THE ENZYMATIC SOLUBILIZATION OF CRYSTALLINE CELLULOSE

by

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Dedicated to Dr. Elwyn T. Reese, whose continued interest in the fundamental problem of cellulose degradation has provided a stimulus for active investigations in this field. His discipline and insight in formulating the truly significant questions and in developing valid experimental approaches to their answers has influenced the thinking of this and many other laboratories. Of particular significance to the present research was his early demonstration that enzyme activity (C_1) distinct from the known hydrolases (C_x) must play a key role in degradation of native cellulose.

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I. STATEMENT OF THE PROBLEM

This study was initiated on the premise that "in general, it is not possible enzymatically to achieve quantitative conversion of natural cellulose aggregates to soluble sugars in spite of the fact that growing cultures (of microorganisms) accomplish complete degradation quite rapidly" (21). In more specific terms as set forth in the dissertation research proposal, it is proposed to investigate the nature and action of enzyme activity (C_1) promoting the degradation of native crystalline cellulose, since its action and even its existence is at best supported by indirect evidence. The stated objective was to determine whether this activity represents a distinct enzyme with defined action which is separable from the hydrolytic (C_x) group of enzymes.

II. INTRODUCTION AND REVIEW OF THE LITERATURE

In the past decade the microbial and enzymatic degradation of cellulose has been reviewed by Siu (52), Whelan (74), Gascoigne and Gascoigne (10), King (21), and Norkrans (33), and has been the subject of a Friday Harbor Symposium (37) and an American Chemical Society Symposium (40). Cowling (4) has prepared a review of the literature on the enzymatic degradation of cellulose and wood. The material and bibliographies contained in the references above provide a comprehensive survey of literature through the year 1961.

Conspicuously absent from this body of literature is demonstrated evidence of significant enzymatic hydrolysis of native crystalline cellulose or native cotton fiber by cell-free enzyme systems. In general, enzymatic studies relating to cellulose degradation have not utilized natural cellulosic substrates, but have instead utilized chemically swollen or modified insoluble celluloses (alkali swollen, H_3PO_4 swollen, regenerated, extensively ball-milled, etc.) and soluble cellulose derivatives (carboxymethyl cellulose, methylcellulose, cellulodextrins, etc.). It has been indicated quite often that modified cellulose and soluble derivatives of cellulose are much more susceptible to enzymatic attack than are the natural forms of cellulose. Many enzyme systems which are highly active toward the former are almost completely inactive toward the latter, influenced to a large extent by the particular organism from which the enzyme system was obtained. Therefore, specific citation of cellulolytic enzyme studies shall be reserved for the most part to those

demonstrating significant degradation of native forms of cellulose, to those relating the particular organism, Trichoderma viride, from which the crude enzyme used in this study was derived, and to those reports suggesting or indicating the manner by which native cellulose is enzymatically degraded.

Numerous forms of cellulose and cellulose derivatives have been utilized as substrates for cellulolytic enzyme studies, and summaries of the various substrates reported in the literature have been compiled (10, 21, 33). Halliwell (13) has discussed the measurement of cellulolytic activity, reviewing in some detail the methods of assay and factors which affect activity.

Many opinions have been expressed as to why the insoluble celluloses, particularly native cellulose, are more resistant to enzymatic degradation. Cowling (5) has discussed the structural features of cellulose in some detail as they influence susceptibility to enzymatic degradation. The structural features were considered within the concept of "accessibility"; any structural feature which limits the accessibility of the cellulose to enzyme will diminish the susceptibility of the cellulose to enzymatic degradation (and vice versa). Reese (39) has stated that, "changes in the physical and chemical nature of cellulose lead to changes in its susceptibility to enzyme hydrolysis," and Whitaker (75,76) has expressed the opinion that "accessibility" factors play the major role in determining the susceptibility of cellulose to degradation.

From the viewpoint of accessibility, a highly modified amorphous cellulose is more reactive than a native cellulose, and it becomes quite

obvious why soluble cellulosic substrates are readily attacked. Many of the cellulase investigators using the rationale of "accessibility" have found it advantageous for a number of reasons to modify cellulose by various procedures to make it more reactive (i.e. more accessible to enzyme). The treatments necessary to make cellulose more reactive almost inadvertently result in modification of the native crystalline form. Although swelling and regeneration treatments may be desirable for studying certain aspects of cellulose degradation, as for example, the mechanism of bond hydrolysis at the anhydroglucose chain (molecular) level, the use of such cellulosic substrates does not permit the study of cellulose degradation in relation to its supermolecular, highly oriented, crystalline-compact, and sterically rigid structure. Many investigators have tended to disregard this aspect of cellulose degradation, for they have used highly modified substrates and have abandoned the enzymatic studies after the easily hydrolyzable amorphous material was removed leaving only the "dense" or "inaccessible" crystalline core of the substrate. It would appear to be of much greater concern, in view of the fact that the cellulose degradation process is accomplished in the natural hydrobiotic environment without the necessity of harsh swelling or regeneration treatments, to consider the enzymatic degradation of cellulose in respect to its native form.

It has become increasingly apparent that a great deal of uncertainty regarding the existence and significance of enzymes promoting the degradation of native cellulose stems from the multiplicity of physical forms which cellulose can assume.

Extensive reviews of the chemistry and physics of cellulose have been edited by Ott and Spurlin (34) and Honeyman (15). Among the topics discussed are those relating to the occurrence of cellulose, the molecular structure, the amorphous and crystalline structure, and the fine structure of cellulose. Marsden (25) has an interesting discussion on crystalline and amorphous regions in fibers with an emphasis on cellulose. Cowling (5) and King (21) have considered some of the structural features of cellulose in relation to the enzymolysis of cellulose and cellulose fibers.

Cellulosic materials have quite a wide distribution in nature being a primary constituent of all higher plants and a product of certain of the microorganisms. Cotton fibers represent one of the purest forms of native cellulose known, while the cellulose of woody tissue, plant stems, and leaves occurs in intimate association with significant amounts of lignin, hemicellulose, and other polysaccharides.

Cellulose can be described as a macromolecular polymer consisting of linear chains of D-glucopyranosides in the C1 (chair) conformation having β-1,4 linkage. The individual anhydroglucose chains of cellulose fibers vary in degree of polymerization; however, an average of D.P. of 3000 glucose units appears reasonable for native cellulose materials. Within microfibrils the individual anhydroglucose chains in the crystalline regions lie parallel to one another with alternate chains running in opposite directions, but in the amorphous regions the chains are disordered and only approximately parallel to the microfibril axis.

Crystalline regions of the microfibrils have been referred to as micelles, having dimensions of about 60 Å in diameter by 600 Å in length.

Individual chains are of such length that they can pass through several crystalline or micelle regions. Frey-Wyssling and Muhlethaler (9) believe that so-called elementary fibrils of cellulose have a diameter of 35 Å consisting of approximately 36 chain molecules and that almost all the antiparallel chain pairs are situated at the surface of the elementary fibril. The elementary fibrils behave as rather brittle crystalline needles, indicating a high degree of crystallinity, and aggregates of the elementary fibrils form the higher microfibril unit.

The crystallinity of cotton has been determined by X-ray diffraction to be at least 70%. The criticism of the X-ray diffraction method for determining crystallinity has been that only crystallites larger than a certain size contribute to the diffraction pattern, while smaller crystallites and amorphous regions contribute to background scattering, making it difficult to determine crystallinity reliably. Accessibility measurements have shown that only 5 to 13% of the material in cotton fiber is accessible to acid hydrolysis, the remaining cellulose being "resistant." Davidson (6) and Nickerson and Harble (30) have indicated that during acid hydrolysis of cellulose the disordered inter-crystalline amorphous networks linking the crystallites are hydrolyzed most readily. Sharples (50, 51) believes that the so-called "acid-sensitive linkages" in cotton are confined to the amorphous regions and that the enhanced rate at which these bonds are hydrolyzed (compared to normal glucosidic bonds) results either from strains set up in the amorphous regions or from local variations in the normal state of

hydrogen bonding. Sharples (49) also points out that the accessibility of undegraded cotton may decrease as hydrolysis proceeds since chains in amorphous regions that are cleaved may orient with respect to each other and crystallize. Regardless of the experimental approach it is evident that cotton and other native celluloses contain a large amount of crystalline material.

