prolonged incubation and, in the case of sprucewood holocellulose, no arabinose is liberated.

The mannosaccharides solubilized by the action of the three cellulases on sprucewood holocellulose were found to be high oligo- or polysaccharides, but their DP was not determined. Therefore, it cannot be decided whether they are mannan fragments resulting from mannanolytic activities of the cellulase preparations or largely intact mannan molecules detached during the hydrolysis of cellulose and xylan. Assuming the electrophoretically homogeneous cellobiohydrolase C to be free from mannanolytic enzyme contaminations, it could be concluded that the cellulases solubilize mannan during cellulose hydrolysis.

The combined action of xylanase and mannanase on sprucewood holocellulose increased the hydrolysis of hemicelluloses without any detectable attack of cellulose. At the end of the experiments—i.e., after 48 hr of xylanase incubation followed by 32 hr of combined xylanase–mannanase incubation—about half the hemicelluloses present in the starting material were selectively converted into low-molecular-weight sugars. The amount of mannan removed was two times higher than after 80 hr of incubation with mannanase only. Unexpectedly, the xylan dissolution was scarcely increased by the combined action of the two hemicellulases.

The addition of mannanase after 48 hr of incubation with one of the three cellulases revived cellulose hydrolysis. The increase of the degradation rate was not as pronounced as in the corresponding treatments of delignified beechwood with cellulases and xylanase (*10*), but the cellulose degradation reached about the same values at the end of the experiments (about 50–65%).

Most of the mannan in the sprucewood holocellulose was hydrolyzed by the cellulase–mannanase treatments, and only about one-third of the xylan was dissolved and partly hydrolyzed into low-molecular-weight sugars.

About a quarter of the mannan in the sprucewood holocellulose seems to be accessible to the mannanase. More mannan can be hydrolyzed only when the second hemicellulose becomes at least partly dissolved. The remaining mannan—less than half the initial amount—appears to be removable only together with cellulose degradation. In beechwood holocellulose, most of the main hemicellulose—i.e., the xylan—can be hydrolyzed rather selectively by the sole action of xylanase. This phenomenon could be due to the different molecular size of the two hemicellulases, mannanase about 24 Å, xylanase about 18 Å (*10*), resulting in better diffusing conditions for the xylanase. However, the porosity of the

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sprucewood holocellulose should be higher than in the case of beechwood because more lignin is removed by chlorite treatment. Moreover, the difference in molecular size of the two enzymes is small, and similar, selective xylan degradations were achieved for delignified beechwood with xylanases of about the same size as that of the mannanase (*10, 22*). Therefore, it seems more likely that the phenomenon is caused by micromorphological or topochemical differences. It might be considered that the mannan in sprucewood is more tightly associated with the cellulose than is the xylan in delignified beechwood. There could also be some kind of association between mannan and xylan. This assumption is based on the observations that mannan or mannan fragments are dissolved during enzymatic xylan hydrolysis and that much more mannan is hydrolyzed by the mannanase when some xylan has been removed previously.

A portion of the xylan seems to be tightly associated with cellulose. Only part of the xylan was dissolved away even when most of the mannan and more than half the cellulose became hydrolyzed by the combined action of cellulase and mannanase.

The foregoing observations confirm the conclusions derived from former experiments with beechwood holocellulose (*10*): (1) A partial degradation of the hemicelluloses is imperative before the cellulose fibrils can be attacked. (2) The hemicelluloses seem to be deposited between the cellulose fibrils or even to be encrusting them. (3) The enzymatic hydrolysis of the cellulose is governed by the porosity of the tissue (enzyme diffusion), the impediment of the hemicelluloses, and the properties of the cellulose (e.g., crystallinity).

The results are consistent with many of the reports on the arrangement of hemicelluloses and cellulose in cell walls (e.g., *20, 21, 24–34*). The microfibril model, in which the hemicelluloses are arranged—perhaps together with loosely packed ("amorphous") cellulose—around a highly ordered, densely packed cellulose core ("crystalline") (cf. *24, 28*), combined with the interrupted lamellae model proposed for wood by Kerr and Goring (*31*) fits well with the chemical results of the enzymatic removal of cell-wall components obtained in the present and in former studies. Scallan's honeycomb concentric lamellae model for wood (*35*), which is based on ultrastructural observations combined with physical experiments (*36, 37*), could also be included in model speculations. Thus the microfibrils (consisting of from 2 to 4 protofibrils bonded on their radial surfaces) arranged in a honeycomb fashion, with interrupted lamellae-preferentially coplanar and parallel to the middle lamella, would be surrounded, at least on their tangential surfaces, by a cortex of oriented hemicelluloses, including perhaps amorphous cellulose, and the spaces between these interrupted lamellae would be filled with lignin or a lignin–hemicellulose matrix.

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The electron micrographs of the enzyme-treated sprucewood holocellulose revealed the loci of the removed substances when compared with untreated samples. The relative intensity of degradation at the ultrastructural level corresponded to the results obtained by chemical analysis of the dissolved carbohydrates.

The current observations confirm previous studies on beechwood and sprucewood holocellulose (*7, 10, 19*). The attack of the hemicellulose proceeds from the primary wall/S_1 as well as from the tertiary wall into S_2; the pit chambers constitute preferred paths of enzyme diffusion into the walls. Also, substances of the middle lamella, especially in the cell corners, are removed by the xylanase and the mannanase treatments. Parallel to the removal of hemicelluloses, the fibrillar structure of the cellulose and its lamellar arrangement in transections of cell walls became obvious. In samples treated with cellulases, the cellulose fibrils were often completely hydrolyzed in the S_1 layer, occasionally accompanied by complete dissolution of cell-wall portions. This is also in conformity with the previous conclusion that the cellulases hydrolyze highly ordered zones of cellulose and remove hemicelluloses by hydrolysis or by detachment.

The loss of mannan caused by the mannanase treatment of sprucewood holocellulose seems to be similar in the S_1 and T layers. This has also been reported by Hoffmann and Parameswaran (*19*).

The removal of xylan by the xylanase appeared more pronounced in T than in S_1. This is in disagreement with microdensitometrical evaluations of substance losses in cell walls of spruce- and pinewood holocellulose reported by Boutelje and co-workers (*5, 6*). They found that the hydrolysis of xylan by xylanase treatment occurred mainly in the S_2 layer; the S_1 and in some cases also the S_3 (T) layer appeared not to be affected. In the same way, they showed that the S_1 layer is more resistant to cellulolytic attack than the other cell wall layers.

Based on the present results, an almost equal concentration of mannan in the S_1 and T layers of sprucewood cell walls (tracheides) and a higher concentration of xylan in the T than in the S_1 layer could be deduced, assuming a linear correlation between local concentration of the hemicelluloses in wood and their enzymatic removal from holocellulose. This interpretation would agree with the distribution of mannan in young tracheids of spruce- and pinewood and that of xylan in those of pinewood reported by Meier and co-workers (*38–40*). Hoffmann and Parameswaran (*19*), however, concluded from electron microscopic studies that in sprucewood holocellulose the hemicellulose concentration is highest in the S_1 layer and that it is about as low in T as in the middle part of S_2. They used different staining reagents to contrast acidic groups originally present in the tissue with those developed from the reducing

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carbohydrate end groups during the sodium chlorite delignification. The experimental results available so far on the distribution of xylan and mannan across the cell walls of sprucewood are too scarce and too divergent to make a final conclusion at this juncture.

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The Cellulolytic Enzyme System of *Thermoactinomyces*[1]

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The CM-cellulase and Avicelase activities in a culture of Thermoactinomyces sp. *are extracellular throughout the entire growth phase and are produced simultaneously with cell growth. The β-glucosidase activity, however, appears to be intracellular. Three major fractions with equal proportions of CM-cellulase and Avicelase activity can be separated from the crude culture filtrate by preparative isoelectric focusing in the pH range 3–5. Analytical isoelectric focusing of culture filtrate samples taken throughout the growth phase of* Thermoactinomyces *indicates that all extracellular proteins are produced and released into the culture filtrate simultaneously. The stability of cellulolytic activities as a function of temperature and time is in the order CM-cellulase > Avicelase > β-glucosidase. Saccharifications using the enzyme system from* Thermoactinomyces *are discussed.*

In view of the current energy crisis, nonoil sources have to be considered for the production of liquid fuels and chemicals. The greatest renewable resource is cellulose, from which glucose can be produced either by enzymatic or acid hydrolysis. The glucose can be further processed to

[1] On the basis of cell wall analysis and other characteristics (H. A. Lechevalier, personal communication, 1979), this organism has now been correctly identified as belonging to the genus *Thermomonospora*.

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fuels and oil-sparing materials. One advantage of an enzymatic hydrolysis of cellulose over an acid hydrolysis is better control of unwanted products, such as hydroxymethylfurfural.

Several microorganisms have been studied with respect to the production of a cellulolytic enzyme system for the saccharification of cellulosic materials, the most thoroughly investigated organism and best producer of cellulase being *Trichoderma viride* (*1*). Recently, good saccharification data have been reported using a strain of *Penicillium* (*2*).

The thermophilic filamentous bacteria, *Thermoactinomyces sp.*, have been studied primarily for the production of single-cell protein (SCP) from cellulose (*3*), because such a culture grows rapidly and degrades up to 70% of cellulosic substrates within 24 hr (Figure 1). The fast growth rate of this organism indicates that its cellulolytic enzyme system is very efficient and that it might be a good source of enzyme for saccharification of cellulosic materials (*4*). It has recently been established that extracellular cellulolytic activity measured as filter-paper activity occurs simultaneously with cell growth in a culture of *Thermoactinomyces sp.* (Figure 1; (*5*)).

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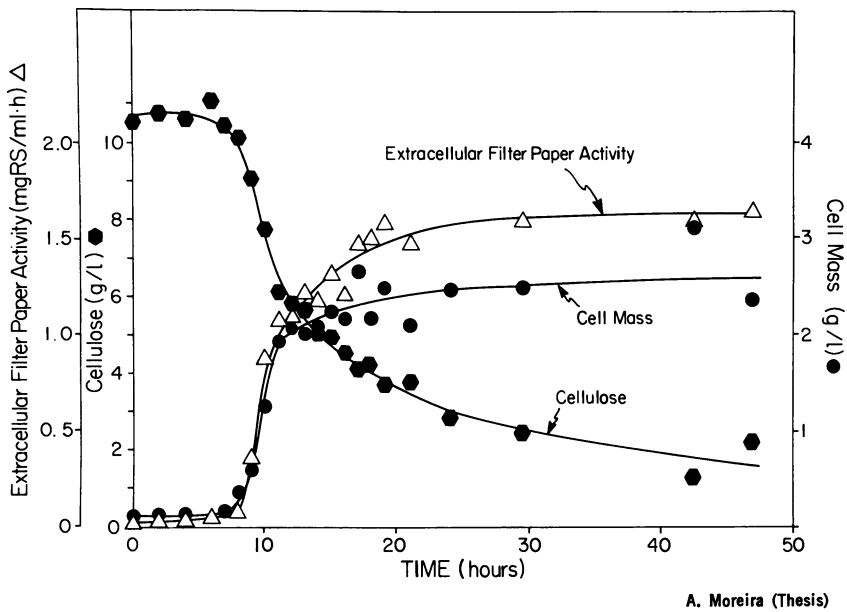

Figure 1. Cellulose degradation, cell growth and extracellular filter-paper activity in a culture of Thermoactinomyces sp., *strain YX. 40 L batch fermentor, 55°C, pH 7.2* (*5*).

This chapter deals with three aspects of the cellulolytic enzyme system of *Thermoactinomyces sp.*: the location of the CM-cellulase, Avicelase, and β-glucosidase (cellobiase) activities in the culture, the multiplicity of the extracellular enzyme system, and the stability of the different activities as a function of pH, temperature, and time. The results are discussed with reference to saccharification of cellulosic materials.

Location and Development of Cellulolytic Activities in a Culture of Thermoactinomyces sp.

The cellulolytic activities were studied in a culture of *Thermoactinomyces sp.* grown on microcrystalline cellulose (Avicel, PH 102). Culture conditions, fractionation of the culture, and enzyme assays are described elsewhere (*6*). The different fractions of the culture were assayed for CM-cellulase, Avicelase, and β-glucosidase activity. It was found for *Trichoderma viride* that the measurement of cellulolytic activity as reducing sugar produced from carboxymethylcellulase (CMC) and Avicel distinguished endoglucanases (CM-cellulase) from exoglucanases/cellobiohydrolases (Avicelases) (*7*). The assay for β-glucosidase activity with *p*-nitrophenol-β-D-glucoside as a substrate has frequently been used to screen for cellobiase activity (*7–10*).

