A REVIEW OF LITERATURE ON THE ENZYMATIC DEGRADATION OF CELLULOSE AND WOOD

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Introduction

Enzymatic degradation of cellulose by fungi is of great biologic as well as economic importance. It constitutes one of the necessary steps in the balance of opposing synthetic and degradative forces in the carbon cycle, and is a major limitation to the usefulness of wood, paper, pulp, cotton, rayon, cellophane, and a host of other cellulosic materials of great and diverse utility. It is a subject of considerable practical as well as fundamental interest, particularly for plant pathologists.

The present review summarizes the principal findings in an intensive investigation of literature on the enzymes that decompose cellulose. This investigation was made in preparation for research on the enzymes of wood-destroying fungi. A brief introduction to the nature of enzymes and their action and to the structure and properties of cellulosic materials in relation to their susceptibility to enzymatic degradation is given by way of orientation. A detailed report of the present status of knowledge regarding the cellulolytic enzymes of fungi is presented.

For more authoritative and comprehensive reviews of this subject, the works of Reese (53), Siu and Reese (70), Jennison (33), and the monograph by Siu (68) are particularly suggested. 2 The earlier reviews of Waksman (80), Boswell (9),

1-Maintained at Madison, Wis., in cooperation with the University of Wisconsin.
2-The numbers in parentheses refer to Literature Cited at the end of this report.
and Nord and Vitucci (49) may also be of interest. A review of the historical development of knowledge of cellulolytic enzymes is given in a thesis by Walseth (76). The more specialized subjects of enzymatic decomposition of cellulose in sewage and in the rumen of cattle have been reviewed by Huntgate (32), McBee (46), and Sijpesteijn (67). Literature on cellulolytic enzyme production by insects has been reviewed by Mansour and Mansour-Bek (42), and by wood-destroying fungi by Bose and Sarkar (7) and by Garren (21, 22). Prevention of deterioration of cellulosic materials has been presented in detail by Siu (68) and Greathouse and Wessel (28). Further information regarding the chemistry of cellulose and its derivatives is contained in the 2-volume work of Wise and Jahn (83). General references on enzyme chemistry are the texts by Sumner and Somers (71, 72). A series of review articles on recent advances in enzymology is edited by Nord (48).

The Nature of Enzymes and Their Action

Enzymes are organic catalysts produced by living cells. They make it possible for the biochemical reactions necessary for physiological processes to take place at the restricted pH, pressure, temperature, and other conditions that exist in living cells. It is the particular enzymes present that determine the chemical transformations that will take place in a given organism. Enzymes are in general heat-labile, water-soluble, protein molecules with molecular weights ranging from 20,000 to 500,000. They may or may not also contain a nonprotein prosthetic group.

Enzymes are named by adding the suffix "ase" to the root designating the substrate upon which they act or to the type of reaction they catalyze. For example, cellulase refers to an enzyme that catalyzes the hydrolysis of cellulose, and polyphenol oxidase refers to one catalyzing the oxidation of polyhydroxy phenols. There is an additional group of enzymes for which traditional names such as emulsin or papain are commonly used.

The reactions catalyzed by enzymes follow the usual laws governing chemical equilibria and kinetics (72). In common with inorganic catalysts, enzymes possess the following properties:

1. They increase the rate of a chemical reaction without being permanently altered in the process. Actual contact between an enzyme and the substance (substrate) upon which it acts is necessary for catalysis. Thus, the following general reaction sequence has been postulated for enzyme-catalyzed reactions:

\[
\text{Enzyme} + \text{Substrate} \rightarrow \text{Enzyme-Substrate Complex} \rightarrow \text{Enzyme} + \text{Products}
\]
2. They are active in very low concentrations. A given enzyme molecule is able to catalyze the transformation of from 100 to 3,000,000 substrate molecules per minute at optimum conditions, depending on the enzyme involved.

3. They do not determine the course of a reversible chemical reaction, but serve to hasten the attainment of equilibrium conditions from either direction. Many reversible biochemical reactions take place at appreciable rates only in the presence of a suitable enzyme. Enzymes achieve this result by reducing the energy required to initiate the reaction. The course of an enzyme-catalyzed reaction is determined by the concentration of substrate and product molecules in relation to the equilibrium constant for the reaction involved. It is unaffected by the concentration of enzyme present.

4. The rate of the reactions they catalyze increases directly with temperature. This relationship holds for enzyme-catalyzed reactions only until denaturation of the enzyme by heat offsets the increase in rate due to an increase in temperature.

The outstanding characteristic of enzymes that distinguishes them from most inorganic catalysts is their specificity. A given enzyme is restricted to catalysis of a particular kind of reaction, and that, often, on a particular molecular species. A notable example of substrate specificity is provided by the α and β specific glycosidases used by Emil Fischer to distinguish the α- and β-glycosides of glucose in his proof of the structure of glucose.