The generally accepted crystalline forms of cellulose, characterized by X-ray diffraction methods, possess different lattice structures which have been designated as celluloses I, II, III, and IV. Ellefsen and Norman (8) have demonstrated the existence of a fifth crystalline form, designated as cellulose x, which was obtained in cellulose precipitates from strong HCl and H_3PO_4, and they indicated possible transitions between the different crystalline forms. Cellulose I is the crystal form in native cellulose materials. Celluloses II, III, IV, and x result from modifications of the native form; II and x are obtained by swelling and reprecipitation treatments, and III and IV are obtained by other treatments.

Hydrocellulose is a term that often has been used loosely in referring to all types of acid-modified celluloses, which have varied in form, according to the extent of acid treatment, from fibers to powders. Among cellulose chemists, however, the term hydrocellulose has been reserved for the product resulting from acid hydrolysis of cellulosic materials which has relatively much more resistance to further hydrolysis, and has been described as the crystalline residue of cellulose, or cellulose freed of amorphous material.

The preparation and characteristics of hydrocellulose have been described by Davidson (6) and by Millett and coworkers (28). Davidson steeped purified cotton linters in 10 N HCl and found that initially there was a progressive change in properties, but that a stage was reached when little further change occurred. The powdery hydrocellulose obtained had an X-ray diffraction pattern identical with that of the original cotton. Davidson suggested that cellulose was composed of easily and difficultly-penetrable phases (i.e. crystalline and amorphous regions), and that the difficultly penetrable region is responsible for the X-ray characteristics of cellulose.

McBurney (27) has good presentation on the acid hydrolytic degradation of cellulose and the properties of hydrocellulose. He concluded his review by stating that all of the observations support the conclusion that the hydrolytic reaction is in the main confined to the amorphous region of the fiber during the heterogeneous hydrolysis (i.e. the rapid phase of hydrolysis).

The one single factor most frequently mentioned as limiting the enzymatic degradation of cellulose has been its possession of a highly organized crystalline structure. Reese, Segal, and Tripp (43) have found that highly crystalline celluloses (hydrocelluloses) were more resistant to enzymic degradation than were the cotton fibers of varying degrees of crystallinity from which the hydrocelluloses were derived. Norkrans (32) has found a correlation between increased degree of crystallinity and increased resistance toward enzymatic attack. Both groups have indicated that the crystallinity of cellulose substrates increased with enzymatic

attack because of preferential degradation of the more accessible amorphous regions.

The manner by which crystalline cellulose residues are degraded by enzymes has been the subject of much speculation. One of the earlier postulates on the degradative process was expressed by Pringsheim and Baur (36), who were of the opinion that the enzymatic cleavage of cellulose took place in three steps--a disintegration of the micelles into anhydroglucose molecules, followed by cleavage to cellobiose, and then further cleavage to glucose. Even prior to this postulate Pringsheim (35) had proposed that cellulose was converted to cellobiose by the action "cellulase."

Reese and coworkers, Siu and Levinson, (39, 42, 44) have postulated that a multi-enzyme system is necessary to catalyze the conversion of native cellulose to soluble sugars.

$$\text{Native cellulose} \xrightarrow{C_1} \underset{\text{"Reactive cellulose" (24)}}{\overset{\text{"Modified cellulose"}}{\text{Linear anhydroglucose chains}}} \xrightarrow{C_X} \text{Soluble low molecular weight sugars}$$

The postulate was based on the fact that certain organisms were not able to utilize native cellulose for growth but were able to grow on cellulodextrins or modified celluloses. Other organisms could utilize native cellulose. It was suggested that these latter were able to do so because they possessed a factor, termed C_1, which made the native cellulose available to this group. Those utilizing modified cellulose and soluble derivatives were thought to have the C_X enzymes which effectively catalyze the breakdown of these substances. Those organisms whose growth could only be supported by cellobiose were considered non-cellulolytic.

Reese (39) has observed the correlation between susceptibility to enzymatic attack and the capacity for a cellulose to take up water, or the "hydratability", and he has expressed the idea that prior to hydrolysis of an insoluble material a solvation to some degree of insoluble substrate material must occur. However, he finds the usual definition of solubility too narrow, as it may be entirely possible for a loose chain end of a tightly bound cellulose aggregate to become completely hydrated and susceptible to hydrolysis, yet still be unable to diffuse freely from the aggregate surface. One possible action of C_1 would be to produce free chain ends for subsequent C_X attack. Mandels and Reese (24) have ventured the opinions that C_1 may be an extremely random acting hydrolytic enzyme, or that C_1 may be non-hydrolytic enzyme acting in a manner to relax intramolecular bonds of cellulose and allowing C_X to act.

Assuming that native cellulose can only be degraded in the presence of C_1 factor, then subsequent C_X activity must be dependent on the action of C_1. Gilligan and Reese (11) reported finding a synergistic action between two component activities of the T. viride enzyme system, one having greater relative activity on a H_3PO_4 swollen insoluble cellulose, the other having a greater relative activity on soluble carboxymethyl cellulose (CMC). No indication was made as to whether the former represented a C_1 type of activity. Recently, however, Mandels and Reese (24) have obtained evidence that C_1 is in fact an enzymatic activity of T. viride, and can be distinguished from the C_X enzymes by several criteria, perhaps the most pertinent observation being that C_1 action on cotton was markedly increased by the addition of C_X enzymes but

that the addition of C_1 to C_x did not stimulate the activity of C_x toward CMC.

King (21), in considering the resistance of crystalline cellulose to enzymatic hydrolysis, has felt that more thought should be given to the steric rigidity of the crystalline material when contrasted with the notion that an enzyme and a highly solvated amorphous chain can assume a number of positions relative to each other. Unless enzymes were able to penetrate the crystalline matrix only surface attack would be possible and steric factors would have to be considered. He has expressed the opinion that an enzymatic "disaggregation" of highly organized cellulose must precede the hydrolytic degradation.

The failure of many culture filtrates to achieve extensive solubilization of native cellulose may result from the absence of the disaggregating system in the culture filtrates. The disaggregating system may not be produced by all so-called cellulolytic organisms or may be ineffective or lost in the process of separating cellulolytic enzymes from the organism.

Siu (53) has suggested that a "hydrogen-bondase" type of activity may be responsible for the disaggregation of highly crystalline cellulose.

Marsh and coworkers (26) introduced the concept of a swelling factor (S-factor) acting on native cellulose as it occurs in cotton fibers to produce an increased degree of swelling of the cellulose when subsequently treated with 18% sodium hydroxide. Reese and Gilligan (41) have found that S-factor:C_x ratios of culture filtrates vary with the organism, with conditions of growth, and with purification of the cellulolytic

enzymes, this being an indication that they are separate entities. Youatt (77) on the other hand finds no experimental support for the suggestion that S-factor is distinct from the group of C_x enzymes. Reese and Gilligan and Youatt have discussed the possible role of S-factor in the degradative process of cellulose, but there is no evidence that S-factor will promote or facilitate the complete hydrolytic degradation of native cellulose. Possibly the action of S-factor is the modification of the primary cell wall of cotton fibers.

Halliwell (12), reviewing in 1959 enzyme preparations which cause significant degradation of insoluble cellulose, does not report any instances of significant degradation of native forms of cellulose. Walseth (72,73) has obtained better than a 50% degradation of cotton linters swollen with phosphoric acid using commercial enzyme preparations. Reese (38) obtained better than a 50% degradation of a wood cellulose (Solka Floc), manufactured by a pulping process, using a cell-free filtrate of T. viride. These two reports, because of the modified nature of the substrates used, are not considered as direct evidence of significant degradation of native forms of cellulose.