Extracellular Cellulolytic Activities. The appearance of the CM-cellulase activity in a culture of *Thermoactinomyces* grown on 1% microcrystalline cellulose is shown in Figure 2. The extracellular CM-cellulase activity approached a maximum of 14–16 mg reducing sugar (RS) mL^{-1} min^{-1} within 18–24 hr. The Avicelase activity of the culture filtrate developed simultaneously with the CM-cellulase activity and amounted to 3 mg RS mL^{-1} hr^{-1}. The extracellular protein concentration reached 1.7 mg/mL in the stationary phase (*6*).

The CM-cellulase activity of the solids fraction shows a skewed curve over the period of 4–24 hr with a maximum of 3 mg RS mL^{-1} min^{-1} around 8 hr, at which point it makes up about 50% of the activity in the whole culture broth (Figure 2). No activity could be detected in the solids fraction in the late stationary growth phase. Within experimental error, the CMC activity of the culture filtrate plus that of the culture solids equals the activity of the whole broth. Similarly, it was found for *Thermoactinomyces*, strain MJØr, grown on 0.5% microcrystalline cellulose, that there was a lag before an appearance of extracellular cellulolytic activity, as compared with the activity in the whole culture broth (*4*). In a culture of *Thermoactinomyces*, strain YX, the CM-cellulase activity can be desorbed readily by washing the solids fraction with water. These wash fractions also show Avicelase activity (*6*). This result, and the fact

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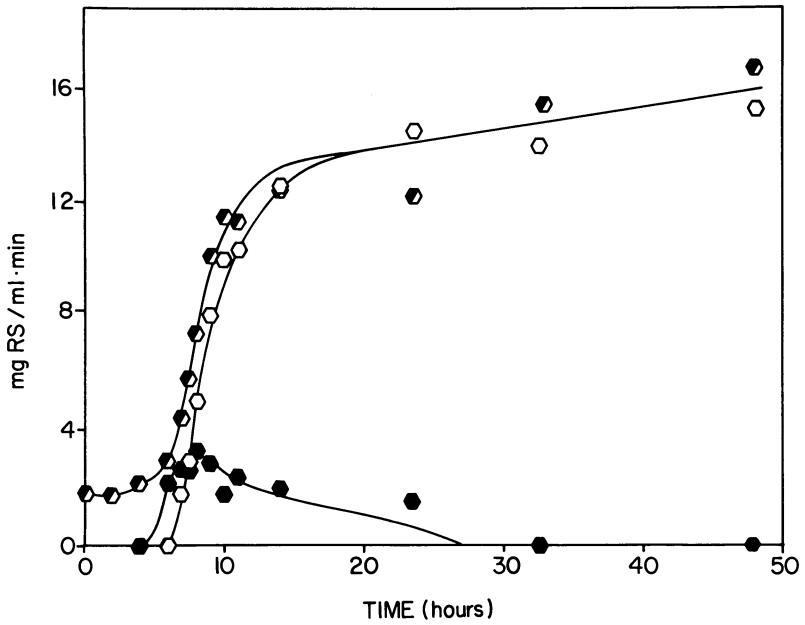

Figure 2. Development of CM-cellulase activity in a culture of Thermoactinomyces sp., *grown on 1% Avicel (PH 102) at 55°C in a 14 L batch fermentor. Uncontrolled pH; 7% shake flask inoculum. (◐) Whole culture broth; (○) culture filtrate; (●) culture solids* (6).

that the CM-cellulase activity of the solids fraction results in a skewed curve that decreases to zero in the stationary growth phase, after the major part of the substrate has been digested, indicates that the activity is adsorbed to the cellulosic substrate rather than to the cells. Such a relationship was predicted in a mathematical model for the growth of *Thermoactinomyces* on cellulosic substrates (*5*). Thus the CM-cellulase and Avicelase activities are truly extracellular and not cell-wall-bound. They are produced rapidly, and the culture can be harvested within 24 hr to produce enzymes for saccharification of cellulose. By comparison, *Trichoderma viride* produces extracellular cellulolytic activity only after the substrate has been degraded, and the activity reaches a maximum after about 7 days (*11*). Berg and Pettersson have suggested that this is because the cellulolytic enzymes of *Trichoderma viride* are part of the cell wall during the exponential growth phase and are not released into the culture fluid until the entire substrate has been degraded (*12*).

Cell-Associated Cellulolytic Activities. The β-glucosidase activity is associated with the culture solids throughout the entire growth period, and no activity is found in the culture filtrate (Figure 3). A maximum

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activity of about 0.17 μmol *p*-nitrophenol (PNP) mL^{-1} min^{-1} appears in the late exponential growth phase, after which the activity levels out to about 0.14 μmol mL^{-1} min^{-1}.

Various washing agents, such as high salt concentrations, detergent, buffer, and water, did not desorb more than trace amounts of the β-glucosidase activity from the culture solids (*10*). However, when the culture solids were sonicated for 10 min and then filtered, the β-glucosidase activity was released into the filtrate; less than 5% of the activity was left with the solids after sonication (Table I). The β-glucosidase activity released by sonication was found to be almost doubled, to 0.25 μmol PNP mL^{-1} min^{-1}, as compared with that of the intact cultures.

When the postsonication filtrate was centrifuged at 120,000 ×g for 90 min, the supernatant fraction contained over 95% of the original activity. This indicates that the β-glucosidase is a highly soluble enzyme which is not attached to any readily precipitable cell fragments.

The whole culture broth and its fractions were also assayed for cellobiase activity. This activity followed closely the β-glucosidase activity, so that it was found in the culture solids and the filtrate after sonication (Table I). Only trace amounts of cellobiase activity appeared

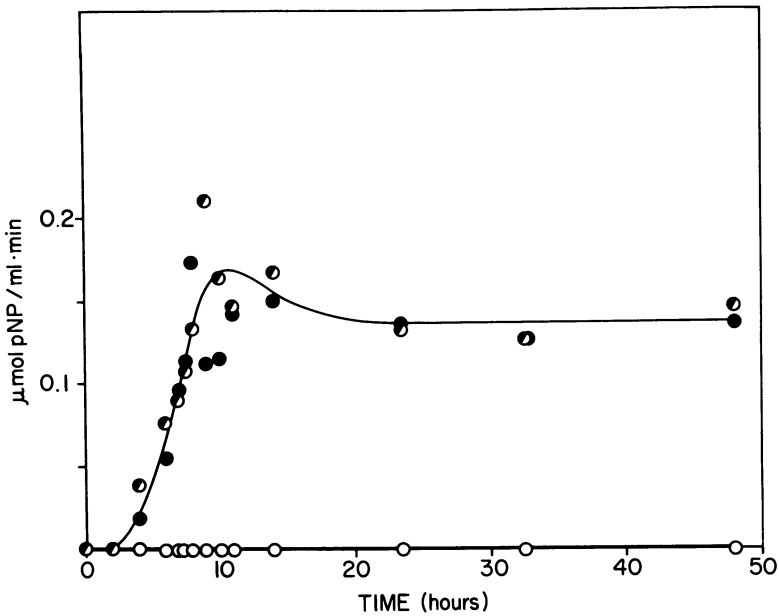

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Figure 3. Development of β-glucosidase activity. Same conditions as Figure 2. (◐) Whole culture broth; (○) culture filtrate; (●) culture solids (6).

Table I. β-Glucosidase and Cellobiase Activities in Whole Culture Broth, Culture Filtrate, Culture Solids, Filtrate, and Solids After Sonication for 10 Min[a]

	p-*Nitrophenol* ($\mu mol\ mL^{-1}\ min^{-1}$)	*Glucose* ($\mu mol\ mL^{-1}\ min^{-1}$)
Whole broth	0.14	0.16
Culture filtrate	0	0.04
Culture solids	0.18	0.19
Filtrate after sonication	0.25	0.95
Solids after sonication	0.03	0.09

[a] β-Glucosidase activity as the amount of *p*-nitrophenol released from 10 mmol *p*-nitrophenol-β-D-glucoside. Cellobiase activity as the amount of glucose released from 20 mmol cellobiose.

in the culture filtrate and solids after sonication. However, when the enzyme was released from the culture solids by sonication, the cellobiase activity increased about sixfold, as compared with a twofold increase in β-glucosidase activity; this might reflect a difference in the way the substrates and/or their products are transported across the cell membrane in intact cells.

Thus the cellulolytic enzyme system of *Thermoactinomyces* is compartmentalized so that the CM-cellulase and Avicelase activities are extracellular and the β-glucosidase (cellobiase) activity is intracellular. This may be a practical advantage in cellulose saccharification, since it has been shown for *Trichoderma viride* that glucose production can be greatly enhanced by increasing the β-glucosidase activity (*13*). In that case it was done by adding β-glucosidase from another microorganism, *Aspergillus phoenicie*. The intracellular location of the β-glucosidase of *Thermoactinomyces* provides the opportunity to choose in a saccharification the optimal proportion between this cell-associated activity and the extracellular activities, without having to use an external enzyme source.

Multiplicity of the Extracellular Cellulolytic Enzymes of Thermoactinomyces sp.

Cellulolytic enzymes have been found to be produced as varying numbers of isoenzymes (*14–17*). Preparative isoelectric focusing therefore appears to be a method of separating and identifying the enzymes in a crude culture filtrate (*18*). Figure 4 shows such a separation of lyophilized, desalted culture filtrate harvested in the late exponential growth phase. The isoelectric focusing was carried out in the narrow pH range of 3.1–4.8, as it had been found in preliminary experiments that no cellulolytic activity was found outside this pH range. The separated fractions were assayed for CM-cellulase and Avicelase activity.

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Three major fractions with cellulolytic activity were found at pH 3.5, 3.7, and 4.6. The CM-cellulase and Avicelase activities were parallel, so the proportions of these activities in the separated fractions were the same. Although CMC and Avicel have been used to distinguish endoglucanases from exoglucanases/cellobiohydrolases produces by *Trichoderma viride* (*7*), this distinction cannot be made for *Thermoactinomyces.* Thus the extracellular cellulolytic enzymes of *Thermoactinomyces* may be less specific in their catalytic action than those produced by *Trichoderma viride.* However, because endo- and exoglucanases have close isoelectric points, it might not be possible to separate them in a one-step purification procedure, such as preparative isoelectric focusing. A combination of ion exchange chromatography and preparative isoelectric focusing could probably overcome this problem and distinguish endo- and exoglucanases from *Thermoactinomyces* as well (*19*). These findings, however, also indicate that more specific assays, such as change in viscosity of CMC (*20*) and identification of soluble hydrolysis products by HPLC (*17*), may have to be used to characterize the different cellulolytic activities produced by *Thermoactinomyces.*

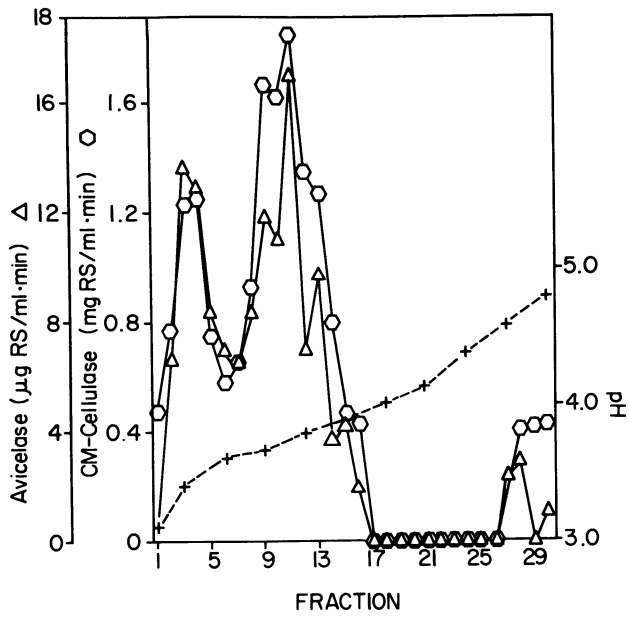

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Figure 4. Preparative isoelectric focusing in a granulated gel of a desalted lyophilized culture filtrate (50 mg protein) from the late exponential growth phase of Thermoactinomyces sp. *Separation carried out for 48 hr at a constant power of 8 W (29). (⬡) CM-cellulase activity; (△) Avicelase activity (6).*