Some enzymes are secreted by the cells in which they are formed and act extracellularly; others are active within the cells that produce them and are called intracellular enzymes. The degradation of cellulose by fungi involves enzymes of both types. Digestion of cellulose takes place outside the cell by hydrolysis of its large molecules into water-soluble sugars of low molecular weight. These sugars are then taken within the cell and are there transformed by intracellular enzymes to give various degradation products from which the organism derives the energy and substance needed for its growth (40).

Some enzymes are produced in demonstrable quantities only in the presence of the substrate upon which they are active. These are called adaptive enzymes in contrast to constitutive enzymes that are produced by cells grown on any substrate that will support their development. The cellulases produced by fungi are in general adaptive; those produced by bacteria are usually constitutive (70).

Methods of Studying Enzymes

Both in vivo and in vitro methods are used for the investigation of enzyme reactions. The utility of both techniques depends upon the availability of suitably...
characterized substrates and methods of determining the alterations in their properties caused by enzymes. The limited availability of suitable substrates has been a primary obstacle to acquisition of further knowledge of the mechanism of cellulose decomposition by enzymes (70).

In vivo methods involve growing the organism on a substrate of known composition; alterations in the substrate detected by subsequent analysis are then attributed to the enzymes of the organism. This technique has been widely used in investigations of the enzymes of wood-destroying fungi (21, 22, 63 to 66), since the enzymes of these organisms are apparently active at measurable rates only in relatively close proximity to the hyphae. The method has the advantage of minimal artificiality, but it has several disadvantages as follows:

1. The reactions of specific enzymes are difficult or impossible to identify.

2. Intermediate products of reaction are found in mixture with final products and usually cannot be easily separated or identified.

3. Separation of the effects of extracellular and intracellular enzymes is difficult.

4. The presence of the cells of the organism in mixture with the substrate to be analyzed may lead to erroneous conclusions in interpreting the results of chemical analyses unless care is taken to separate the cells from the substrate or to account for their effect on the analysis.

In vitro methods involve separating the enzymes from the organism that produces them and then testing their activity in vitro. This technique avoids many of the disadvantages of the first method but presents the difficulties of artificiality, our inability to reproduce all of the effects of the organism itself, and often, limited rates of action compared to those obtained when the organism is present. To overcome the latter two difficulties, modified substrates and concentrated enzyme preparations are used and this all the more contributes to the artificiality of the reactions investigated. However, when used with caution, much can be learned using these methods that would otherwise remain unknown.

In vitro techniques usually involve growing the organism in an agitated liquid culture medium, extraction of the enzyme under observation from the culture fluid or cells of the organism, partial or rigorous purification of the enzyme, subjection of a known substrate to the enzyme under conditions of pH, temperature, and chemical association that are favorable for their reaction, and analysis of the substrate to detect changes induced by the enzyme.

The shake-culture technique commonly employed (33) insures rapid growth of the organism in a homogenous environment (19). Extracellular enzymes are

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obtained from such a culture by filtration of the culture fluid. Intracellular enzymes are obtained by thoroughly washing the cells of the organism and then fracturing their cell walls either sonically or mechanically to release the cell contents. Filtration or centrifugation then insures that the fluid thus obtained is free of living cells.

Methods of purification may incorporate the following procedures:

1. Precipitation in organic solvents such as acetone or alcohol (30).
2. Fractional precipitation with organic solvents and water (20).
3. Drying under vacuum (40).
4. Dialysis to remove salts and other water-soluble molecules of low molecular weight (26).
5. Differential adsorption on various substrata such as carbon, aluminum compounds, kaolin, and others (73).
6. Elution by differential solvents from an adsorptive substrate (26).
7. Physical separation by various chromatographic and electrophoretic techniques (47, 55).
8. Differential inactivation of other enzymes in a given filtrate by heat, chemical inhibitors, or specific conditions of pH (52).

The most complete purifications of cellulolytic enzymes have been accomplished by Whitaker (81, 82) who determined an approximate molecular weight of 63,000 and by Higa and others (30) whose purification technique yielded two crystalline forms. However, much has been learned about cellulolytic and the analogous starch-hydrolizing enzyme systems with relatively impure enzyme preparations (70).