Reese, Segal, and Tripp (43) have reported a degradation on the order of 20% for a scoured cotton fiber and the hydrocellulose derived from it by a T. viride culture filtrate, and also have shown that a decrystallized sample of the same cotton and its hydrocellulose gave weight losses of 67% and 27% respectively when subjected to the same enzyme treatment. Mandels and Reese (24) have reported recently a 59% weight loss for dewaxed cotton sliver treated with a culture filtrate of

T. viride for 45 days, and up to 80% weight loss with 30 changes of fresh enzyme solution.

Selby and coworkers (48) have reported a 30% weight loss of a scoured cotton yarn treated daily for 33 days with fresh culture filtrate of Myrothecium verrucaria. The daily changes of the enzyme solution were necessary because of the apparent "exhaustion" of the activity responsible for extensive degradation of cotton.

Wakayawa and coworkers (71) have obtained a 24.5% weight loss of native cotton linter in 120 hours using crude cellulase preparations of T. viride. Under similar conditions cotton linter receiving an alkali was more readily attacked (39.4% weight loss), and wood pulp and well beaten filter paper were decomposed to the extent of about 90%. Toyama and Ogawa (68) have demonstrated 18% weight loss of cotton gauze using a T. viride crude enzyme preparation.

Under the most favorable conditions it would appear the native celluloses are only slowly solubilized by enzyme preparations; however, a significant degree of degradation can be achieved. The observations on the degradation of modified celluloses are included for comparative purposes when such materials were subjected to identical treatments.

Trichoderma viride, classified among the fungi imperfecti, has been shown by the above survey of enzyme preparations which cause significant degradation of native cellulose to be one of the more potent cellulolytic enzyme producing organisms. It has been placed among the organisms which, according to Reese and coworkers, have the complete spectrum of enzymes necessary for efficient degradation of cellulose (24,39,42).

Toyama has done considerable work with T. viride (T. koningi), investigating conditions of culture as influencing cellulolytic enzyme production, the cellulosic substrates attacked, and the application of the extra-cellular enzymes (including cellulolytic) of this organism in the decomposition of biological materials (58,61,63). Although his enzyme assays are somewhat unique from those commonly employed, he has been able to demonstrate potent cellulolytic activity, has partially resolved the cellulolytic enzymes demonstrating several cellulolytic activities, and has presented evidence for the existence of a C_1, several C_X's, and β-glucosidase activity (56,57,59,60,62,67,69). His use of the terminology for these activities differs somewhat from Reese's usage, and Toyama suggests that it is the property of C_1 to decompose natural cellulose, C_2 to decompose modified cellulose (filter paper and H_3PO_4 swollen cellulose), and C_3 to decompose water soluble derivatives of cellulose (CMC).

Mandels and Reese (24) have found that, of 12 organisms tested, the cellulolytic enzyme system of T. viride was the most effective in hydrolyzing cotton sliver. T. viride also had the richest content of the C_1 factor. The C_1 factor was produced by most T. viride cultures and of 71 culture filtrates tested, 58 were active on cotton. The ratio of C_1 factor:C_X activity produced by T. viride grown on various substrates was on the following order: cotton sliver > cotton duck > Solka Floc > filter paper. In addition, they describe for the T. viride cellulolytic enzyme system the relative activity towards various substrates, the cellulolytic enzyme characteristics and purification of C_1 and C_X components, and discuss cellulose degradation in regard to the C_1-C_X enzyme concept.

The kinetic studies of cellulolytic enzyme hydrolysis of insoluble substrates have presented only meager evidence that the solubilization process proceeds according to classical enzyme kinetics. In most instances no direct relationship has been found between extent of hydrolysis and the concentration of enzyme, the concentration of substrate, or the time of incubation, this being most likely a reflection of the insoluble and heterogeneous nature of the substrates. Karrer and coworkers (18,19) have observed that hydrolysis of cellulose followed the monomolecular law for a time, then followed Schutz's Rule. Van Sumere and coworkers (70) reported that the hydrolysis of CMC adhered to Schutz's Rule. Walseth (72) found that hydrolysis of swollen cellulose followed neither the monomolecular law or Schutz's Rule; however, a log-log plot of the extent of hydrolysis versus time yielded a straight line. Dixon and Webb (7) and Moelwyn-Hughes (29) have discussed the kinetic form referred to as Schutz's Rule.

Whitaker (76) has presented criteria for characterizing cellulases and had discussed the definition and naming of cellulases. "Cellulase" is a trivial term according to the Report of the Commission on Enzymes of the International Union of Biochemistry (45), and the systematic name and code number β-1,4 glucan 4-glucanohydrolase, 3.2.1.4 is given. Whitaker points out that the systematic name defines the enzyme action as the hydrolysis of β 1,4 glucosidic linkages of cellulose, but does not include the hydrolysis of specific linkages at chain ends. Until a standard substrate is defined, units of enzyme activity cannot follow the recommendation of the Commission on Enzymes. As yet no substrate or unit of activity

has been proposed which is free of objection and as Whitaker states, "...until this matter is resolved, arbitrary units will probably continue to flourish."

There has been every indication, however, that multiple enzyme components which vary in substrate specificity and mode of action are involved in cellulose degradation (11,24,39,69). It is unreasonable to expect that the systematic name, β-1,4 glucan 4-glucanohydrolase, can be applied to the various activities, but the introduction of new terminology should await the time that their action on cellulose is definitely defined. For this reason it will be necessary to designate the cellulolytic activities reported in this study in descriptive terms, indicative of the relative substrate specificity of the particular enzymatic fraction.

Hydrocellulose, the crystalline residue of acid hydrolysis of cotton, has been demonstrated to be relatively more resistant to further hydrolysis by acid and enzymes than are forms of cellulose having appreciable amorphous material. By means of an assay technique using hydrocellulose as the substrate, it was possible to assess the ability of enzyme fractions to solubilize hydrocellulose in a convenient manner. The fact that this assay method did not respond to several supposedly potent sources of "cellulase"[1] (as determined by their ability to hydrolyze CMC) supported the idea that the assay was measuring the ability of an enzyme or enzyme system capable of degrading the crystalline form of

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[1]Cellase 1000 (Wallerstein Company) derived from Aspergillus niger.
Takamine Cellulase 4000 (Miles Chemical Company) derived from Aspergillus niger.
Culture filtrate of Myrothecium verrucaria.

native cellulose. Late in this investigation, however, it became apparent that this assay was not completely insensitive to certain types of enzymes hydrolyzing CMC because some CMCase acted synergistically with "hydrocellulase."

"Hydrocellulase" is used as a trivial term in the place of the more cumbersome phrase, hydrocellulose solubilizing activity. The term, CMCase, is used to refer to the enzyme activity which reduces the viscosity of CMC solutions.

III. MATERIALS AND METHODS

Crude Enzyme

Cellulase Meiji (Meicellase) was produced commercially (Meiji Seika Co. Ltd., Pharmaceutical Division, Tokyo, Japan) by the culture of Trichoderma viride by the Koji process (64,65). Toyama has indicated (66) that the following steps in the preparation of Cellulase Meiji have been performed: 1) water extraction of the T. viride Koji ferment, 2) precipitation with $(NH_4)_2 SO_4$, 3) desalting with an ion exchange resin, 4) precipitation with isopropyl alcohol, and 5) spray drying. As it was not possible to ascertain what purification of Cellulase Meiji has been achieved by the above processing procedure, "crude enzyme" here refers to the product as received.

Substrates

Native Cotton. Raw cotton fiber was provided by the U. S. Department of Agriculture, Southern Utilization Research and Development Laboratory, New Orleans, Louisiana.

Purified Cotton Linters. Cleaned and dewaxed cotton linter prepared with a minimum of cellulose degradation (type A-1000-PSF), was provided by Hercules Powder Company, Hopewell, Virginia.