A recent study indicated that the multiplicity of cellulolytic enzymes produced by *Trichoderma viride* might be due in part to proteolytic degradation as a result of the presence of proteolytic enzymes in the culture broth (*21*). Culture filtrate samples taken throughout the exponential and stationary growth phase were investigated by analytical isoelectric focusing to see if this phenomenon also applied to the extracellular enzymes produced by *Thermoactinomyces*. The culture filtrate samples were lyophilized immediately after separation from the culture solids. They were desalted in the cold, frozen on dry ice, and thawed just prior to applying them to the polyacrylamide gel slabs. Figure 5 shows that the culture filtrate sample taken from a 6-hr culture contains only a few proteins in the acidic region. This sample had no cellulolytic activity (Figure 2). However, the 7.5-hr sample, which has only 20% of the activity found in the stationary phase, already contained all the extracellular proteins that were found in samples taken later in the

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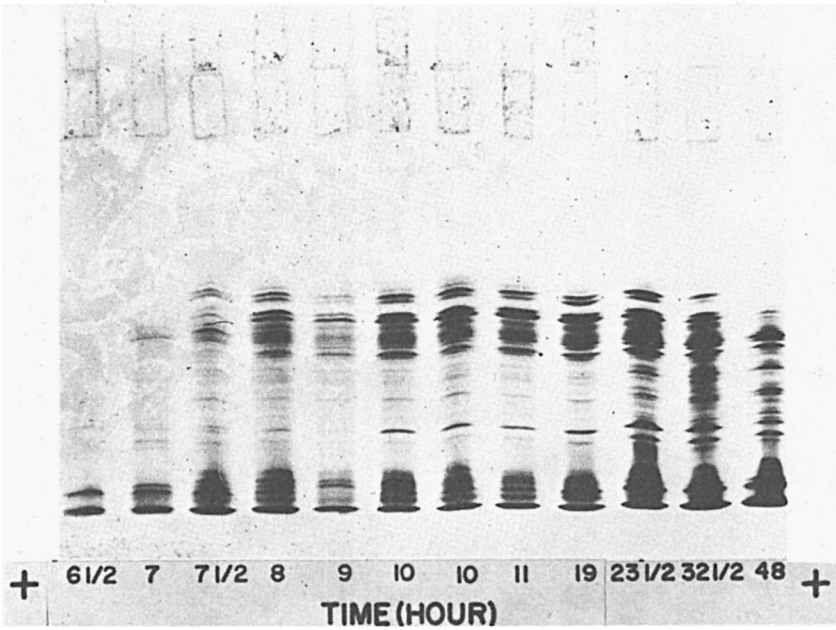

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Figure 5. Analytical isoelectric focusing in the pH range 3.5–9.5 (Ampholine PAG Plate, LKB-Produkter AB, Bromma, Sweden) of filtrate samples from a culture of Thermoactinomyces sp. *Separation carried out for 1.5 hr at a constant power of 1 W/cm (30). Protein concentration 0.5 mg mL^{-1}; 50 μL applied with glass filter paper (Whatman GF/A) (6).*

exponential and stationary growth phase. Thus no evidence was found of a sequential appearance of the extracellular proteins in a culture filtrate of *Thermoactinomyces.*

Figure 5 also shows two 10-hr samples, 10a and 10b. Sample 10a was stored in solution at 4°C for one week, while sample 10b was stored frozen and then thawed immediately before application to the polyacrylamide gel. Both samples show the same protein band pattern. If proteolytic enzymes in the culture filtrate had acted on and partially degraded the extracellular proteins, a different band pattern would have been expected. Thus no product–precursor relationship appeared to exist between the various extracellular proteins in a culture filtrate of *Thermoactinomyces.* Moreover, it seems as if this organism produces at least three different extracellular cellulolytic enzymes simultaneously.

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Stability of Cellulolytic Activities of Thermoactinomyces *with Respect to pH and Temperature*

To obtain high glucose concentrations, saccharifications have been carried out for at least 24 hr at temperatures at or above 50°C (*1, 2, 4, 13, 22*). Furthermore, it has been suggested that in order to make the process economically feasible, the cellulolytic enzymes have to be recovered after saccharification (*23*). Such conditions require that the cellulolytic enzymes retain their stability over an extended period of time and at temperatures where proteins usually undergo significant heat denaturation (*24*). Recently, the stability of the cellulolytic enzyme system from *Trichoderma viride,* QM 9414, a mesophile, has been reported (25). It was found that only 60% of the activity remained when the enzyme system was maintained at 50°C for 24 hr. Only when the temperature was lowered to 30°C did the activity remain unchanged over 24 hr. Since it has been found that enzymes of thermophiles often have better stability at higher temperatures than those of mesophiles (*26*), it is reasonable to expect that the cellulolytic enzymes of *Thermoactinomyces,* a thermophile, would exhibit greater stability.

We have studied the stability of the CM-cellulase, Avicelase, and β-glucosidase activities of *Thermoactinomyces* over 24 hr in the pH range of 6.0–7.3 and at 55°C, 60°C, and 65°C. The pH of the various enzyme preparations was adjusted to 6.0, 6.6, and 7.3. The preparations were incubated in the absence of substrate at three different temperatures: 55°C, 60°C, and 65°C. Samples were taken out at 0, 1, 2, 4, 6, and 24 hr and immediately placed on ice and stored in the cold until the series were completed and the activities were measured (27). The pH and temperature optima of the three cellulolytic activities were first studied under assay conditions, i.e., up to 0.5 hr (Figures 6 and 7). The β-gluco-

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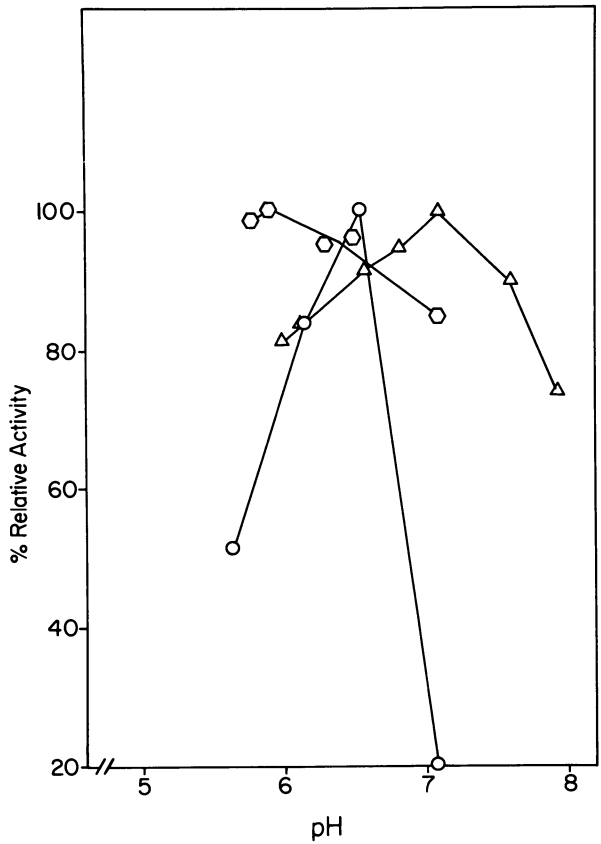

Figure 6. pH–activity profile of cellulolytic enzyme activities from Thermoactinomyces sp. *under assay conditions. (⬡) CM-cellulase, incubation time 10 min; (△) Avicelase, incubation time 20 min; (○) β-glucosidase, incubation time 30 min.*

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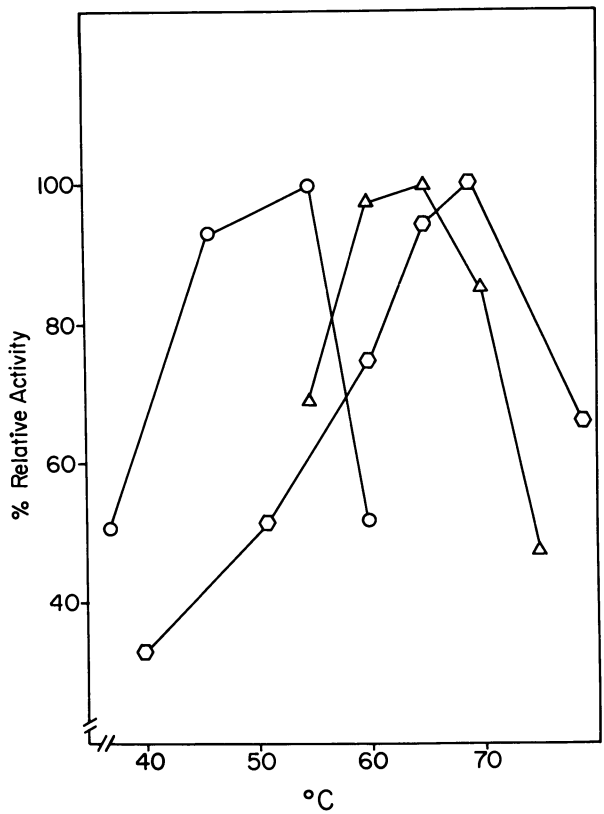

Figure 7. Temperature–activity profile for cellulolytic activities from Thermoactinomyces sp. *under assay conditions. Same symbols and conditions as Figure 6.*

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sidase activity has a sharp optimum at pH 6.55, while the CM-cellulase and Avicelase activities retain 90% of their maximum in a range of more than 1 pH unit. The temperature optima of the three activities show a similar pattern, with the β-glucosidase activity being the most temperature sensitive and having an optimum at 55°C, while the Avicelase and CM-cellulase activities have optima at 65°C and 70°C, respectively.

The CM-cellulase activity in a culture filtrate of *Thermoactinomyces* is stable over a wide pH range at elevated temperatures (Figure 8). At 55°C and 60°C, the pH range studied does not influence the stability, and only about 15% of the activity is lost over 24 hr at 60°C. At 65°C, a pH of 7.3 destabilizes the activity more than a pH in the range of 6.0–6.6. In this latter pH range, 60% of the CM-cellulase activity is retained over 24 hr.

The Avicelase activity is less stable than the CM-cellulase activity (Figure 9), and, similar to the CM-cellulase activity, the stability is lowest at pH 7.3. The stability of the activity at 55°C over 24 hr is only marginally affected. At 60°C, 50% of the activity is left after 24 hr, and at 65°C, the half-life of the enzyme activity is 3–4 hr.

The stability of the β-glucosidase activity in the whole culture broth of *Thermoactinomyces* was studied at 55°C and 60°C (Figure 10). The destabilizing effect of a pH of 7.3 is even more pronounced for this

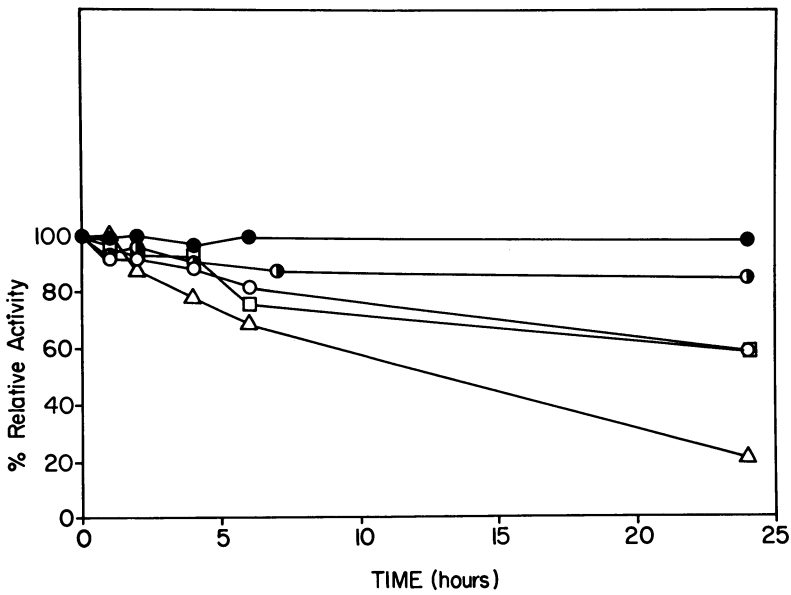

Figure 8. Stability of CM-cellulase activity in culture filtrate from Thermoactinomyces sp. (□) *pH 6.0;* (○) *pH 6.6;* (△) *pH 7.3; filled symbols 55°C; half open symbols 60°C; open symbols 65°C.*