In determining the activity of enzymes, it is important to recognize that the conditions of pH, Eh, temperature, substrate, nutrient balance, and so forth, which are most favorable for the growth of an organism are not necessarily optimal for greatest production of enzymes or for their maximum activity in vitro. For example, the temperature and pH optima for growth of Polyporus versicolor are 28° C. and 5.5 pH (33), whereas the optimum conditions for activity of the enzyme cellulase taken from this same organism are about 50° C. and 4.5 pH (58).
Determination of the specific effects of a given enzyme is usually accomplished by judicious choice of substrate. A hypothetical mechanism of enzymatic action may be tested by choosing a substrate that will permit the assumed path to be inspected by means of appropriate physical or chemical assays.

In the case of cellulolytic enzymes, the extent of hydrolysis of cellulose may be determined by the following measurements:

1. Increase in reducing sugar value. This would indicate the increase in the number of cellulose-chain ends in the substrate. The colorimetric method of Sumner and Somers (71) has been widely used.

2. Decrease in the viscosity of soluble cellulose derivatives (60) or of purified natural celluloses (39). Decrease in viscosity is proportional to the decrease in average degree of polymerization of the cellulose.

3. Decrease in the tensile strength of cotton fiber (6, 79) or in the bursting strength of cellophane film (33).

4. Loss in the weight of an insoluble substrate such as cotton, wood, or rayon, (77, 78).

5. Chromatographic separation and identification of end products such as cellobiose and glucose (38).

6. Increase in susceptibility of the substrate to another enzyme of known specific activity (70).

7. Increase in swelling on exposure to dilute alkali (43).

8. Increase in dye uptake (44).

9. Decrease in opaqueness of an agar medium containing an insoluble cellulosic material (33).

Structure and Properties of Cellulosic Materials in Relation to Their Susceptibility to Enzymatic Degradation

Cotton and wood are the two most prevalent forms in which cellulose is found in nature. In the manufacture of certain cellulosic end products, alterations are made that yield a variety of additional modified forms of cellulose. Cotton and wood are decomposed by organisms that are usually restricted in nature to one substrate or the other. However, modified cellulosics are commonly
degraded by the organisms that attack cotton and wood and by an additional
group that is incapable of utilizing either wood or cotton. These differences
in susceptibility of cellulosic materials to various organisms are in large
measure due to differences in the enzymes of the organisms and correlated
differences in the noncellulosic constituents and structure of these materials.
The following section will explain some of the structural and chemical differ-
ences between cotton and wood cellulose, indicate the general means by which
Cellulose from either source may be modified, and show the bearing these fac-
tors have on the susceptibility of various cellulosic materials to enzymatic
degradation by fungi. A more complete review of the structure and properties
of cellulosic materials with special emphasis on wood cellulose is given by
Wise and Jahn (83). This reference is the primary source for the report on
cellulose structure that follows.

Cotton Cellulose

Cotton is the purest form of cellulose found in nature. Since it contains only
minor amounts of extraneous materials such as pectins, waxes, and others,
it has been adopted as a standard for research work. The cotton fiber consists
of an outer, very thin primary wall that contains most of the noncellulosic con-
stituents of the fiber and a thicker secondary wall composed almost entirely of
pure cellulose. Fortunately for experimental work on the structure of cellu-
lose, the relatively impure primary wall can be removed readily, for example
by shredding in a Waring blender, leaving the secondary wall unaffected.

Structure of Cellulose

The cellulose molecule is an unbranched linear polymer of glucose. This is
shown by its empirical formula \( \left( C_6 H_{10} O_5 \right)_n \), and its structural formula:

\[
\text{O} \quad \text{CH}_2\text{OH} \\
\text{C} \quad \text{CH} \quad \text{O} \\
\text{OH} \quad \text{C} \quad \text{H} \quad \text{H} \\
\text{H} \quad \text{C} \quad \text{O} \\
\text{CH}_2\text{OH} \\
\text{H} \quad \text{C} \quad \text{OH} \\
\text{H} \\
\text{OH}
\]

The glucose units are linked at their 1- and 4-carbon positions by \( \beta \)-glycosidic
bonds into molecular chains varying in length from a few glucose units to as
many as 3,000 or more. From this we have the more technical name for cellu-
lose, 1, 4-\( \beta \)-polyanhydroglucopyranose.

The linear molecules of cellulose are bound laterally by hydrogen bonds or Van
der Wall forces into linear fibrils of 50 to 100 chains. The individual chains
in these fibrils are associated in various degrees of parallelism. Regions containing highly oriented chains are called crystallites; those in which the chains are more randomly oriented are termed amorphous. These two regions are interspersed, one with the other, and are not discrete but grade gradually from one into the other. Due to the greater molecular surface exposed in the amorphous regions, reactions, including enzymatic hydrolysis, that require penetration of the cellulose matrix for completion generally proceed more rapidly in these regions than in those which are more crystalline.