Hydrocelluloses. The hydrocellulose used in enzymatic assays was prepared in the following manner modified from the procedure of Hungate (17). Purified cotton linters were packed into a flask containing 270 ml of concentrated HCl diluted to 300 ml (10.8 N) so that all free

liquid was taken up. This mixture remained for 24 hours at room temperature, with intermittent agitation to ensure good contact between the disintegrating linter and the acid. The resulting slurry of hydrocellulose was poured into a large excess of water, washed several times by decantation, and neutralized with $NaHCO_3$. Exhaustive dialysis against a slow flow of distilled water was performed until the contents of the dialysis bag were negative when tested for the presence of chloride ion. A one minute Waring blendor treatment yielded the well dispersed stock hydrocellulose suspension which was stored under refrigeration. The concentration of the hydrocellulose in the stock suspension was determined from the dry weight of aliquots.

When diluted in distilled water, the hydrocellulose formed a uniform turbid suspension of finely divided particles which settled out of suspension over a matter of hours. However, in the presence of electrolytes, such as the assay buffer, the particles had a tendency to flocculate and settle from suspension much more rapidly.

Avicel, a commercial hydrocellulose, was obtained from the American Viscose Corporation, Marcus Hook, Pennsylvania. It is described as being a microcrystalline cellulose prepared by treating a very pure α-cellulose with 2.5 N HCl for 15 minutes at 105° (1).

Amorphous Cellulose. Amorphous cellulose was prepared by wetting 15 g of Avicel with 100 ml of concentrated HCl, then pouring the slurry into 400 ml of concentrated HCl at a temperature below -30°. When most of the particulate cellulose disappeared, the resulting viscous

solution was filtered through a mat of glass wool on a Buchner funnel into 3 liters of vigorously agitating water. The precipitated cellulose was washed with distilled water until free of acid, then blended for 5 minutes to yield a uniform stock suspension.

Other Substrates. Carboxymethylcellulose, CMC-70-Premium High and CMC-70-Premium Low (Hercules Powder Company) were used in the viscosimetric and reducing sugar assays respectively.

The cellulodextrins, cellotriose, cellotetraose, and cellopentaose, were prepared and characterized by Cole (3), and kindly supplied by him.

p-Nitrophenyl-β-D-glucopyranoside (A grade, California Corporation for Biochemical Research) was used for the aryl-β-glucosidase assay.

Enzyme Assays

Hydrocellulose Assay. This assay, measuring the solubilization of a finely dispersed hydrocellulose, was developed in principle from the turbidmetric assay of Norkrans (31), who had used a regenerated cellulose sol as substrate.

Optically matched colorimeter tubes were used, and to each tube was added 3 ml of 0.1 M citrate buffer, pH 4.8, and 2 ml of water-plus-enzyme. The reaction was initiated by the addition of 1 ml of the assay hydrocellulose suspension (approximately 5 mg/ml hydrocellulose). Each tube was vigorously mixed after the substrate addition with the aid of an eccentric stirrer, and the initial turbidity was determined against a non-substrate blank using a Klett-Summerson colorimeter with the number 66 filter. For routine assays, incubation was for one hour at 40° in a

water bath, after which the tube was again mixed well, and the turbidity was determined. The decrease in turbidity (Δ Klett) was related to enzyme activity by reference to a standard curve.

The standard curve (Fig. 1) was obtained by using a series of enzyme levels and determining the Δ Klett values at intervals of one, two, and three hours. Δ Klett values were plotted on the ordinate and enzyme aliquot volume x hours on the abscissa. To obtain units of activity the abscissa scale was conveniently subdivided so that one unit of activity corresponded to a Δ Klett of 10 in one hour. It was found that the shape of the standard curve did not vary significantly during purification, and all subsequent standard curves were related to the original standard curve on the basis that a Δ Klett of 22 represented 3.3 units. That the standard curve was analytically reliable can be illustrated by the fact that when a series of appropriate aliquot volumes from any enzyme solution, either crude or in some stage of purification, were assayed according to the stated procedure and plotted as indicated, the standard curve would be reproduced. For accurate quantitation of enzyme activity it was found desirable to have the Δ Klett value fall in the range 15 to 25 since reproduction of readings on the instrument was on the order of plus or minus one Klett unit.

As can be seen in Fig. 2, there was a direct relationship between a decrease in turbidity and an increase in soluble carbohydrate. Thus Δ Klett values represented a true solubilization of the substrate, and the term solubilization will be used frequently in the text.

Fig. 1. A typical enzyme-time standard curve for "hydrocellulase" activity. The details of preparing a standard curve are presented in the text, p. 26.