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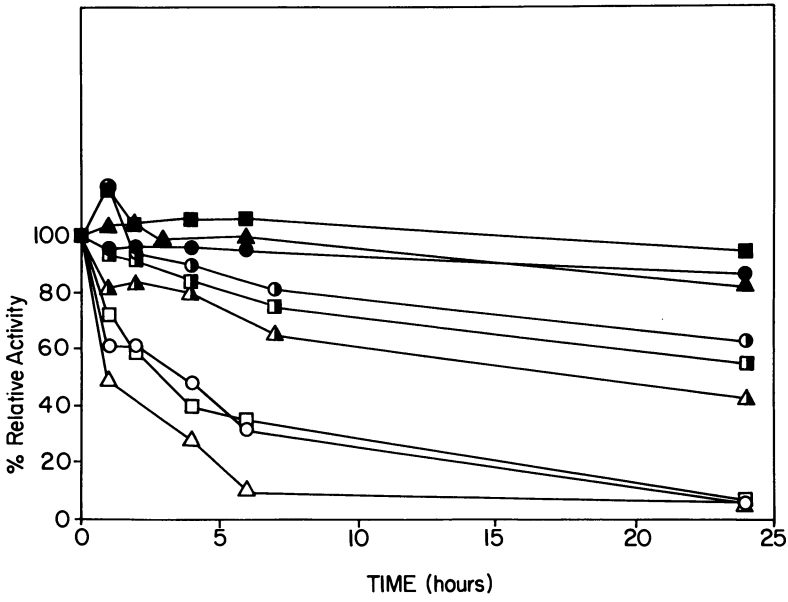

Figure 9. Stability of Avicelase activity in culture filtrate from Thermoactinomyces sp. *Same symbols as Figure 8.*

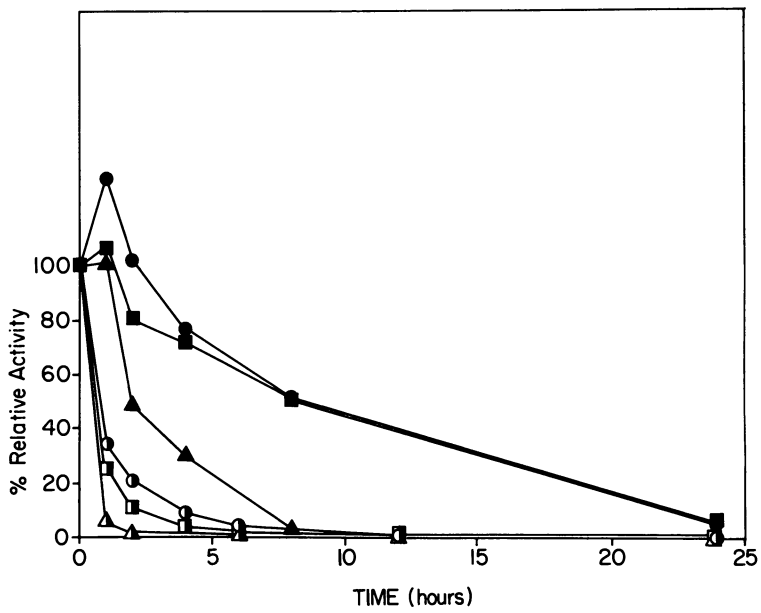

Figure 10. Stability of β-glucosidase activity in whole culture broth from Thermoactinomyces sp. *Same symbols as Figure 8.*

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activity than for the Avicelase activity. The half-life is 8 hr at 55°C and less than 1 hr at 60°C. Thus the sharp pH profile and the relatively low temperature optimum of the β-glucosidase activity under the assay conditions are functions of the stability of this enzyme.

Conclusions

Despite the fact that the β-glucosidase is sensitive to high temperatures, the cellulolytic enzyme system of *Thermoactinomyces* has several advantages that have to be considered in the choice of an enzyme system for saccharifications.

1. Optimum cellulolytic activity is produced within 24 hr of growth, at which time the culture can be harvested to obtain enzyme for saccharification.
2. The extracellular cellulolytic activities, CM-cellulase and Avicelase, are stable over 24 hr at 55°C in the pH range of 6.0–7.3, which suggests that no enzymatic activity would be lost in a saccharification under these conditions and that the enzymes would be recoverable.
3. The enzyme system is compartmentalized into extracellular CM-cellulase and Avicelase activities and cell-associated β-glucosidase activity, which permits optimal proportions of these activities to be used in saccharifications.
4. Saccharifications can be carried out with the enzyme system of *Thermoactinomyces* at 55°C and pH 6.6, under the following conditions: (a) The β-glucosidase activity is added in increments about every 8 hr or continuously. In such a process, up to 2% glucose has been produced in 24 hr (unpublished results). However, the addition of β-glucosidase activity might not be economically feasible. (b) The β-glucosidase activity is immobilized; immobilization has been found to increase the half-life of the β-glucosidase activity from *Aspergillus phoenicis* considerably (*28*). A similarly improved stability might therefore be expected for the β-glucosidase of *Thermoactinomyces*.

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Two Pea Cellulases Display the Same Catalytic Mechanism Despite Major Differences in Physical Properties

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Hormone-treated pea seedlings generate two physically distinct cellulases (EC 3.2.1.4), with similar substrate specificities, K_m values, and inhibitor sensitivities. They may be effectively separated by sequential extraction with buffer and salt and they appear to possess identical active sites but different apoprotein structures. The question arises of why this tissue should elaborate two hydrolases which catalyze the same reactions. The cellulase that forms first is synthesized by and accumulates in vesicles, where it would never encounter cellulose, while the other is concentrated on the inner wall microfibrils. It is suggested that only the latter cellulase functions to hydrolyze cellulose. A precursor/product relationship between them could explain their distribution and developmental kinetics, but physical and chemical differences mitigate against this interpretation.

When microorganisms encounter cellulose, they often generate and secrete a group of enzymes that digest it. This process permits them to survive saprophytically on plant debris and/or to penetrate and parasitize living plants. The induced enzymes usually include several glycosidases that act sequentially or synergistically on native cellulose microfibrils, and collectively degrade them to products which are nutritionally acceptable to the microorganism (*1,2*). In higher plants, in contrast, there are no reports of substrate-induced glycosidases. Most plants are nutritionally self-sufficient, and few are parasitic. The glucose

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that is incorporated into cellulose in seedlings stays there until the plant dies. Hence, cellulose has generally been regarded as a metabolically inert excretory product of the plant.

Nevertheless, there are occasions in the life of most plant cells, and in specific tissues and even in regions of individual cells, when it is necessary for the plant to at least partially digest the cellulose already deposited in its own walls. Plant cellulases have been found concentrated, for example, in specialized differentiating tissues such as abscission zones, perforating cell plates, fusing tracheids, and senescing and softening fruits (*3*). Some of the earliest observations (*4, 5*) of cellulase activity in plants reported that it was also located in very young tissues. The reason for searching for such a potentially suicidal enzyme in growing cells was the possibility that limited hydrolysis of cellulose microfibrils in the primary cell wall was necessary to "loosen" the restrictive wall framework for cell expansion to take place (*6*). In fact, recent work (*7, 8*) has shown that low concentrations of purified plant cellulases, paradoxically, can stimulate the deposition of cellulose in young tissue. Thus, the functions of cellulase in such tissue may be more complex than in fungi. For example, the enzyme could introduce chain ends along microfibrils of pre-existing cellulose so that new sugar units can be inserted into the structure. In this case limited cellulolytic action may be a prerequisite to both extension of the wall and cellulose deposition (*7, 9*). This interpretation does not fit well with most current interpretations of the appearance of plasma membrane–wall interfaces, where putative synthetase complexes appear to be associated with microfibril ends (*10*). If primer β-glucan chain ends within a fibril are not required for cellulose synthesis, the stimulation of synthesis by extracellular cellulase may have to be explained by effects on other essential components, e.g., intermediates, of the complex itself (*7*).

The formation of plant cellulases has been found to be closely regulated by different growth hormones, particularly auxin (*6, 11*), steroids (*12*), or ethylene gas (*13*). The hormones act in different tissues under different circumstances, and they seldom lead to such high cellulase activity that there is a net decline in total cellulose. Indeed, cellulose biosynthesis usually continues even while partial hydrolysis occurs, and net cellulose deposition often keeps pace with growth under all of these conditions (*14*).

When cellulase activity is induced in higher plants, analysis shows it to be present in multiple forms, which differ in electrophoretic and/or chromatographic mobility (*3, 13, 15–18*). These forms are analogous to those found in fungi in at least one respect, namely, that they include two or more endohydrolases which can attack CMC (CMCases). There is only one report of a plant exocellulase (*16*) and none of the synergistic

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"C_1" activity (*1, 2*), but β-glucosidase and cellobiase activities are probably ubiquitous (e.g., *see* Ref. *14* and *16*). Whether or not these together constitute a "cellulase complex" as in fungi is unknown. The possibility seems unlikely since there are so few occasions in plants when whole cellulose microfibrils are autolyzed, and there is no need for it on nutritional grounds. Nevertheless, the question remains of why specific plant cells or tissues often elaborate more than one endocellulase.

This question is asked here of the endocellulases found in growing regions of etiolated pea seedlings. In pea tissue, treatment with the auxin type of growth hormone evokes cell expansion and a concurrent increase in cellulase activity (*6, 11, 14*), as well as net cellulose deposition (*14*). The activity is due to two endohydrolases that have been purified to homogeneity and shown to be very different physically (*3*). Nevertheless, these two enzymes appear to hydrolyze cellulose powder, CMC, and lower cellodextrins with remarkably similar reaction characteristics (*19*). In this report, further details are given on the fractionation of pea cellulases, the relative rates with which they hydrolyze various substrates when acting alone or together, and their susceptibilities to inhibitors. They are compared with respect to subcellular distribution between cell organelles and compartments, and their separate development is followed after hormone treatment. It is concluded that multiple forms of endocellulase, which are identified in extracts or secretions, can not be assumed to all be functional as part of an active cellulolytic complex, but some may be processed forms of a single precursor and some may have different functions altogether.

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Methods

Enzyme Source and Hormonal Treatment. Crude buffer-soluble and -insoluble cellulases were extracted from the third internode (1 cm long, 7–8 days) of etiolated pea epicotyls as described previously (*3*). Cellulase activity was routinely assayed viscometrically, with one unit of cellulase activity defined here as the amount which, in 2 hr at 35°C, causes a 1% decrease in η_{rel} of 1.1 mL 6.5% (w/v) CM-cellulose in 20m*M* sodium phosphate, pH 6.2, containing 0.2% NaF (*19*). Reducing power was measured with Somogyi–Nelson reagent in aliquots of reaction mixtures in which substrates and cellulases (200–600 viscosity units/mL) were incubated at 35°C in the same buffer containing 0.03 sodium azide (*19*).

For hormonal treatments, whole apices of intact epicotyls were sprayed with 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D), or they were decapitated, i.e., the plumule and hook were detached (*6*), followed by application of 2.5 mg lanolin paste containing a suspension of indole-acetic acid (IAA, 0.5%, w/w) on each cut apex. Control tissue received lanolin paste alone. Both regulators caused massive swelling of the

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apices (*6, 14*). Effects of a DNA-synthesis inhibitor on the development of cellulases was studied by treatment with 5-fluorodeoxyuridine (FUdR, 0.1%, w/w) plus IAA (0.5%, w/w) in lanolin paste on decapitated epicotyls.

Enzyme Fractionation. Buffer-soluble and -insoluble cellulases from auxin-treated apices of pea epicotyls were extracted in a crude form and purified to homogeneity as previously described (*3*). For fractionation, a Sephadex G-100 column (1.6 × 100 cm) was prepared and equilibrated with 20m*M* sodium phosphate, pH 6.2, containing 5% glycerol and 0.03% sodium azide at 2°C. Crude enzyme preparations (1.5 mL) containing 1,000 to 3,000 viscosity units of activity (1–3 mg protein) were applied to the column and developed at flow rate of approximately 13 mL/hr using the same buffer. The column was calibrated for molecular weight with standard known proteins.