Norkrans (50) and Walseth (78) have shown that resistance of celluloses to enzymatic breakdown is a function of their degree of crystallinity. Norkrans (50) showed that a crystalline residue with an average DP of 50 and particle dimensions of approximately 300 by 150 Angstrom units was left after exhaustive incubation of a cellulose sol with cellulolytic enzymes.

It is important to recognize that both wood and cotton celluloses are found in nature as aggregations of linear molecules of variable length. The length of cellulose molecules is commonly described by the term average degree of polymerization (DP), which refers to the average number of glucose units in the molecules of a given sample of cellulose. Celluloses of different average DP's have different solubility properties but are, in general, similar in other chemical properties. Differences in solubility properties provide a crude means of separating long, intermediate, and short chains into what are called alpha, beta, and gamma celluloses (1).

Wood Cellulose

Wood cellulose is structurally identical with cotton cellulose, except that it is associated with polymers of basic sugar units other than glucose and has a lower proportion of crystalline material. However, many organisms virulently cellulolytic against cotton are unable to utilize wood for food. This is primarily due to the nature of association of the cellulose with the lignin of wood. A chemical bond between cellulose and lignin has been postulated but evidence for its existence remains inconclusive (83). Present thought suggests that these two substances occur in wood as mutually interpenetrating polymer systems of such intimate association that lignin provides a "protective barrier" against degradation by any except specialized wood-destroying organisms the enzymes of which are capable of degrading wood cellulose in spite of the lignin "barrier." When the lignin has been removed from wood, as in the pulping processes, wood cellulose is then susceptible to organisms that commonly degrade cotton (33).

A short introduction to the special problem of enzymatic degradation of wood is given in the final section of this report.
Modified Celluloses

Natural celluloses, as described above, may be changed in susceptibility to enzymatic degradation by physical and chemical modification. Much of our present understanding of the mechanism of action of cellulolytic enzymes has resulted from the use of cellulosic substrates that have been modified in specific known ways. Modified cellulosic materials may be prepared from natural celluloses by five general methods: (a) hydrolysis, (b) substitution, (c) regeneration, (d) mechanical degradation, and (e) irradiation.

Hydrolysis. -- Hydrolysis by strong mineral acids or weak organic acids decreases the susceptibility of the residual cellulose to enzymatic degradation by removing the more easily hydrolyzed amorphous material, and possibly also by forming substituted derivatives of cellulose.

Substituted cellulose derivatives. -- Substituted cellulose derivatives are formed by replacing the hydrogen of the primary and secondary hydroxyl groups of cellulose by such reactive groups as methyl, ethyl, carboxymethyl, and so forth. The addition of these groups makes cellulose less crystalline and more soluble in water in proportion to its degree of substitution (DS). DS refers to the average number of substituent groups attached to each glucose unit in the cellulose. The substitution level at which complete solubility is usually attained ranges from a DS of 0.5 to 0.7 (60), depending on the solvating capacity of the substituents and also the degree of polymerization of the cellulose.

The susceptibility of substituted celluloses to enzymatic hydrolysis increases as they become more water soluble and less crystalline up to the point of complete solubility (54). After this point, susceptibility decreases with increasing DS until complete immunity to enzymatic action results, which is usually at a DS somewhat greater than 1. Apparently, a substituent group on each glucose unit in a cellulose chain protects the chain from hydrolysis by cellulolytic enzymes. 3 There is some evidence that substituent groups of large molecular dimensions are more effective in contributing resistance to enzymatic degradation than are small groups (54).

Since there are three potential sites for substitution, that is, three hydroxyl groups on each glucose unit, the average DS for a particular sample of cellulose does not necessarily indicate that that number of substituents has been added to each and every glucose unit. This is of particular importance when such fiber products as cotton or wood are substituted to insure resistance to

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3 Actually a substituent group on every other glucose unit insures resistance to enzymatic degradation. However, selective substitution of alternate glucose units cannot be achieved practically.
Penetration of the fiber is necessary for substitution of its interior portions. A determination of the average DS of a fiber does not indicate the distribution of substituents within it so that the proportion of unsubstituted glucose units is a better index of resistance than DS alone (54).

Regenerated cellulose. --Cellulose regenerated after swelling or complete dissolution in cupriethylene diamine, cuprammonium hydroxide, strong acids, such as 72 percent sulfuric or 85 percent phosphoric, or in strong alkali is much more susceptible to enzymatic hydrolysis than unregenerated forms (53). This is due to the greater proportion of amorphous material in the regenerated product and to the different unit cell dimensions in the crystallites that are formed. Where increased susceptibility to enzymes is a desired end result, phosphoric acid treatment (77) is especially useful, since it does not involve the partial substitution that accompanies treatment with sulfuric acid, nor the decrease in susceptibility of alkali cellulose produced by contact with air (53).