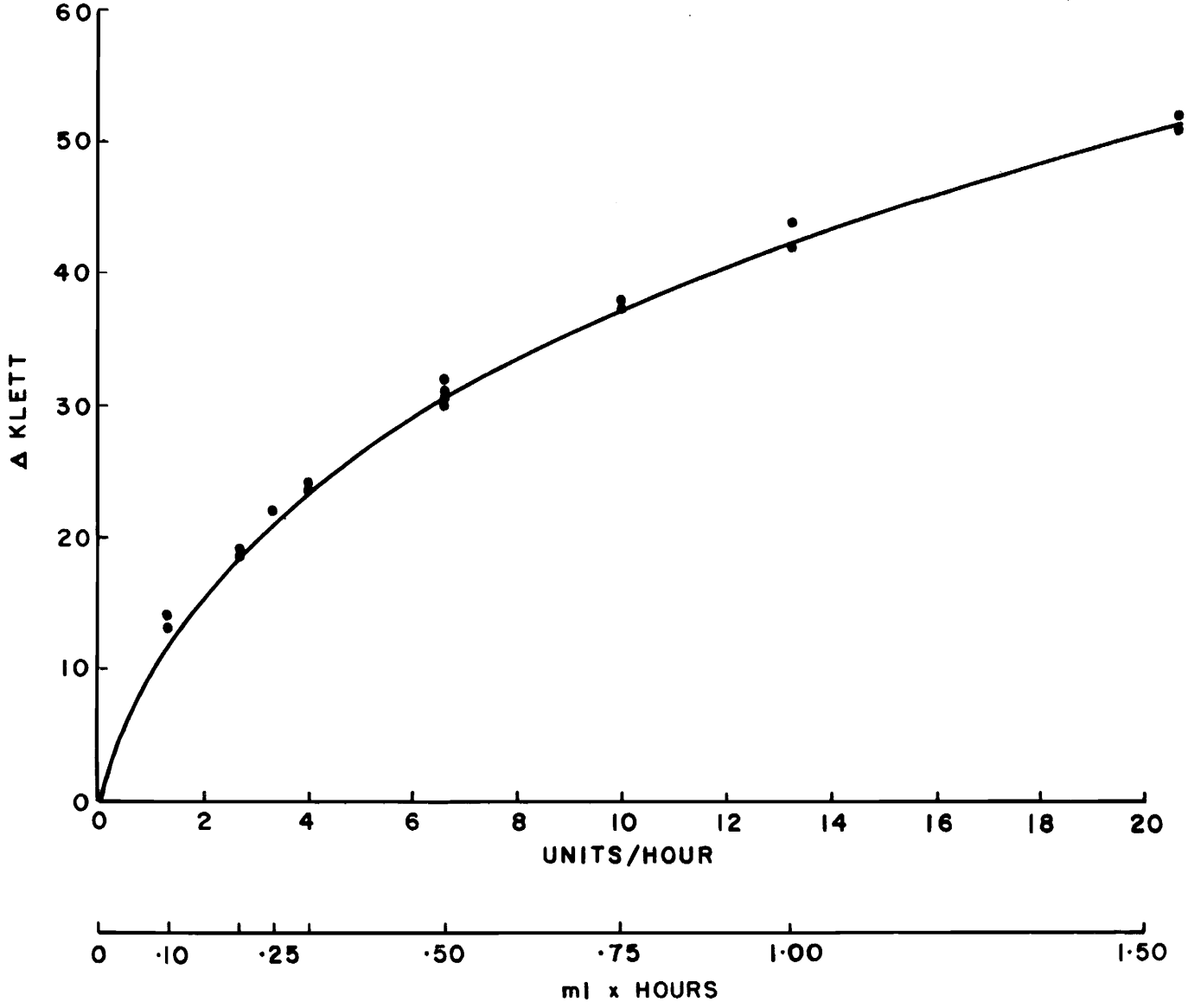
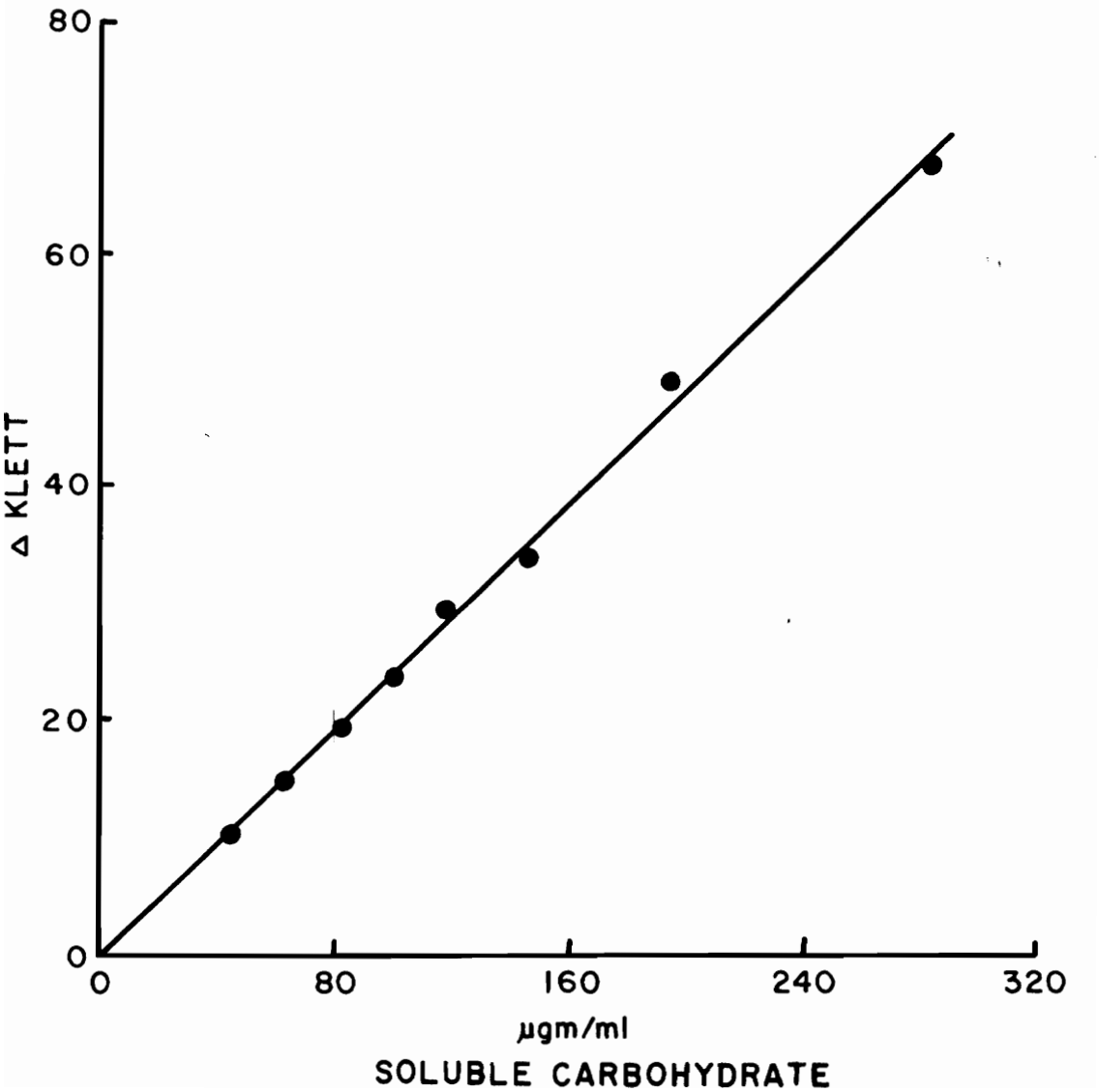

60
50
40
30
20
10
0
Δ KLETT
0
2
4
6
8
10
12
14
16
18
20
UNITS/HOUR
0
·10
·25
·50
·75
1·00
1·50
ml x HOURS

Fig. 2. Proportionality between soluble carbohydrate production and the decrease in turbidity (Δ Klett) determined by the hydrocellulose assay method. Analysis of the soluble carbohydrate was by the phenol-H_2SO_4 method on aliquots taken from reaction mixtures that were measured turbidimetrically. Soluble carbohydrate was separated routinely from insoluble substrate by Millipore filtration (pore size 0.47 μ). Each point represents an average of duplicate determinations.


80
60
40
20
0
Δ KLETT
0
80
160
240
320
µgm/ml
SOLUBLE CARBOHYDRATE

Carboxymethylcellulose (CMC) Assay. The viscosimetric assay, measuring the reduction in viscosity of a CMC solution, was based essentially upon the method used previously in this laboratory (20). The stock substrate was a 0.25% solution of CMC-70-High adjusted to a pH of 4.6 with acetic acid. Five ml aliquots of the substrate were pre-incubated in 13 x 100 mm tubes at the assay temperature of 40°. Enzyme was added (usually 10 µl or less) at zero time, and the tube was mixed well by inversion and returned to the constant temperature bath. After a fixed time interval of incubation (30 minutes routinely) the drain time was measured with a "viscosimeter," which can be described as follows.

The "viscosimeter" was constructed from a section of straight heavy wall capillary tubing with an expanded portion of about 3 ml capacity blown out in the middle. Joined to the capillary tube above the expanded portion was a section of large bore tubing which served as a reservoir. A rubber aspirator bulb having a small hole at the top for finger-tip control of suction was mounted on the upper end of the reservoir tube and was used for drawing reaction mixtures into the "viscosimeter." Drain time, measured with a stop-watch calibrated in hundredths of a minute, was the time required for the level of liquid to pass between a mark above the expanded portion to a mark below. With the particular "viscosimeter" used, the drain time for water at 40° was 0.117 minute, and that of the substrate was between 1.15 and 1.30 minutes varying upon the stock substrate solution. The use of the modified

"viscosimeter" provided a means of convenient and rapid assay of a sequence of enzyme fractions.