Cell walls, total membrane-bound components, and ribosomes were separated and assayed for cellulase activity to study the subcellular localization of the enzymes as follows. Segments (approx. 5 g fresh wt) were ground in two volumes of extraction medium containing 0.4*M* sucrose (ribonuclease-free), 5m*M* Mg acetate, 10m*M* Tris–HCl (pH 7.5 at 22°C), 20m*M* KCl and 5m*M* β-mercaptoethanol. The brei was filtered and the filtrate centrifuged at 500 ×g for 20 min. The post-500 ×g supernatant was fractionated essentially as previously described (*28*). Aliquots (7 mL) of the supernatant were layered on a discontinuous gradient composed of 2 mL 70% (w/v) sucrose and 3 mL 15% (w/v) sucrose both in 10m*M* Tris–HCl (pH 7.5 at 22°C), 10m*M* KCl, 2.5m*M* Mg acetate and 1m*M* β-mercaptoethanol. The tubes were centrifuged at 190,000 ×g for 24 hr, resulting in a clear supernatant overlying the 15%-sucrose layer, a membrane band at the 15%–17% sucrose interface, and a translucent pellet. The supernatant was recovered and assayed as soluble enzyme. The upper one-third of the 15%-sucrose layer contained no cellulase activity and was discarded, and the lower two-thirds together with the membrane layer were recovered, centrifuged (190,000 ×g for 1 hr) and the pellet was assayed as total membrane-bound enzyme. The wall fraction was prepared from the residue of initial tissue extraction, which was first homogenized (polytron) and washed (centrifugation) twice with four volumes of 20m*M* sodium phosphate buffer, pH 6.2. Buffer-insoluble cellulase activity was extracted from the pellet with 1*M* NaCl, and buffer-soluble cellulase activity is the sum of that recovered in cell washings.

Inhibition Studies. A number of compounds were employed to study the amino acid residue(s) that are important for cellulase activity. Samples of enzyme (0.1 mL, 500 units) were pre-incubated with 0.1 mL of inhibitor in semimicroviscometers for 8 min at 35°C. CM-cellulose solution (0.8%, w/v), which had been separately equilibrated at 35°C for 20 min was added to the viscometers and initial viscosity losses were measured after 15 min. Inhibitors were replaced by buffer in control experiments. Compounds that are insoluble in buffer, e.g., *N*-ethylmaleimide, diisopropyl fluorophosphate, and succinic anhydride, were dissolved in a small volume of 95% ethanol before assay. *p*-Chloromercuribenzoate (*p*-CMB) was first dissolved in 0.2*M* NaOH and the pH adjusted to eight prior to pre-incubation with cellulases.

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Results

Physical Properties. All of the cellulase (CMCase) activity which develops in auxin-treated pea apices dissolves in salt solutions (e.g., phosphate buffer, 20m*M*, pH 6.2, containing 1*M* NaCl). Gel chromatography of such extracts indicates the presence of two cellulase components with similar levels of activity and elution volumes corresponding to molecular weights of about 20,000 and 70,000 (Figure 1). If the tissue is extracted with buffer alone, only the smaller cellulase dissolves (referred to as buffer-soluble or BS cellulase). The larger buffer-insoluble (BI) cellulase can then be extracted from the residue by salt solutions. This simple extraction procedure effectively separates the two cellulases, and can be used as an initial step for their estimation or purification.

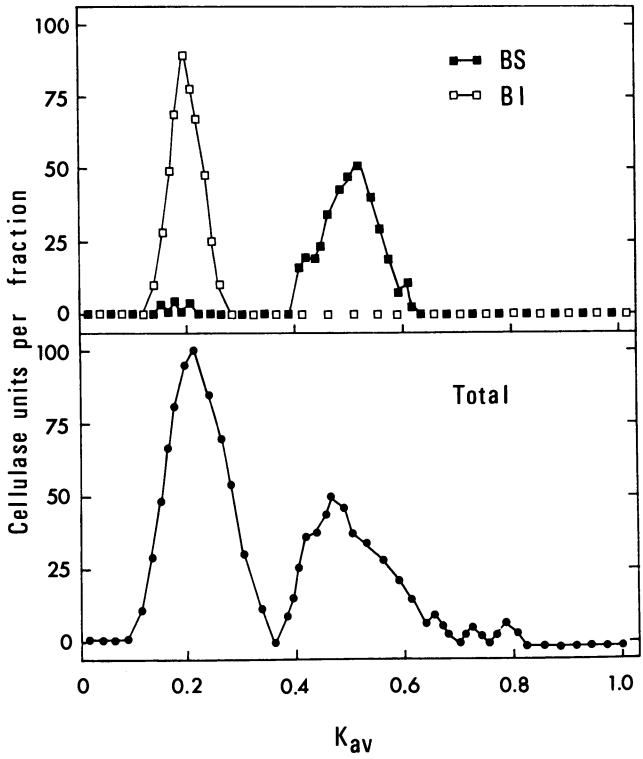

Figure 1. Elution profiles of cellulase activity from Sephadex G-100 gel chromatographs of crude extracts of auxin-treated pea apices. BS cellulase activity has an elution volume corresponding to a molecular weight of 20,000. BI cellulase activity dissolves in 1M NaCl and elutes with a molecular weight of 70,000. These values correspond to those observed for purified cellulases (3), indicating that the enzymes were not altered in molecular weight during purification, and could be effectively separated by differential extraction.

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The two cellulases were purified (*3, 19*) by a variety of fractionation procedures, including differential precipitation, affinity and gel chromatography, and ultrafiltration. The final preparations are homogeneous proteins as judged by SDS-gel electrophoresis and ultracentrifuge profiles. The purified cellulases possess very different isoelectric points (determined by isoelectric focusing), sedimentation and diffusion coefficients, partial specific volumes, and amino acid composition. The smaller cellulase contains more residues per molecule of certain amino acids (alanine, arginine, histidine, valine) than the large cellulase, implying that the smaller can not be merely a subunit of the larger. Rabbit antisera raised against the two purified cellulases yields single immunoprecipitation bands with no apparent cross-reactivity, indicating that antigenic properties of the two proteins are completely different.

On the basis of these observations, it was tentatively concluded (*3*) that auxin-treated pea tissue elaborates two cellulases which are physically so distinct that it is unlikely that one could have derived from the other. Of course, if two forms of cellulase arise from genetically determined differences in protein structure, it would be legitimate to refer to them as isozymes (*21*). But in the absence of proof that the pea or any other plant cellulases are under separate genetic control, we will continue to refer to them as multiple forms.

Enzymic Properties. Figure 2 shows progress curves for hydrolysis of [^{14}C]-cellohexaose (G_6) by purified BS and BI cellulases. Patterns of degradation catalyzed by the two cellulases show no significant differences. K_m values (3.8 mg/mL) for this substrate are identical (*19*). Cellobiose (G_2) and slightly lesser amounts of glucose (G_1) are the main products that accumulate in reaction mixtures, but these are not the earliest products: cellopentaose (G_5), -tetraose (G_4) and -triose (G_3) are all generated first. It is only after a lag period that cellobiose and finally glucose appear. Such a reaction pattern is typical of endohydrolase activity, with preferential hydrolysis of internal linkages and avoidance of at least one of the terminal linkages. Similar studies of the degradation of other cellodextrins (C_3–C_5), and of reduced [H^3]-cellodextrins, confirm (*19*) that both pea cellulases hydrolyze internal linkages but not the linkage closest to the reducing end.

As shown in Table I, of all the potential substrates for cellulases that were tested with these enzymes, cellohexaose was the most rapidly degraded, with lower cellodextrins hydrolyzed at a gradually decreasing rate. The reaction velocity is proportional to log DP (DP is degree of polymerization) (*19*). Substituted derivatives (e.g., CM-cellulose) were also rapidly degraded, provided preparations were used with a relatively low DS (degree of substitution). Highly substituted derivatives (e.g., DEAE-cellulose) were not attacked, although they adsorb the enzymes

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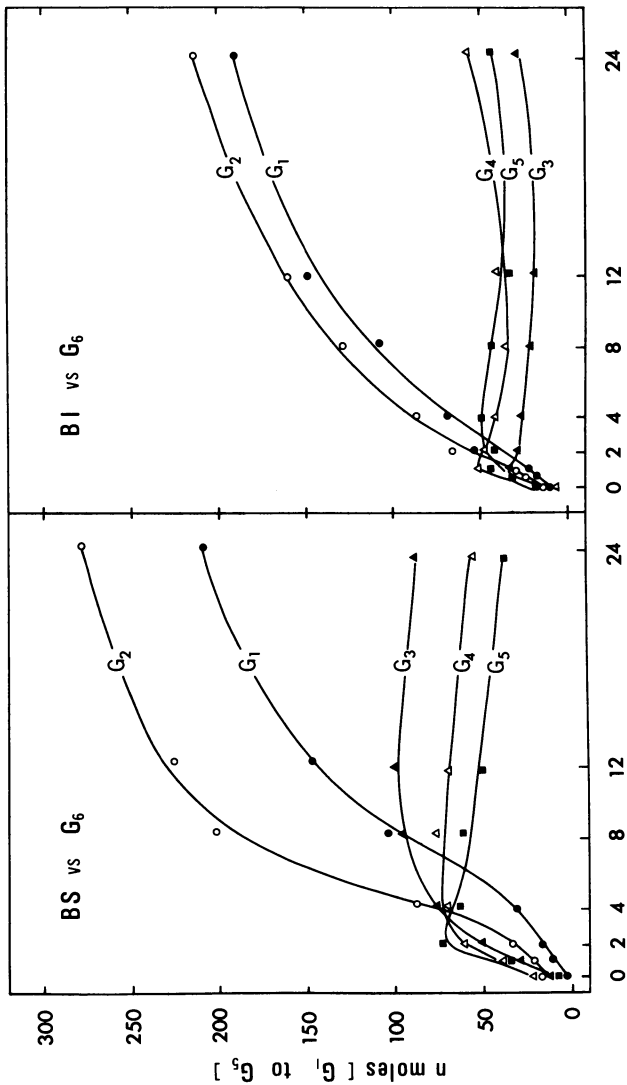

Figure 2. Hydrolysis of ^{14}C-cellohexaose (G_6) by BS and BI cellulases. The enzymes were purified to homogeneity (3) and incubated with pure uniformly-labeled cellohexaose as described in earlier tests with other cellodextrins (19). Reaction mixtures (0.3 mL) contained 1 mg substrate and cellulase (200 units) in 20mM sodium phosphate, pH 6.2, 0.03% sodium azide. They were incubated at 35°C and at intervals aliquots were removed and chromatographed. Cellodextrins with lower DP (G_1–G_5) were located by radioautography and estimated by scintillation spectrometry (19).

effectively (3). Mixed-linkage β-glucans may be hydrolyzed, but only if they contain stretches of more than one contiguous 1,4-linkage, e.g., barley β-glucan is hydrolyzed at these linkage points, but not reduced SIII polysaccharide, which is a strictly alternating β-1,3/1,4-linked glucan (22). Cellulose fibers that have been swollen by acid or alkali pretreatment are good substrates, but polysaccharides containing other sugars or linkages are not.

On a molar basis, V_{max} values with cellohexaose as substrate were about twice as high for BI as for BS cellulase (Table I), suggesting that BI was the more efficient catalyst. Relative V_{max} values using other substrates remained close to 1:2 for BS vs. BI, indicating no differential effects of substrate structure on enzyme affinities. Susceptibility to inhibition by heavy-metal ions and sulfhydryl-binding agents varied with the agent and concentration employed, but the relative degree of inhibition was generally similar for the two cellulases (Table II). An exception was observed with agents that bind hydroxyl groups, which did not inhibit BS cellulase effectively at concentrations that abolished BI cellulase activity. This may be related to the fact that BI possesses six times the number of hydroxyl-containing amino acid residues than does BS cellulase (3).

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Table I. Relative Activities of BS and BI Cellulases vs. Potential Substrates Relative to Cellohexaose[a]

Substrate	*BS*	*BI*
Cellodextrins		
cellohexaose	100	100
cellopentaose	43	53
cellotetraose	26	20
cellotriose	12	9
cellobiose	0	0
β-Glucans		
CMC (DS 0.7)	54	52
barley β-glucan	54	46
alkali-swollen cellulose	16	24
acid-swollen cellulose	13	22
CM pachyman (DS 0.4)	0	0
RS III polysaccharide	0	0
laminarin	0	0

[a] Purified cellulases (400 CMCase units in 0.2 mL buffer, pH 6.2) were incubated with cellodextrins (10m*M*), CMC or polysaccharide suspensions (1.2% w/v) for 2 hr at 35°C, and initial rates of generation of reducing power were measured. V_{max} values for cellohexaose were 173 and 389 mmol glc equivalents/min/μmol BS and BI cellulases, respectively (*19*). No synergism was observed when both BS and BI were incubated with these substrates.