In celluloses regenerated under tension, as in the case of rayon or cellophane, molecular alinement is greater and susceptibility to enzymatic hydrolysis is less than for celluloses regenerated without stress (29). Acetate rayon, which is both substituted and regenerated under tension, is highly resistant to enzymic hydrolysis (70).

Mechanical disintegration. --Mechanical disintegration of cellulosic materials increases their susceptibility to enzymatic hydrolysis. This increase is apparently more than can be accounted for by increase in surface area alone. It would appear that cut surfaces, particularly of natural fibers, are more susceptible than normal surfaces (53). Extensive mechanical disintegration, as achieved by a vibratory ball mill, results in depolymerization as well as increased surface area and, consequently, still further increased susceptibility to enzymatic hydrolysis (51).

Irradiation. --Irradiation of cellulose with cathode rays from a van der Graaf accelerator was shown by Reese (53) to decrease susceptibility with increasing dosages of radiation up to about 30 megarads. (A megarad is that radiation dose which produces an energy absorption of $93 \times 10^6$ ergs per gram of tissue (25).) Above 30 megarads, susceptibility was reported to increase, reaching a level with unirradiated samples at about 60 megarads. These observations, as well as those of Wagner and others (79) for irradiation using ultraviolet light, are in contrast to expectations from the results of Saeman and others (61), and Lawton and others (36). They concluded that no crosslinking of cellulose chains is induced by radiation and that its major effect is random depolymerization of cellulose in both the crystalline and amorphous regions. Irradiation is unique among cellulose treatments in its random, rather than preferential, effect on the amorphous regions of cellulose.
This effect would be expected to increase the susceptibility of cellulose to enzymes in the same way that ball milling has been shown to do under the preceding section entitled "Mechanical Disintegration." The effects of radiation on cellulose structure need further clarification before resultant alteration in susceptibility to enzymes can be satisfactorily explained. A review of recent work on the effects of high energy radiation on the chemical and nutrition properties of wood is given by Mater (45).

Present Knowledge Regarding the Mechanism of Enzymatic Digestion of Cellulose by Fungi

The mechanism of fungal degradation of cellulosic materials now generally accepted provides that the actively growing hyphae secrete extracellular enzymes that diffuse through a film of water coating the surfaces of the material. These enzymes hydrolyze the long cellulose molecules to cellobiose. This product diffuses into the fungous cells where it is further hydrolyzed to glucose and metabolized by intracellular enzymes to provide the energy and substance needed by the organism for continued growth.

The present review is restricted to this earlier hydrolytic phase of the process. For a report on the oxidative metabolism of glucose by fungi, the work by Lilly and Barnett (40) is suggested.

Fortunately, the enzymatic mechanism of cellulose hydrolysis is similar in nearly all fungi. This makes it possible to investigate the enzymes of a few type organisms and to obtain thereby, information applicable to fungi generally. Reese and his associates (23, 38, 39, 41, 53 to 58, 60), whose earlier work included a wide variety of organisms, have concentrated their recent work on two fungi, Myrothecium verrucaria and Trichoderma viride. Other workers have also used these organisms so that the following presentation is based primarily on investigations of these two representative fungi.

The hydrolysis of cellulose is accomplished by several distinct enzymes in a series of steps. Each step is attributed to a particular enzyme as shown in Table 1.

The mechanism depicted graphically in Table 1 is described somewhat more completely by the following:

1. The cellulose of wood or other ligno-cellulosic materials may be digested by an unknown factor "X," presumably enzymatic, possessed only by fungi adapted to this type of substrate. Absence of this factor accounts for the inability of nonwood-destroying fungi to utilize wood for food.

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Table 1

Enzymes responsible for the degradation of various cellulosic materials and representative fungi that possess them.

<table>
<thead>
<tr>
<th>Extracelluar reactions</th>
<th>Intracellular reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural cellulosics</strong></td>
<td><strong>Modified cellulosics</strong></td>
</tr>
<tr>
<td>Wood and other ligno-cellulosic materials</td>
<td>Wood pulp</td>
</tr>
<tr>
<td>Native cellulose as in cotton</td>
<td>Linear anhydro-glucose chains</td>
</tr>
</tbody>
</table>

I. Organisms producing the enzymes "X," C_{x}, and β-glucosidase

- Wood-destroying basidiomycetes such as *Poria monticola*, *Polyporus versicolor*, and others
- *Myrothecium verrucaria, Trichoderma viride, Aspergillus fumigatus* and others

II. Organisms producing the enzymes C_{1}, C_{x}, and β-glucosidase

III. Organisms producing the enzymes C_{x} and β-glucosidase

- *A. flavus, A. sydowi, A. tamarii, A. niger*, and others

IV. Organisms producing the enzyme β-glucosidase

Most fungi

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1. Taken in part from *Reese and Levinson* (58).
2. "X" is a convenient designation for a hypothetical factor, presumably enzymatic, that enables wood-rotting fungi to utilize the cellulose in wood in spite of its association with lignin.
2. The laterally bonded cellulose chains occurring in cotton are apparently separated from one another by an enzyme designated $C_1$. Organisms that do not possess this enzyme, or the factor "X," are unable to utilize natural cellulosics for food and must, therefore, be considered noncellulolytic.