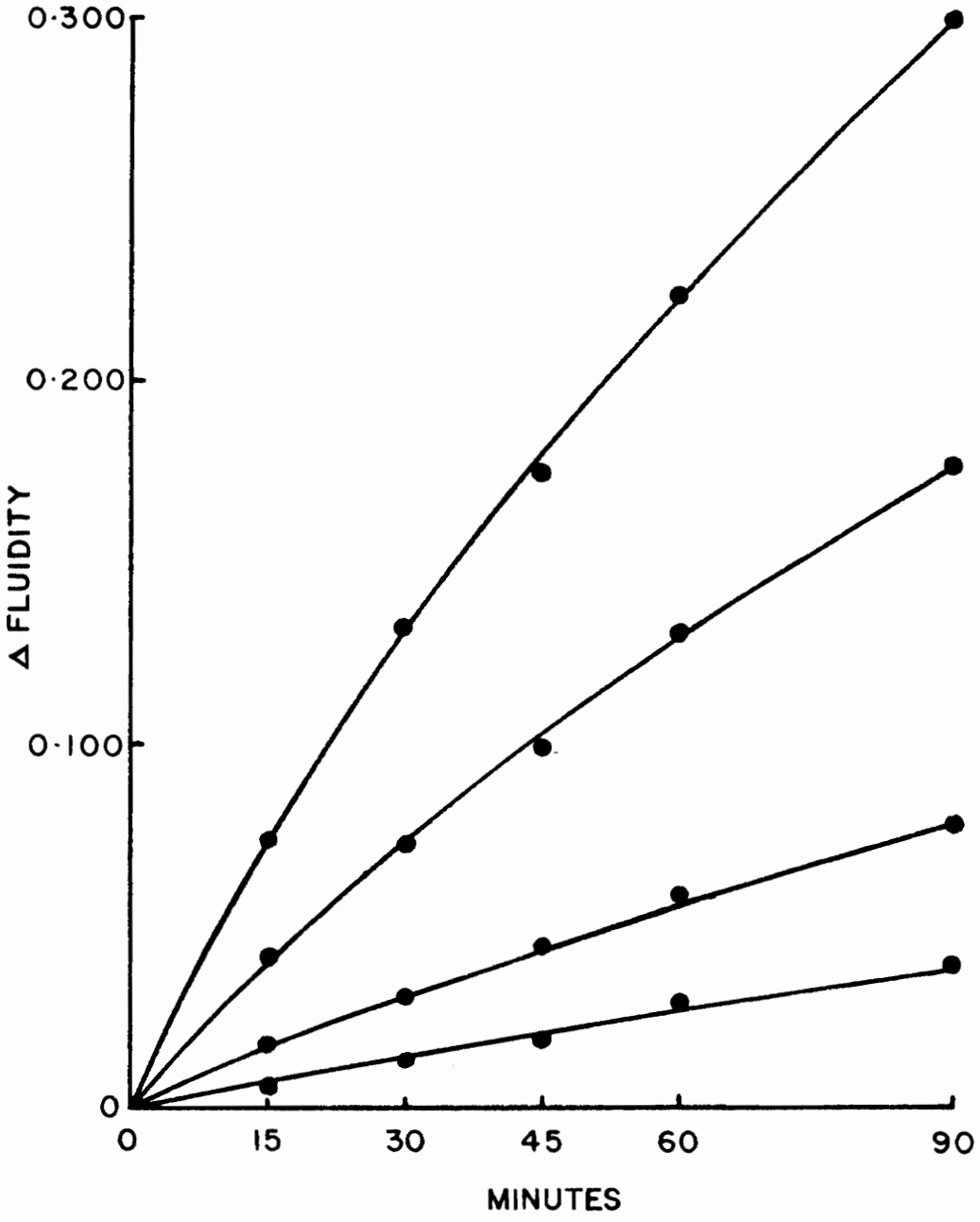
The decrease in specific viscosity resulting from enzyme action, determined from the drain time measurement, was expressed as the increase in fluidity (Δ F), according to Thomas (54). The assay of a series of enzyme levels (Fig. 3) indicated that the assay method was only approximately quantitative; however, the results have been used for comparative purposes when the Δ F values obtained were less than 0.200.

Reducing Sugar Assay. This assay using CMC-70-Low as substrate was performed according to the procedure of Hash and King (14) and measured the increase in reducing groups produced by enzymatic action. No attempt was made to define a unit of activity and the assay was used on a comparative basis only.

Amorphous Cellulose Assay. This assay was used to determine the relative ability of enzyme fractions to solubilize an amorphous cellulose. The procedure was similar to that of the hydrocellulose assay, the only difference being that the reaction mixture contained 2 ml of the stock amorphous cellulose preparation (4.4 mg cellulose per ml on a dry weight basis). Turbidities were determined in the same manner as in the hydrocellulose assay, and the Δ Klett values obtained after incubation were used as a semi-quantitative measure of activity.

Cellotriose Assay. A semi-quantitative assay was used to compare the relative ability of enzyme fractions to yield glucose from cellotriose. The reaction mixture consisted of 1 ml of a buffered cellotriose solution (1 mg/ml in 0.02 M citrate buffer, pH 4.8) to which was added a small

Fig. 3. Relationship between incubation time and the fluidity change as determined by the CMC viscosimetric assay. Four enzyme levels were used, 1, 2, 5, and 10 µl, and viscosity measurements were taken at the intervals shown. The relative viscosity values were converted to fluidity, and the decrease in fluidity (Δ fluidity) was plotted versus incubation time. Each point represents the average of two separate determinations.


0·300
0·200
0·100
0
Δ FLUIDITY
0
15
30
45
60
90
MINUTES

enzyme aliquot (10 µl or less) at zero time. After incubation for one hour at 40°, the reaction was stopped by placing the tubes in boiling water for 10 minutes. After cooling, the glucose produced was determined by the enzymatic glucose procedure (see Analytical Procedures, below).

Aryl-β-Glucosidase Assay. p-Nitrophenyl-β-D-glucopyranoside was used as substrate for determining aryl-β-glucosidase activity according to the procedure of Hash and King (14). Results obtained from this assay were used in a comparative manner.

Analytical Procedures

Protein (range, 0 to 300 µg, bovine serum albumin standard) was determined according to the method of Lowry, et al. (23), and specific activities were based upon the values obtained by this procedure. Ultra-violet absorbancy at 280 mµ was used for rapid scanning of column fractions.

Total carbohydrate material (range, 0 to 150 µg as glucose) was determined by the phenol-sulfuric acid method described by Timell (55). In some of the early experiments, total carbohydrate was determined by the 0.2% anthrone-sulfuric acid method.

Degree of polymerization of soluble reaction products from cellulose degradation was determined by the procedure of Timell (55).

Reducing sugars (range, 0 to 100 µg as glucose) were determined using the Somogyi copper reagent and the Nelson arsenomolybdate reagent described by Hash and King (14).

Glucose (range, 0 to 100 μg) was determined by an enzymatic (notatin) method modified from Saifer and Gerstenfeld (46). The reagent contained 20 mg glucose oxidase (Sigma Chemical Co., Type III), 15 mg peroxidase (Worthington Biochemical Corp., HPO-D), and 30 mg o-dianisidine (Eastman Organic Chemical Co.), in 200 ml of 0.1 M phosphate buffer. Two ml of reagent were added to 1 ml of sample aliquot-plus-water and incubated 20 minutes at 40°. The reaction was stopped by addition of 2 ml of 0.5 N HCl, decreasing the pH to between 1 and 2 and causing a spectral absorbancy shift of the chromogen (2). Absorbancy was determined with a Klett-Summerson colorimeter (420 mμ filter), and unknowns were compared to a series of glucose standards run simultaneously.

IV. RESULTS AND DISCUSSION

Preliminary Studies of the Crude Cellulase System

The initial studies on an enzyme system exhibiting "hydrocellulase" activity made use of cell-free culture filtrates of Cellvibrio gilvus (see Appendix), from which background information was derived and applied to the T. viride crude enzyme when it became available. It was observed that solutions of Cellulase Meiji behaved similar to filtrates of C. gilvus in the hydrocellulose assay.

An enzyme-time study was performed to detect the presence of any inhibitor of, or a co-factor requirement for, the "hydrocellulase" activity (Fig. 4). Over the 50-fold range of crude enzyme concentration used no evidence for either was found, the slight decrease in activity observed at the lower enzyme levels being attributable to enzyme inactivation during the 12.5 hours of incubation.

The optimum pH for "hydrocellulase" activity was determined over the pH range of 2.95 to 9.42 and was found to fall between 4.6 and 5.0 (Fig. 5-A). Citrate and acetate buffers were used to repeat the pH optimum determination over a narrow range, and the optima were found to be at pH 4.8 and 4.7 respectively. Citrate buffer at a pH of 4.8 was used routinely for "hydrocellulase" studies. The "hydrocellulase" activity was found to be relatively stable over the pH range 3.8 to 7.0 for a period of three hours at room temperature (Fig. 5-B).