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Table II. Inhibition of BS and BI Cellulases[a]

Inhibitor	*Conc. (mM)*	*Percentage Inhibition* BS	BI
Metal ions			
Mg^{++}	5	3	1
Cu^{++}	5	46	50
Hg^{++}	5	100	100
Sulfhydryl agents			
iodoacetamide	10	22	26
pCMB	2	88	98
N-ethylmaleimide	2	100	100
OH-binding agents			
diisopropylfluoro-P	10% (v/v)	4	24
succinic anhydride	15	9	100

[a] Purified enzymes were incubated with CMC ± inhibitors as described in Methods. With respect to effects of agents which react with hydroxyl groups, note that BS contains 8 serine and 8 threonine residues vs. 35 and 65, respectively, in BI (*3*).

The possibility that BS and BI cellulase could act synergistically, as has been recorded for many components of fungal cellulolytic complexes (*1, 2*), was tested in several assay systems by adding the enzymes separately or together at the same total activity levels (CMCase units). The assays included the hydrolysis of CMC, cellohexaose, and cellulose powder. The results (not shown here) indicated that the pea cellulases were no more or less effective when added together than when added singly, i.e., there is no indication of any interaction between the enzymes, or any preference by one for the products generated by the other.

It appears that, while BI cellulase may be a more efficient and readily inhibited catalyst than BS cellulase, the modes of action and affinities of the two enzymes are so similar that they must operate via similar reaction mechanisms. By implication, therefore, they may contain the same amino acid sequence and configuration at their active sites, despite physical differences in the rest of the molecules.

Intracellular Distribution and Development. When pea segments are homogenized and centrifuged to separate fractions containing wall material from particulate material and soluble supernatant, most of BS cellulase is found in the supernatant fraction while most BI cellulase is adsorbed to the wall (Table III). A minor part of both enzymes is particulate, i.e., retained in intact cell organelles and "microsomes" derived by vesiculation from cell membranes. In time-course studies of the distribution between cell fractions of total cellulase following auxin treatment (*23*), the activity appears first in the microsomes, and only

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Table III. Subcellular Distribution of Pea Cellulases[a]

	Cellulase Activity (units/segment)	
Fraction	*BS*	*BI*
Wall	10	122
Particulate	20	7
Supernatant	93	0

[a] Segments of epicotyl (1 cm) were collected 3 days after treatment of intact peas with 0.1% 2,4-D. They were homogenized and fractionated as described in Methods.

later in the supernatant and wall. This implies that the microsomes include organelles that synthesize one or both cellulases.

Microsomal cellulase sediments in sucrose gradients showing a major sharp peak at a density of 1.11, coincident with endoplasmic reticulum (ER) markers, and a small part at a density coincident with Golgi apparatus (*7*). Manipulation of [Mg^{++}] during isolation and centrifugation can separate rough (ribosome-bound) ER from smooth ER; the cellulase activity remains with the latter (*24*). Using ferritin-labeled antibodies to the purified cellulases, it was also demonstrated (*24*) that BS cellulase is present mainly within smooth ER vesicles. Antibody–ferritin–BI cellulase, however, is concentrated along the inner wall surface. BS in the supernatant of homogenates (Table III) presumably results from a rupturing of the ER vesicles during extraction, since little or no ferritin–antibody–BS cellulase is associated with the cytoplasmic matrix or intercellular spaces.

These observations are consistent with the conclusion that auxin treatment leads to the synthesis of BS cellulase, which then accumulates in smooth ER vesicles. There is direct evidence that the synthesis occurs in rough ER vesicles (*11*). Cellulase activity was shown (*25*) to increase in RNA-rich pea microsomes, provided these were isolated from auxin-treated tissue, when the preparations were incubated with ingredients necessary for carrying out protein synthesis in vitro. Messenger RNA (mRNA) from these microsomes has been translated in a different ribosomal system and shown to synthesize BS cellulase protein (*11*). Thus, it is legitimate to use the term "induction" to apply to the ability of auxin to evoke the appearance of mRNA for BS cellulase.

Antibodies to BI cellulase failed to detect any of this protein among the translation products of pea mRNA in experiments where BS cellulase was clearly synthesized (*11*). There are several possible reasons for this failure, including the alternative that no message for BI cellulase exists as such. Structural modifications often occur during processing of extracellular proteins, and these may be so extensive that antibodies to the final form of the protein do not recognize the precursor. Such modifica-

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tions may include selective excision of part of a precursor polypeptide, as in the activation of zymogens, covalent association with other polypeptides, as in condensation via disulfide linkages, and introduction of nonpeptide components, as in glycosylation or phosphorylation reactions. This may alter dramatically the amino acid composition and molecular weight of the final product from the original polypeptide, and it could conceivably lead to multiple enzymic forms of a single active site. Such modifications have been suggested before (*26*) as explanations for the multiple forms of cellulase observed in fungal sources. Clearly, such enzymic forms would not be considered isoenzymes in a genetic sense (*21*).

If the pea cellulases are related as an inactive intracellular precursor (BS) to an active wall-bound enzyme (BI), a delay would be expected between the appearance of BS after auxin induction and its secretion as BI. Such a delay has been observed repeatedly in tests of the relative rates of development of the two enzymes following auxin treatment of decapitated or intact tissue (Figure 3). During the first day after treatment, BS cellulase activity increases up to 18-fold over controls, while BI cellulase activity barely changes. By 2 days, although the BS cellulase activity level has increased further, BI cellulase activity surpasses it. The ascendancy of BI continues for several days, i.e., until effects of the hormone subside and levels of both cellulases begin to decline. Similar

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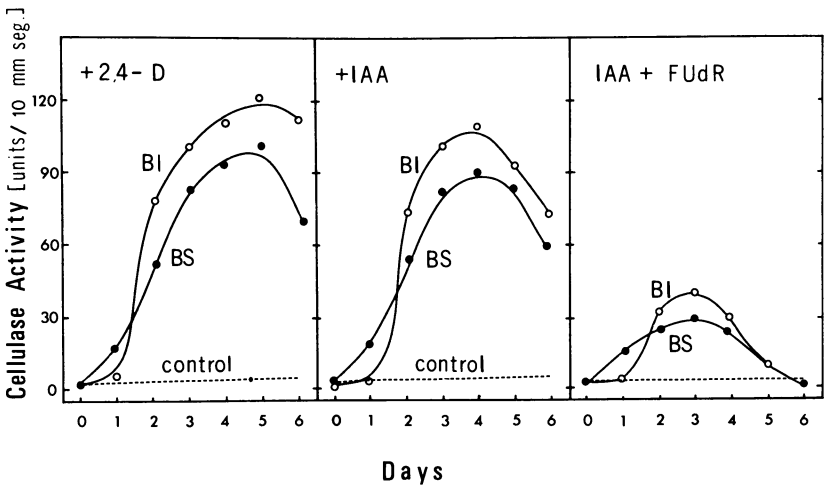

Figure 3. Development of BS and BI cellulase activity in apices of pea seedlings. Intact seedlings were sprayed with the auxin analogue 2,4-D and decapitated seedlings were painted with the natural auxin IAA with or without an inhibitor of DNA synthesis, FUdR. All treatments resulted in massive swelling at the pea apex because of cell expansion; cell divisions also occurred, but not in the presence of FUdR (6). Cellulases were extracted as described in Figure 1 and assayed in unpurified form.

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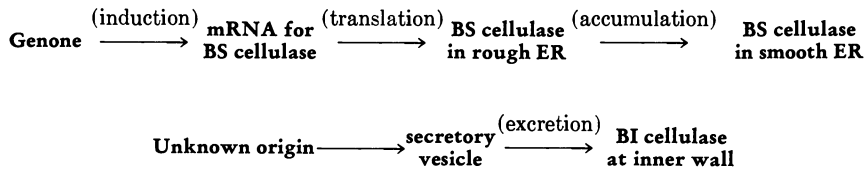
but less marked developmental patterns are observed when FUdR is added along with auxin. Since FUdR is an effective inhibitor of DNA synthesis and cell division in peas (*6*), this indicates that BS cellulase is generated before BI cellulase in expanding cells, as well as in new cells.

Discussion

Our present knowledge of the mechanism of biosynthesis of the two pea cellulases may be summarized diagrammatically as follows, where the organelles involved are indicated in bold type, the processes required are in parentheses, and the physical transfer of template, intermediates, or products is designated by arrows.

Biosynthesis of Two Pea Cellulases



The origin of BI cellulase is not established, nor is it certain that it passes through the usual sequences of organelles associated with the process of secretion, though the fact that it eventually accumulates outside the cell at the inner wall surface is not in doubt (Table III and Ref. *24*). During excretion and under natural conditions in turgid cells, BI would be expected to come into close contact with the plasma membrane and, indeed, under certain isolation procedures, part of it may be recovered buried within plasma membrane fragments (*17*). Thus the subcellular distribution of cellulase activity in growing peas resembles that of invertase in yeast, where there is a small intracellular invertase and a much larger plasma membrane-bound form which is precursor to the glycosylated wall-bound invertase (*27*). Whether or not the internal invertase is processed to form the plasma membrane invertase is in dispute.

Cell-surface BI cellulase is envisaged as the form which is active against cellulose in peas in vivo, with a function that may be constructive in that it can act synergistically with plasma membrane-bound β-glucan synthetase complexes to enhance the rate of cellulose deposition (*7, 8, 9*). BS cellulase never appears to reach the wall in vivo in a form recognized by a BS antiserum (*11*). BS cellulase does not even bind readily to wall material in homogenates (Table III) despite its ability to bind to cellulose (*3*) and hydrolyze it (Table I). It is possible that BS cellulase functions intracellularly to hydrolyze a noncellulosic organelle-bound polysac-

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charide such as xyloglucan (*28*), although a value to the plant for such a reaction is difficult to envisage. The alternative is that BS cellulase represents a transient form of the enzyme that is inactive in vivo and processed during secretion in such a way as to retain its active site and acquire the physical properties of BI cellulase. The lag period between appearance of BS and BI (Figure 3) could then be explained and, in the above scheme, the two flow lines for the synthesis of BS and BI cellulase would be connected.

However, any proposal that the intracellular form of pea cellulase (BS) is a precursor of wall cellulase (BI) must contend with the assembled physical and chemical evidence against this possibility (*3*). Thus, BS is much smaller than BI, and would have to be processed in such a way during secretion that a high-molecular-weight moiety is covalently associated with it. This moiety could not be a carbohydrate, since BI is not a glycoprotein, nor could it be a polypeptide attached by S–S linkages, since BI does not dissociate during SDS electrophoresis in the presence of β-mercaptoethanol (*3*). Moreover, BS contains more of certain amino acids then BI, and it would have to be partially degraded before attachment to another polypeptide to account for such differences in amino acid composition. The two are also antigenically distinct (*3*), though immunological experiments were conducted with undissociated antigens, and the antibodies may not have been specific to the active sites of the two proteins. The weight of evidence to date therefore mitigates against a precursor/product relationship between the two cellulases, though it does not completely rule it out.

The point to be emphasized in relation to reports of multiple cellulases in plants or microorganisms, is that not all of these are necessarily functional components of an extracellular "cellulase complex" that are needed for optimal or complete cellulose breakdown. Though all of the forms may show a capacity for hydrolyzing β-1,4-linkages in vitro, in vivo they could function in different intra- or extracellular loci on different substrates, and some could represent processed forms of inactive precursors. In general, not enough is known about the mechanisms whereby these enzymes are synthesized and excreted to enable an informed decision to be made on the roles that they perform.

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18

Structural and Mechanistic Comparisons of Some β-(1 → 4) Glycoside Hydrolases

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The modes of action and chemical characteristics of several β-(1 → 4) glycoside hydrolases suggest similarities in their catalytic and substrate binding mechanisms. A xylanase from Schizophyllum commune *produced an approximately 10^9 fold increase in the rate of xylan hydrolysis when compared with acid hydrolysis. This increase is similar to a rate enhancement of more than 10^{10} for lysozyme catalysis. Xylanases from various microorganisms and lysozyme show some similarities in amino acid composition—notably a high content of aspartic and aromatic amino acid residues. However, their amino acid compositions are not similar enough to suggest extensive homologies. Also, the partial amino acid sequence of the xylanase from* Schizophyllum commune *shows no homology with any known protein sequence, with a possible exception of α-amylase from* Bacillus subtilis.

The β-(1 → 4) glycoside hydrolases (e.g. cellulase, xylanase, and lysozyme) are classified into groups according to their specificities and modes of action. For example, in cellulose hydrolysis by microorganisms, at least three groups of enzymes are operative: cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21). These three enzyme groups can act synergistically and are thus capable of complete hydrolysis of both amorphous and crystalline cellulose (*1*). In addition, oxidative enzymes play a role in cellulose hydrolysis in at least one microorganism (*2*). A similar array of hydrolases is found to attack other β-(1→4)-linked polysaccharides, such as mannans and xylans (*3*).