3. The linear anhydroglucose chains produced by $C_1$ are hydrolyzed by a series of enzymes designated $C_x$'s, such as $C_{xA}$, $C_{xB}$, $C_{xC}$, to give cellobiose. The $C_x$ enzymes are active not only on chains liberated by the enzyme $C_1$ but also on those provided artificially by the delignification of wood in pulping processes or by regeneration or substitution of cotton or wood cellulose.

4. Cellobiose is hydrolyzed to glucose by the intracellular enzyme $\beta$-glucosidase. This enzyme is partially reversible and may be capable of regenerating saccharides containing up to 3 or 4, or perhaps more, glucose units. $\beta$-glucosidase is produced by many fungi that bear little relation to cellulose digestion in the usual sense of the term.

The enzymes "X," $C_1$, and $C_x$ are active outside the fungus cell; $\beta$-glucosidase acts inside the cell. The enzymes $C_x$ and $\beta$-glucosidase act specifically on the 1, 4-$\beta$-glucosidic linkages in cellulose; the action of the enzymes $C_1$ and "X" is poorly understood and their specific activity remains unknown as yet. Their existence as entities distinct from the $C_x$ enzymes has only been inferred by the differences in the degradative capacities of the organisms in Class III and Classes I or II of table 1. Only the organisms in the first two classes are truly cellulolytic; those in classes III and IV are noncellulolytic, since they are unable to utilize cellulose in either of its natural forms.

From this introduction the evidence supporting each segment of the foregoing mechanism will be examined.

Jennison (33) points out that wood-destroying fungi are unique in their ability to utilize the ligno-cellulosic complex that makes up wood. The symbol "X" has been used in this paper to identify the as-yet-unknown enzymes responsible for this unique capacity. Further details on this subject are given under the section entitled "Enzymatic Degration of Whole Wood" in this report.

Reese and associates (60) showed that the ability to produce enzymes capable of hydrolyzing the 1, 4-$\beta$-glucosidic link in modified celluloses was widespread among micro-organisms, but the ability to utilize cotton cellulose was more restricted. This finding led to a concept of two enzyme components that they designated $C_1$ and $C_x$ and believed necessary for hydrolysis of native cellulose. The classical concept of "cellulase" developed by Pringsheim (52) embodies the combined effects of $C_1$ and $C_x$. 

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Cx was found to be produced by a number of noncellulolytic fungi. Its activity was measured by treating the sodium salt of carboxymethylcellulose (CMC) of low degree of substitution with cell-free enzyme preparations and determining the increase in reducing sugar value of the substrate (58). The effect of time of incubation, pH, and temperature was shown for a wide variety of organismal filtrates. Initial rates of activity were found quite variable. A pH optimum between 4 and 5 was shown for nearly all filtrates and for several modified cellulosics including CMC. Optimum temperatures were found to be between 50° and 60° C. For these reasons the assay conditions adopted as standard for Cx activity are incubation of the culture filtrate with CMC for 2 hours, at 50° C. in citrate buffer at a pH of 5.4. Reese (60) has shown that the Cx activity of these unpurified culture filtrates was independent of the degree of polymerization of the CMC used as test substrate. This had been observed earlier by Greathouse (27) and Siu and others (69).

Levinson and others (38) showed that the end product of Cx activity was cellobiose and that this disaccharide could be assimilated by fungous cells at a rate in excess of the rate of its production from cellulose itself. These conclusions were drawn from the following observations:

1. Low B-glucosidase activity of extracellular enzyme preparations of many cellulolytic fungi as measured by increase in reducing values of a medium containing cellobiose.

2. Accumulation of cellobiose in culture filtrates containing Cx acting on CMC.

3. Rapid and comparable rates of disappearance of glucose and cellobiose from a medium containing actively growing mycelium of cellulolytic and noncellulolytic fungi. This last finding was in accord with an earlier observation of similar rates of penetration of cell membranes by monosaccharides and disaccharides. Reese and associates (57) showed that cellobiose inhibited Cx activity but that glucose did not. This observation also supports the conclusion that cellobiose is the end product of Cx activity. Product inactivation of enzyme reactions is a usual phenomenon, in accord with the law of mass action, and explained on the basis of competition between the product and substrate molecules for the reactive sites on the enzyme surface (72).