Thermal stability decreased markedly above 43°, when determined over a three hour period (Fig. 6-A). The optimum temperature for

Fig. 4. Enzyme-time study of crude enzyme assayed for "hydrocellulase" activity. A 50-fold range of enzyme concentrations was used and each enzyme concentration was incubated for the time interval required to give a constant E x T product. The point ▲ represents the lowest enzyme concentration used incubated without substrate for the interval indicated, then incubated with substrate for the same interval as a control for thermal stability. Each point represents an average of duplicate determinations.

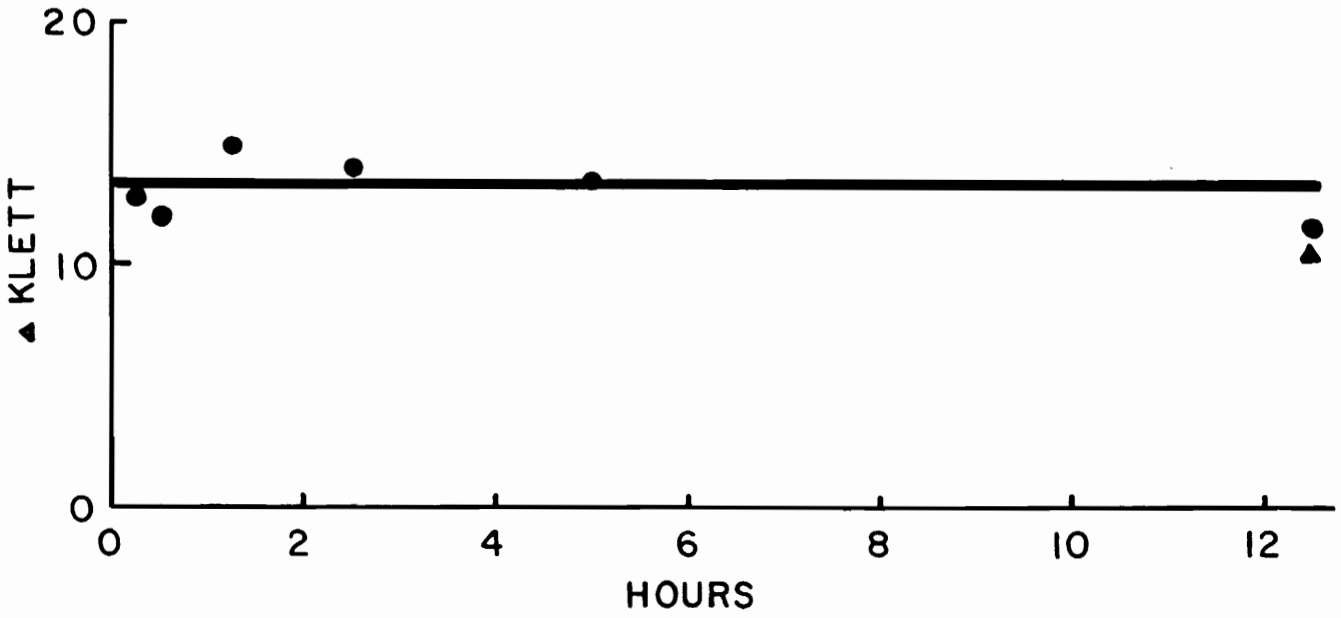

20
10
0
KLETT
0
2
4
6
8
10
12
HOURS

Fig. 5. pH characteristics of "hydrocellulase" activity.

A. Optimum pH for "hydrocellulase" activity. Buffers used to cover the pH range were 0.05 M citrate-phosphate (2.95 to 4.20), 0.10 M acetate (4.39 to 5.43), 0.05 M phosphate (5.77-7.80), and 0.10 M bicarbonate (8.53 to 9.42). Averages of duplicate values are shown.

B. pH stability for "hydrocellulase" activity. The stability data were obtained by measuring the activity remaining after exposure to the indicated conditions of pH for 3 1/2 hours at 25°. Treated enzyme solutions were adjusted to pH 4.8 before assaying. Averages of duplicate values are shown.

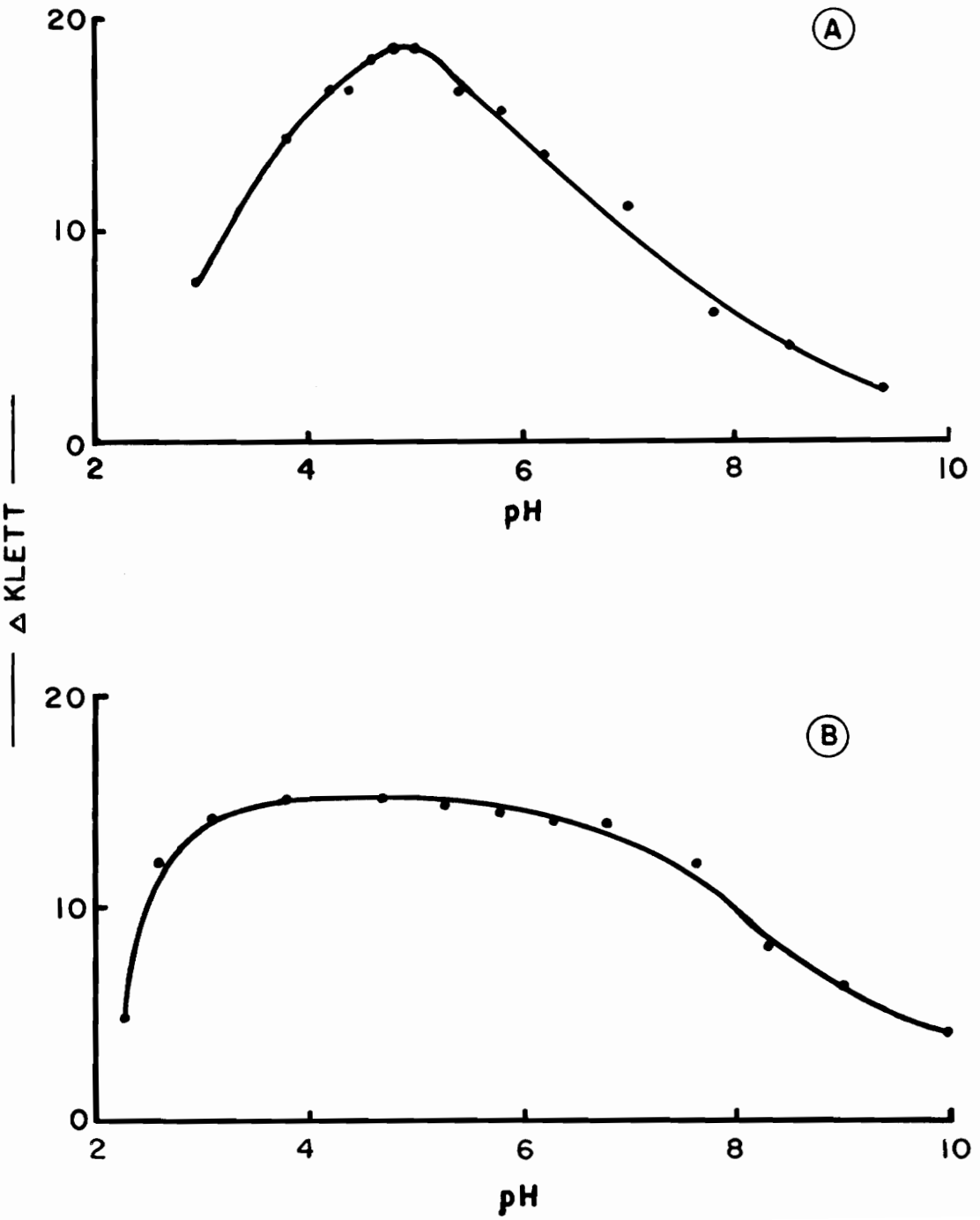
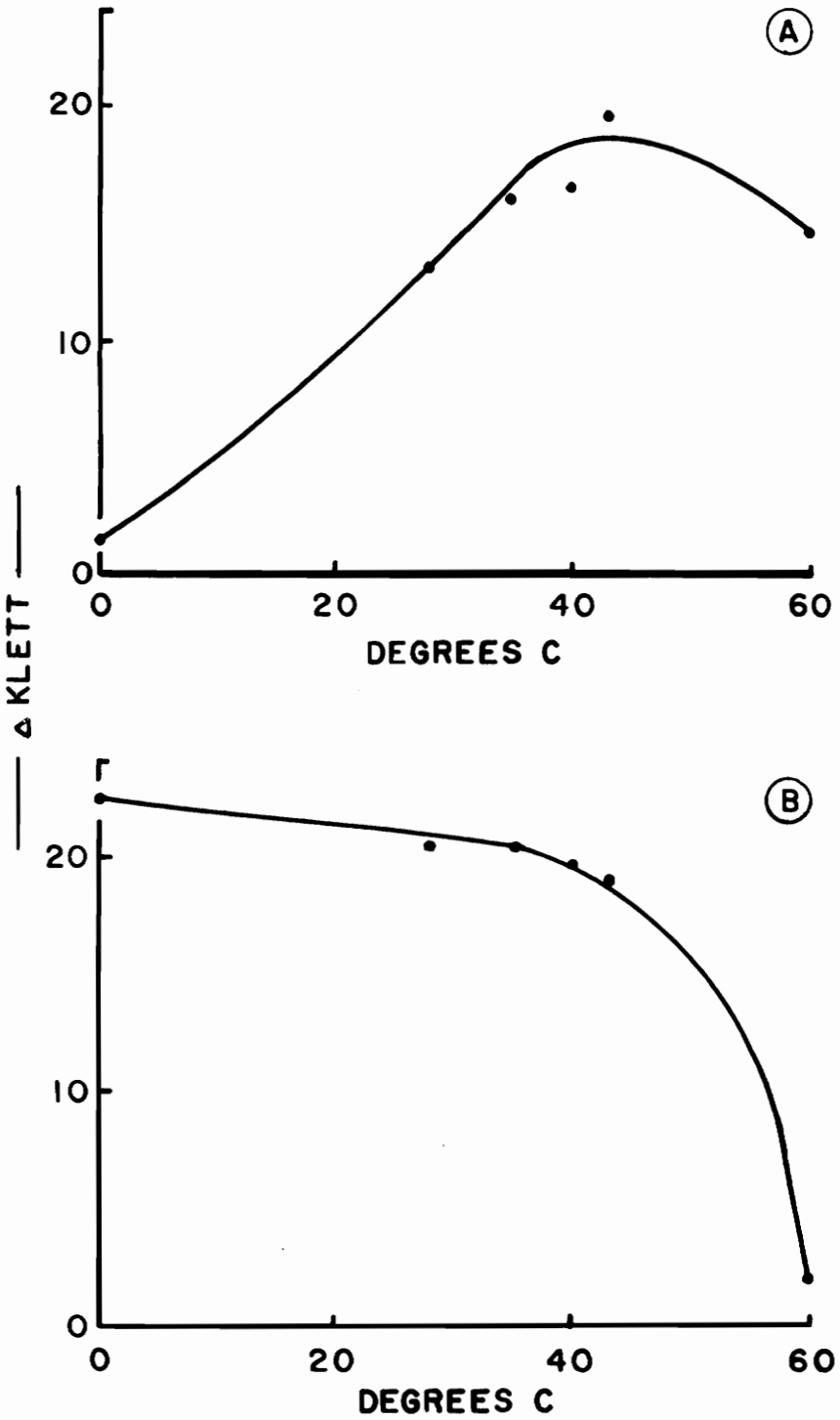