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The characterization of the β-(1 → 4) glycoside hydrolases according to their specificity is now well established. Less well studied, however, is the structure and molecular mechanism of these enzymes—the outstanding exception to this statement being lysozyme. The catalytic mechanism of lysozyme is now well understood through a combination of structural analyses (*4*), binding studies (*5*), and kinetic experiments with model compounds (*6*). It is tempting to hypothesize that the catalytic mechanism of lysozyme is applicable to glycoside hydrolases in general, and this idea has been advanced before (*7*). A precedent for a common mechanism among enzymes with different specificities is found, for example, in the Asp–Ser–Gly serine proteases. Trypsin, chymotrypsin, elastase, alpha-lytic protease, and *Streptomyces griseus* proteases A and B all catalyze protein hydrolysis by a virtually identical charge transfer mechanism involving aspartate, serine, and histidine residues at their catalytic sites. On the other hand, their substrate binding sites differ considerably (for a review *see* Ref. *8*).

In spite of the apparent extreme diversity of the β-(1→4) glycans—from crystalline cellulose to components of bacterial cell walls—the similarity of the environments around their β-(1→4) glycosidic linkage raises the question of a common catalytic site structure in the various β-(1→4) glycoside hydrolases. This chapter will review the evidence for and against a general lysozyme type mechanism.

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Substrate Specificity and Kinetic Comparisons

Lysozyme hydrolyses a polysaccharide present in bacterial cell walls that is a β-(1→4) linked polymer of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues. The enzyme only attacks the NAM→NAG linkage. Certain lysozymes also attack chitin, the β-(1→4) linked linear polymer of NAG (*9*). It was demonstrated by Rupley (*5*), using oligosaccharides of NAG, that the C_1–O linkage was specifically cleaved and β-configuration was retained.

Endo-β-(1→4) glucanases hydrolyze the β-(1→4) linkage of cellulose at a rate dependent on chain length. They are capable of attacking crystalline cellulose (*10*), although the rate of hydrolysis of insoluble substrates is, of course, highly dependent on accessibility. Soluble derivatives such as carboxymethyl cellulose are attacked much faster, although the chain linkages close to carboxymethyl-substituted units are not attacked (*11*). Highly purified endo-β-(1→4) glucanases from various sources hydrolyze both cellulose and β-(1→4) xylan (*12, 13, 14*). This is perhaps not surprising in view of the similarity in molecular structure of cellulose and xylan. Conversely, a purified xylanase from *Schizophyllum*

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commune (*15*) does not hydrolyze carboxymethyl cellulose. Possibly the active site of the xylanase is too small to accommodate the bulkier cellulose chain.

The specificity of endo-β-(1→4) glycanases is not always restricted to β-(1→4) linkages. A *Streptomyces* cellulase was reported to cleave preferentially the β-(1→3) linkages of lichenin rather than its β-(1→4) linkages (*16*). This apparent contradiction is readily explained by a hypothesis advanced by Perlin (*16*), that at least some β-glycanases are specific for the structure of the glycosyl unit that becomes the reducing-end unit of the product liberated, rather than for the type of glycosidic bond that is being broken. Other interesting examples of departures from the specificity for β-(1→4) linkages are several highly purified xylanases capable of hydrolyzing the (1→3)-α-L-arabinofuranosyl branch points of arabinoxylans (reviewed in Ref. *3*).

The specificity of lysozyme can be explained from its three-dimensional structure; binding occurs between the enzyme and *N*-acetyl groups in the substrate. Thus cellulose, for example, is not a substrate for lysozyme (*17*). The exact binding mechanisms in other glycoside hydrolases is not known, and will require more information about enzyme structures. The relative positioning of binding and catalytic sites can be estimated by analyzing the hydrolysis products obtained from small oligosaccharides. In lysozyme, the trimer of *N*-acetylglucosamine is cleaved only slowly and is a competitive inhibitor, whereas the hexamer is cleaved rapidly and specifically between the fourth and fifth residue from the nonreducing end (*5*). From these two facts it can be deduced that the trimer is bound to the enzyme but does not extend over the catalytic site. Therefore, a binding site extends at least over the length of four sugar residues from the catalytic site.

From rate and product studies with xylooligosaccharides it was concluded that a xylanase from *Ceratocystis paradoxa* requires a chain of at least five xylose residues for rapid binding and subsequent hydrolysis (*18*). The catalytic site is assumed to be situated asymmetrically within a row of the five binding subsites. Similar studies on a cellulase from *Aspergillus niger* also suggest the presence of five binding subsites (*14*).

The interpretation of reaction mechanisms from end-product analysis is often complicated by the transferase activity of many of the glycoside hydrolases, especially at high substrate concentrations. In lysozyme and endoglucanases (e.g., cellulase III from *Trichoderma* (*19*)) the oligosaccharides appearing as products of the enzymic reactions are formed both by hydrolase and transferase activity. A general model of carbohydrase action, based on α-amylase degradation of maltotriose, has been proposed (*20*). The model involves multiple transglycosylation reactions and simultaneous binding of two substrate molecules.

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Among the characteristics listed by Jolles (*21*) as mandatory for classification of an enzyme as a lysozyme, are high basicity (isoelectric point around 10.5–11), low molecular weight (18,000 or less), stability to high temperature at low pH, and poor stability at high pH. In contrast to lysozymes, the xylanases are generally acidic, (Ref. 3, p. 332) with stability over a wide range of pH. Xylanase A from *Schizophyllum commune* showed poor stability only at low pH (*15*). The cellulases, too, are generally acidic proteins, as determined by their electrochemical properties (*22, 23*). The molecular weight of cellulases is generally larger than 18,000. These differences between the properties of lysozyme and other β-(1→4) glycoside hydrolases must reflect differences in the tertiary structure of the protein molecules. However, it is worth noting that even lysozymes from different sources, possessing identical catalytic sites, show some variability in these properties. For instance, the stability at higher temperatures is lost as the cystine content of the enzyme decreases (*9*).

As discussed by Vernon (*6*), the rate enhancement produced by lysozyme is greater than 10^{10}. This figure represents the ratio of the enzyme catalytic constant (k_{cat}) for hexa-NAG to the extrapolated first-order rate constant for the acid-catalyzed process at pH 5. Similarly, hydrolysis of the α-(1→4) glycosidic bond in starch by amylolytic enzymes takes place 10^{10}–10^{12} times faster than hydrolysis by mineral acids (*24*). The rate enhancement produced by other β-glycoside hydrolases will be a reflection of their reaction mechanisms. The catalytic constant (k_{cat}) of a xylanase from S. *commune* was found to be 1.4×10^5 min^{-1} per enzyme molecule using soluble larchwood xylan as substrate (*15*). This figure represents the number of xylosidic bonds cleaved per enzyme molecule per minute at 30°C and can be compared with a catalytic constant of 3.1×10^{-3} min^{-1} for catalysis with trifluoroacetic acid at 100°C. If the enzyme figure is extrapolated to 100°C ($E_A = 28.6$ kJ mol^{-1} (*25*)), then the ratio of molecular activities is approximately 10^9. Thus the rate enhancement of this xylanase appears to be almost as great as that of lysozyme. From viscosity measurements with carboxymethyl-cellulose solution, a series of endo-β-(1→4) glucanases was found to have molecular activity ranging only from 3000–42,600 bonds broken min^{-1} $molecule^{-1}$ (*26*). However, these lower values could be caused by the carboxymethyl substitution of cellulose.

Structural Comparisons

The amino acid composition of many β-(1→4)glycoside hydrolases, including cellulases (*27–30*), xylanases (*15, 31, 32, 33*), and mannanases (*34*) have been determined. In general, the enzymes contain higher than

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average numbers of aspartic acid (including its amide) and aromatic (tyrosine, tryptophan, phenylalanine) amino acid residues. This is demonstrated for example by the four xylanases isolated to date (Table I). The average percentages of aspartic acid and aromatic amino acid residues for a pool of 185 proteins are 9.7% and 8.1%, respectively (*36*). Table II shows that the same averages for the four xylanases are 11.4% and 11.6%, respectively, while the figures for lysozyme are also higher than average (16.3% and 9.3%, respectively). There are also significantly fewer basic and hydrophobic amino acid residues in the xylanases than in the average protein, although lysozyme is higher than average in basic residues.

The amino acid compositions in Table I can be used to determine an index of sequence identities. This index (*37*) is an unbiased estimator of the number of loci at which an amino acid in Sequence A is different from

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Table I. Amino Acid Compositions of Lysozyme and β-(1 → 4) Xylanases from *S. commune*, *S. sanguinolentum*, *T. hirsuta*, and *A. niger*

Amino Acid	*Chicken lysozyme*[a]	Schizophyllum commune *xylanase*[b]	Stereum sanguinolenthum *xylanase*[c]	Trametes hirsuta *xylanase*[d]	Aspergillus niger *xylanase*[e]
Aspartic acid	21	32	19	28	30
Threonine	7	30	27	27	32
Serine	10	38	34	26	28
Glutamic acid	5	19	15	18	17
Proline	2	14	11	19	5
Glycine	12	47	26	22	36
Alanine	12	21	18	21	13
Valine	6	14	13	13	1
Cysteine	8	4	3	4	—
Methionine	2	1	2	1	2
Isoleucine	6	12	7	8	5
Leucine	8	11	8	11	5
Tyrosine	3	23	11	7	16
Tryptophan	6	9	9	10	3
Phenylalanine	3	5	7	7	5
Histidine	1	4	2	3	2
Lysine	6	7	4	6	6
Arginine	11	6	2	4	6
Total (n)	129	297	218	235	212
0.42 n	54	124	92	98	89

[a] Data from Ref. *35*.
[b] Data from Ref. *15*.
[c] Data from Ref. *31*.
[d] Data from Ref. *32*.
[e] Data from Ref. *33*.

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Table II. Percent Composition

Amino Acid Group	*Average Protein*	*Chicken lysozyme*[a]
Aromatic (Phe, Tyr, Trp)	8.1	9.3
"Acidic" (Asp, Asn, Glu, Gln)	19.1	20.0
Asp + Asn	9.7	16.3
Glu + Gln	9.5	3.9
Basic (Lys, Arg, His)	13.2	14.0
Hydrophobic (Leu, Val, Ile, Phe, Met)	23.5	19.4

[a] Compiled from Ref. *36* and Table I.

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that in Sequence B. It is defined as half the sum of squares of the differences between the number of residues of each type in the two proteins. For unrelated proteins with n residues, the index exceeds 0.42 $\times$ n in about 95% of comparisons. For proteins of unequal length, n is defined as the smaller length. The index values for pairs of enzymes from Table I are shown in Table III. They suggest that there is no gross homology between any of the pairs since all values exceed 0.42 $\times$ n (Table I).

A similar comparison of cellulases from different organisms (*38*) concluded, using a different index of sequence identity, that there was a high probability of homology between most of the enzymes studied.

Table III. Index of Sequence Identities (*37*) for Lysozyme and Four β-(1 $\rightarrow$ 4) Xylanases

	Schizophyllum commune *xylanase*	Stereum sanguinolentum *xylanase*	Trametes hirsuta *xylanase*	Aspergillus niger *xylanase*
Chicken lysozyme	207	253	169	298
Schizophyllum commune xylanase	—	119	254	167
Stereum sanguinolentum xylanase	—	—	132	300
Trametes hirsuta xylanase	—	—	—	417

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of Amino Acid Groups[a]

Schizophyllum commune *xylanase*	Stereum sanguinolentum *xylanase*	Trametes hirsuta *xylanase*	Aspergillus niger *xylanase*	*Average Xylanase*
12.5	12.4	10.2	11.3	11.6
17.2	15.6	19.7	22.2	18.7
10.8	8.7	12.0	14.2	11.4
6.4	6.9	7.7	8.0	7.2
5.7	3.7	5.6	6.6	5.4
14.5	17.0	17.1	8.5	14.3

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However, this latter index has since been criticized (*37*) because it fails to take into account the dependence of the standard deviation of the index on the length of protein.

Sequence studies with β-(1→4) glycoside hydrolases are only just beginning. Whitaker determined that the *C*-terminal amino acid of a cellulase from *Myrothecium verrucaria* was glycine (*39*). The *N*-terminus appeared blocked, possibly by an acetyl group. A β-(1→4) glucan cellobiohydrolase from *Trichoderma viride* had an alanine residue at the *N*-terminal (*29*). The *N*-terminal and probably the *C*-terminal amino acid in a cellulase from *Penicillium notatum* is also alanine (*40*). The amino terminal sequence of xylanase A from *S. commune* (Table IV) has recently been determined (*15*). The sequence reflects the overall amino acid composition (Table I) but shows no homology with lysozyme nor with any sequence held in the Atlas of Protein Sequence and Structure data bank. Using a predictive model for secondary structure (*41*), it appears that the sequence from residue 11 to 21 inclusive is arranged in a β-sheet conformation. No α-helix was predicted from the same model.