Whitaker (81) claims that there is only a single cellulolytic component in Myrothecium verrucaria cellulase. However, using similar methods Gilligan and Reese (23) have demonstrated several components in the Cx portion of the cellulase of this same organism. The existence of these components as separate entities was demonstrated by fractionating acetone precipitated enzyme preparations by paper chromatography (55), zone electrophoresis on starch columns (47), and partition chromatography on calcium phosphate gel.
columns (23). Assay of the activity of these fractions provided the following evidence for multiple components in fungal cellulases:

1. The fractions showed different rates of activity on CMC and Walseth cellulose.

2. The fractions had different rates of activity on three test substrata of different degrees of polymerization.

3. A synergistic interaction of components was observed when several of the fractions were recombined.

4. Differences were noted in the inhibitory influences of cellobiose and methocel on the rates of activity shown by the fractions.

5. The relative magnitudes of induced changes in viscosity and reducing sugar values were found to be different for the fractions.

The number of components positively identified was two—one active on very long cellulose chains and another active on short chains. Less conclusive evidence was found for as many as five components. Discovery of still larger numbers of components was predicted to follow as more well-defined substrata become available.

The mode of action of the Cx enzymes remains questionable. The observation that cellobiose is the only detectable product of Cx activity and the strongly inhibitory effect of cellobiose on Cx activity, suggests an endwise mode of attack whereby cellobiose units are liberated from the ends of cellulose chains. However, additional evidence supports the alternate concept of random splitting along the chain. This evidence includes the rapid loss of tensile strength of cellophane strips, rapid decrease in viscosity of CMC and rapid depolymerization of regenerated cellulose, each without correspondingly rapid increase in the formation of reducing substances (50). The latter mode of Cx activity apparently could account for cellobiose as the only product, if the overall reaction sequence is such that the initial stages are slow relative to later stages (53).

The production of glucose from cellobiose is accomplished by a separate enzyme, β-glucosidase (58). This was suggested by the same evidence supporting the conclusion that cellobiose is the end product of Cx activity described above. β-glucosidase was found to be present only in small quantities outside the cells of fungi and is believed to be active primarily within the cell. The activity of this enzyme may not be restricted to cellobiose. Jerym (34) and Grassman and others (26) have reported activity of β-glucosidase on chains containing as
many as six glucose units. It appears clear, however, that true \( \beta \)-glucosidase has very little or no activity on chains containing 10 or more glucose units (53). It is not clear from the work of either Jerym or Grassman whether they had entirely eliminated all enzyme components belonging to the Cx group. The relevance of this inadequacy of their data may be seen from the account of the paper by Gilligan and Reese (23) in the following paragraph. Another possible explanation for apparent action of \( \beta \)-glucosidase on saccharides containing more than two glucose units is the possibility of transferase activity of this enzyme. Such activity has been detected by Giri and others (24), Crook and Stone (15), Barker and others (4), and Jerym and Thomas (35).

The mechanism of action of the enzyme \( C_1 \) remains obscure. Siu (68) has suggested the term "hydrogenbondase" that Reese suggests may be properly descriptive for its action but has no known prototype. The occurrence of \( C_1 \) has been inferred from the fact that some organisms that are incapable of hydrolyzing cotton -- strips of cotton duck cloth -- actively hydrolyze such soluble substituted celluloses as CMC and methyl cellulose. The enzymic element these organisms lack has been designated \( C_1 \). The only measurement of enzymatic activity that may be related to the effect attributed to \( C_1 \) is the "swelling factor" or "S factor" proposed by March (43). A very short incubation of cotton fiber with filtrates taken from cultures of cellulolytic organisms induced a marked increase of up to 200 percent in subsequent swelling in dilute alkali. This alteration preceded any detectable change in reducing value, loss in tensile strength, or change in DP of the cotton. Marsh showed that the factor responsible is destroyed by heat, is adaptive, and therefore is presumably enzymatic. Reese and Gilligan (56) further showed that the S factor occurs only in organisms and commercial enzyme preparations in which Cx also occurs. He compared the activities of S factor and Cx and found similar temperature-activity relationships; similar levels of inhibition of activity by cellobiose, glucose, and methocel; and a similar rate of movement, with certain Cx fractions active on long chains, on paper chromatograms. Differences were noted in curves of pH activity for Cx and S factor. However, these differences were associated with the salt concentration of the reaction medium and were not considered indicative of essential differences between the two enzymes. Thus, it would appear that S factor and Cx may be active on the same linkage in the cellulose and that S factor -- or \( C_1 \), if they are the same -- may be merely the Cx component that is active on the longest chains.