Table IV. Partial Amino Acid Sequence of Xylanase A from *S. commune*[a]

NH_2–Ser–Gly–Thr–Pro–Ser–Ser–Thr–Gly–Thr–Asp–Gly–Gly–Tyr–Tyr–Tyr–Ser–Trp–Trp–Thr–Asp–Gly–Ala–Gly–Asp–Ala–Thr–Tyr

[a] Data from Ref. *15*.

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Table V. Partial Amino Acid Sequence of α-Amylase from *B. subtilis*[a]

Val–Asn–Gly–Thr–Leu–Met–Gln–Tyr–Phe–Glu–Trp–Tyr

[a] Data from Ref. *42*.

Several amylases have been partially sequenced (*42–45*). For example, α-amylase from *Bacillus subtilis,* which is composed of two subunits of 24,000 molecular weight each, has an amino terminal sequence as shown in Table V (*42*). Perhaps fortuitously, the sequence of residues 8 through 12 resembles residues 14 through 18 in xylanase A, in that a polar residue is surrounded by four aromatic residues.

The lack of sequence data for β-(1→4) glycoside hydrolases probably is caused by the difficulty in obtaining homogeneous enzymes because of the multiplicity of glycoside hydrolases produced by any microorganism. Also, the frequent occurrence of covalently bound sugars in the enzyme molecules makes sequence analysis difficult. Differently glycosylated forms of the same enzyme have been found in *Trichoderma viride* (*46*). Other factors also seem to contribute to the multiplicity of cellulases with apparently similar function. Partial proteolysis may produce a multiple endocellulase system in *Trichoderma viride* (*47*). Complexing of enzyme and polysaccharide produced electrophoretic heterogeneity in a cellulase solution from *Myrothecium verrucaria* (*48*). Strong noncovalent bonding of sugars also has been observed in xylanase from *S. commune* (*15*), which is not a glycoprotein, and in cellulase from *Sporotrichum pulverulentum* (*49*). Possibly the sugar component of glycoproteins plays an important part in binding, as has been implied by some authors (*10*).

Five endo-β-(1→4) glucanases from *Sporotrichum pulverulentum* (*30*) are enzymes with different amino acid compositions and catalytic properties. This may be compared with two different lysozymes found in duck egg white. These two lysozymes have different chromatographic behavior and slightly different structures, giving rise to differences in catalytic constants (*9*). Even greater differences are found between species—the T4 phage lysozyme has a molecular weight of 18,000 (cf. 15,000 for egg white lysozyme) and it shows no sequence homology with egg white lysozyme, although the distribution of basic, acidic, and hydrophobic amino acids seems to be similar (*9*), and there is some similarity in the three-dimensional structure (*50*).

The Catalytic Site

Catalysis by lysozyme is caused by three main factors (Figure 1): (1) general acid catalysis by glutamic acid residue 35, located in the proximity of the glycosidic bond, initiates the formation of a carbonium

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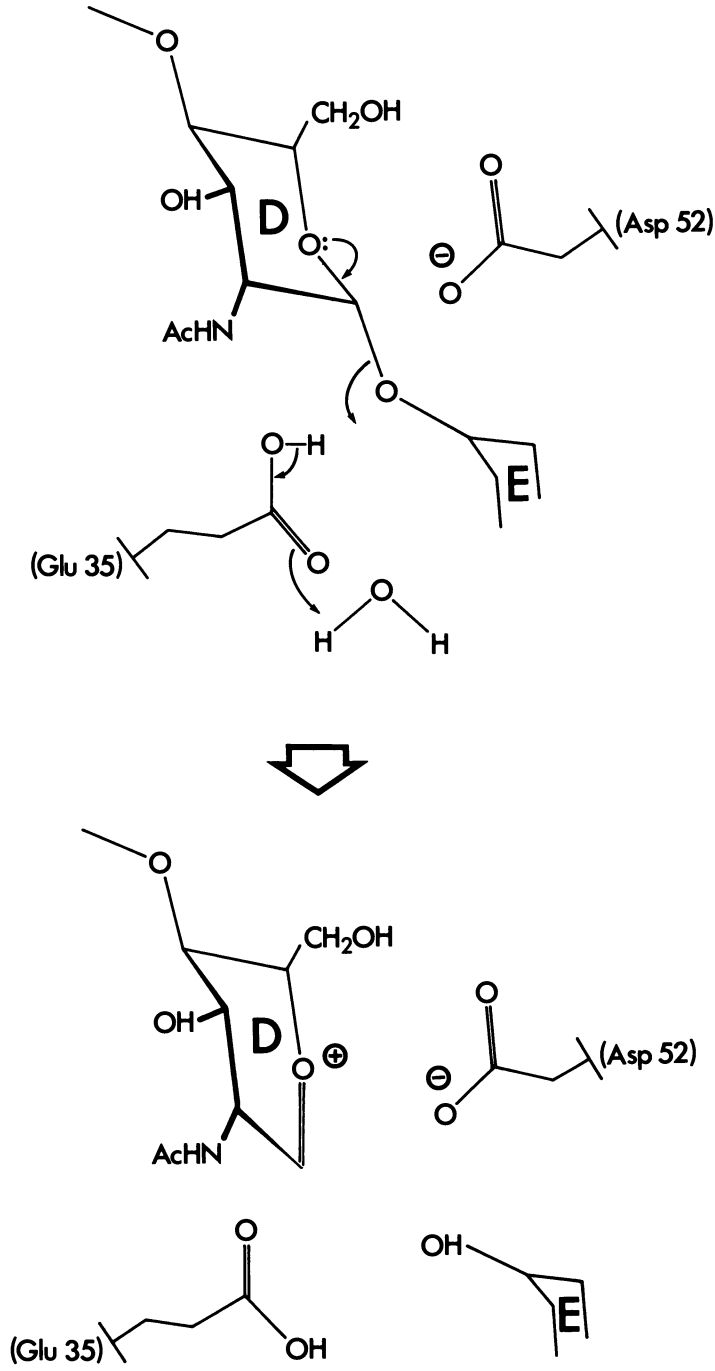

Figure 1. Postulated mechanism of action of lysozyme

ion; (2) the energy required to form the carbonium ion is lowered by flattening of the pyranosyl ring D during binding; (3) the carbonium ion is stabilized by electrostatic interaction with the negatively charged aspartic acid residue 52. The relative magnitude of effects (2) and (3) is still a subject of debate (*51*).

Is there any evidence for these mechanisms in other β-(1→4) glycoside hydrolases? Legler has sequenced the active site of a β-glucosidase from *Aspergillus wentii* (*52*) after labelling the enzyme with the affinity label conduritol-B-epoxide. The label bonded covalently to an aspartic acid residue in the senquence Ser–Asp–Trp. It is interesting to note that in lysozyme both glutamic acid residue 35 and aspartic acid residue 53 are also adjacent to a β-hydroxy amino acid residue on one side and an aromatic residue on the other, which might imply a similarity in the geometry of the active sites. However, Legler also found (*53*) that conduritol-B-epoxide did not inhibit cellulase from *Oxyporus* and *Aspergillus* species and, more surprisingly, it did not inhibit cellobiosidase activity from the *Oxyporus* species. From inhibitor studies with epoxyalkyl oligo-(1→4)-β-D-glucosides (*53*), it was concluded that, in the above cellulases, there are two or three binding subsites adjacent to the catalytic site. These subsites bind the nonreducing end of the substrate chain. Additional binding sites may be present towards the reducing end of the substrate, as in lysozyme. The latter proposal is in accordance with earlier conclusions from enzymic hydrolysis of oligosaccharides from cellulose (*54*).

Recently, the active site sequence of a β-glucosidase from bitter almonds has been determined (*55*). As with the β-glucosidase from *A. wentii,* an active-site-directed inhibitor (in this case 6-bromo-3,4,5-trihydroxycyclohex-l-ene oxide) was bound covalently to an aspartic acid residue. However, no further sequence similarities, either with lysozyme or β-glucosidase from *A. wentii,* were found. In addition, 30% of the inhibitor label was attached to a different part of the enzyme molecule perhaps indicating binding with another carboxyl group, also essential for catalysis. Further evidence for a carboxylate anion at the active site of β-glucosidases from almonds is the stabilization of binding of basic inhibitors compared with their nonbasic analogues (*56*). The additional binding energy can be attributed to electrostatic interaction of the inhibitor cation with the carboxylate ion in a low-polarity environment.

From kinetic (*57*) and chemical modification (*58*) studies, Hurst et al. concluded that the catalytic residues in a cellulase from *Aspergillus niger* are a carboxylate anion (pK_a 4.0–4.5) and a protonated carboxyl group (pK_a 5.0–5.5) with tryptophan and dicarboxylic amino acid residues involved in substrate binding.

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Oxidation of two out of 13 tryptophan residues in a cellulase from *Penicillium notatum* resulted in a complete loss of enzymic activity (*59*). There was an interaction between cellobiose and tryptophan residues in the enzyme. Participation of histidine residues is also suspected in the catalytic mechanism since diazonium-1-H-tetrazole inactivated the enzyme. A xylanase from *Trametes hirsuta* was inactivated by *N*-bromosuccinimide and partially inactivated by *N*-acetylimidazole (*60*), indicating the possible involvement of tryptophan and tyrosine residues in the active site. As with many chemical modification experiments, it is not possible to state definitively that certain residues are involved in the active site since inactivation might be caused by conformational changes in the enzyme molecule produced by the change in properties of residues distant from the active site. However, from a summary of the available evidence it appears that, for many β-(1→4) glycoside hydrolases, acidic and aromatic amino acid residues are involved in the catalytic site, probably at the active and binding sites, respectively.

Both glycosidases (*61*) and amylases (*62*) are inhibited by certain lactones, as is lysozyme, D-glucono-1,5-lactone, for example, is presumed to inhibit amylase by acting as a transition state analog because it closely approximates a half-chair conformation. However, as stated by Laszlo et al. (*62*), lactone inhibition cannot establish whether distortion of substrate occurs during binding, as in lysozyme, or after bond splitting to form the carbonium ion, as in proton catalysis.

Future Considerations

There is still much to be learned about the structure and mechanism of action of this class of enzymes. Their mode of attack in terms of gross effects on substrates is now fairly well understood, especially in the cellulases, and this has resulted in a clearer classification of the purified components of the cellulase system. In order to explain the catalytic effects at a molecular level, it will be necessary first to obtain more information on the primary and, eventually, tertiary structures of the enzymes. The molecular mechanism, defined as a description of the number and structures of intermediates lying on the reaction path (*6*), then can be fully identified and from this the origin of the observed catalytic rate enhancements can be sought.

What will we gain from structural and mechanistic data on the β-(1→4) glycoside hydrolases? First, we will be able to identify evolutionary pathways of these enzymes and determine whether they originated from the same ancestral gene or whether there are several distinctly different classes. Second, it may be possible to modify the enzymes more

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specifically, such as by cross-linking or immobilization, and thus increase their stability and expand their applicability. And thirdly, we may be able to mimic the enzymes with more stable and less expensive synthetic catalysts.

Two examples of the "enzyme analog" approach to glycoside hydrolases are Arai's work, reported in this edition (Chapter 9) and an earlier paper by Painter (*63*), which describes an attempt to devise an artificial endopolysaccharase system. Another example in a different field is a catalyst modelled on the enzyme aryl sulfatase. Histidine has been implicated as an active site residue in this enzyme. A catalyst was synthesized (*64*) by attaching imidazole groups (for catalysis) and dodecyl chains (for hydrophobic bonding) to a polyethylenimine backbone. The observed rate enhancements in the hydrolysis of phenolic sulfate esters with the catalyst were greater than with the native enzyme. Another promising approach in the field of enzyme analog design is to use crown ethers, which contain large hydrophobic pockets, as the basic skeleton for constructing the synthetic enzyme analogs. Stoddart et al. (*65*) substituted carbohydrates into these molecules to introduce chirality and functionality while Cram (*66*) has substituted binaphthyl groups to perform the same functions. The field of polymeric enzyme-modelled catalysts recently has been reviewed (*67*), and appears to be the subject of considerable research interest at present (*68*).

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