The action of S factor was shown to be of the same order of magnitude as that produced by mechanical tearing in a Waring blender (56). This treatment is reported to fracture at least part of the primary wall of cotton fibers (74). The primary wall has a restrictive influence on swelling of cotton in alkali. This suggests that S factor acts on some component of the primary wall of cotton fibers.

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5 Personal communication.
Mandels and Reese (41), in studying the adaptive nature of Cx and S factor, found that these enzymes were produced when the organism was grown on cellulose and lactose but that glucose, cellobiose, and 58 other carbohydrates failed to induce extracellular release of these enzymes. Calcium and magnesium were found essential for production of the enzyme but their presence or absence, in quantities greater than those provided by the inoculum or as contaminants in the carbon source materials, had little effect on mycelium production. Increased yields of these enzymes were found when iron, manganese, zinc, and cobalt were included in the culture medium. Omission of zinc reduced the yield of cellulase but any combination of iron or manganese with zinc or cobalt gave good yields. These trace elements were also found to have little effect on mycelial growth except on highly purified cellulose. No evidence was found that the minerals required for production of these enzymes activated the enzymes themselves.

The promising speculation that chemicals might inhibit cellulases and offer a practical means of protecting cellulosic materials from degradation by fungi led Reese and Mandels (59) to test 175 compounds for their ability to inhibit fungal cellulases and β-glucosidases. Their results showed that several strongly inhibitory compounds were among those tested but that more of every compound was required to inactivate the enzymes than would kill the organism producing them.

**Enzymatic Degradation of Whole Wood**

Though much has been learned regarding the enzymatic degradation of cotton and modified wood celluloses, enzymatic breakdown of whole wood is less well understood. For a somewhat more complete presentation of this subject the review by Campbell (10) and a research proposal by Gowling (14) are suggested. An important obstacle to understanding the mechanism of enzymatic breakdown of wood is the relative complexity of wood compared to other cellulosic materials and the inadequacy of present knowledge of the chemical structure of wood, in particular of the lignin and hemicellulose of wood.

As explained previously, only a relatively restricted group of fungi are able to utilize wood for food. It has been suggested that the nature of the association between cellulose and lignin of wood is primarily responsible for restricting the decay flora of wood to these relatively few organisms. In table 1, the symbol "X" was used to identify the additional enzyme or enzymes that presumably enable wood-destroying fungi to utilize wood cellulose in spite of the "protective barrier" provided by lignin.
Three distinct types of wood decay have been recognized: brown rot, white rot, and soft rot. Soft rot is distinct in that the hyphae of the decaying organism occupy enzymatically eroded, cylindrical cavities within the secondary walls of wood cells, and are not usually evident in the cell lumens as are those of the brown- and white-rot fungi (3). Several white-rot fungi have been shown to utilize both the polysaccharides and lignin of wood approximately in proportion to the amounts originally present in sound wood (11,12,13, 62). A noteworthy exception to this generalization is provided by the work of Hirt (31). Since lignin is readily oxidizable, it may be that the ability of these fungi to cause decay of wood can be explained by their production of oxidative enzymes that can act on lignin in situ (83). Bavendamm (5) and Davidson and associates (16) have shown that white-rot fungi invariably produce polyphenol oxidases. Day and others (17), Van Fliet (75), and Adams and Ledingham (2, 37) have shown that white-rot fungi can utilize isolated lignin as sole source of carbon.

The ability of brown-rot fungi to utilize wood for food is less readily explained since their degradative effects are largely confined to the polysaccharides of wood and apparently do not utilize lignin. Decay by these fungi was considered analogous to acid hydrolysis by Campbell (10). Findlay (18) showed that only hydrolyzing extracellular enzymes were produced by brown-rot fungi. Boswell (8) confirmed this report suggesting that all oxidative effects on decay products occur within the cells of brown-rot fungi. Though lignin is readily oxidizable and resists hydrolysis (83), hydrolysis has been observed and might be induced by the brown-rot enzymes. It is conceivable that a hydrolyzing enzyme might alter the lignin so as to release wood polysaccharides for digestion by cellulolytic enzymes without rendering the lignin metabolizable as well. However, this is conjecture and has little supporting evidence.

It would appear clear that comprehensive research on the mechanism of enzymatic degradation of whole wood must involve an investigation of enzymatic effects on lignin. This is difficult because of our present inadequate knowledge of the structure of lignin. However, certain characterizing determinations (83) now available are applicable to lignin and enzyme studies could be made using these techniques.

The degradation of the noncellulosic polysaccharides of wood presents a simpler problem than does that of lignin since their structures are somewhat better understood (83). However, no significant research on the enzymes involved has been encountered in preparing this review.

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