A
B
20
10
0
2
4
6
8
10
pH
Δ KLETT

Fig. 6. Thermal characteristics of "hydrocellulase" activity.

A. Optimum temperature for "hydrocellulase" activity determined for a one hour assay at the temperature indicated. Averages of duplicate values are shown.

B. Thermal stability data obtained by assay for activity remaining after holding enzyme for three hours at the indicated temperature. Duplicate tubes were then assayed under standard conditions.


A
20
10
0
0
20
40
60
DEGREES C
Δ KLETT
B
20
10
0
0
20
40
60
DEGREES C

activity during a one hour assay was found to be in the vicinity of 43° (Fig. 6-B); however, routine assays and solubilization studies were run at 40°.

Under circumstances of prolonged incubation of reaction mixtures (as in the weight loss experiments) it was desirable to use a bacteriostatic compound to inhibit growth of contaminating microorganisms. The following agents were found to be without effect on the "hydrocellulase" activity; toluene, 0.01%, Dowicide D (chloro-p-phenylphenol), 10^{-3} M sodium fluoride, and 10^{-3} M potassium cyanide. When necessary, a drop of toluene was added to the reaction vessel; however, in its absence contamination growth was seldom a problem.

Cellobiose was found to be several times more inhibitory than glucose when present at equal molar concentrations (Table 1). The presence of 0.05% Methocel (methylcellulose) resulted in 95% inhibition of the crude enzyme activity toward hydrocellulose. Sulfhydryl compounds were found to stimulate the "hydrocellulase" activity. Cysteine was the most effective of three compounds tested (Table 1) providing a maximum stimulation when at concentrations of the order of 0.01 M. The presence of lead acetate in reaction mixtures at 0.0001 M caused a 24% inhibition, but the addition of 0.01 M cysteine completely reversed this inhibition and stimulated the activity 40% above the controls without cysteine. Cysteine was used throughout the preliminary studies in the assay buffer. EDTA (ethylenediamine-tetra-acetic acid) at 0.5% had no effect on the assay for "hydrocellulase" activity.

Table 1

Stimulation and Inhibition of "Hydrocellulase" at Several Levels of Purity

Enzyme Preparation	Compound Tested	Concentration in Assay Tube	Percent of Control Activity
Crude	D-Glucose	0.05 M	52 %
"	"	0.10 M	39
"	Cellobiose	0.025 M	12.5
"	"	0.05 M	8
"	Methocel	0.05 %	5
10X-purified	Cellobiose	0.006 M	18
"	"	0.06 M	4.5
"	CMC-70-high	0.01 %	23
"	"	0.05 %	11.5
"	Methocel	0.01 %	0
Crude	Cysteine	0.0125M	150
"	Glutathione	0.001 M	140
"	Mercaptoethanol	0.0125M	125
10X-purified	Cysteine	0.01 M	115
"	"	0.01 M	105
Crude or 10X-purified	EDTA	0.166 %	100
"	"	0.5 %	98
Crude	Gluconolactone	0.001 M	96.5
10X-purified	"	0.01 M	97
"	"	0.001 M	100

Early in the studies of "hydrocellulase" it was observed that the enzymatic activity could be removed from solution by centrifuging out the hydrocellulose substrate. Such adsorption was more efficient at room temperature than at 0-3°. Table 2-A shows the results when a constant amount of crude enzyme was passed through small columns of Avicel, followed by buffer and water washes. Adsorption of the "hydrocellulase" activity was apparently related to the surface area of the hydrocellulose.

In the hydrocellulose assay procedure Avicel behaved similarly to the substrate hydrocellulose (Table 2-B), but the Δ Klett values obtained were considerably less, a consequence of the larger particle size of the Avicel material. Fine particles of Avicel obtained from water suspensions were very good substrate material.

Little loss of activity of crude enzyme resulted from ammonium sulfate precipitation, or solvent precipitation using either ethanol or acetone if performed at 0-3°. Solutions of crude enzyme were stored up to several months under refrigeration without significant loss of activity and the activity was stable to freezing and thawing.

Purification of "Hydrocellulase"

Observations of the adsorptive characteristics of the "hydrocellulase" onto its substrate, hydrocellulose (Avicel), were the basis for the first major step in purification. Results from a number of trial columns packed with Avicel indicated that "hydrocellulase" would remain bound through continued buffer washing, but could be eluted with urea,

Table 2

Avicel as a "Hydrocellulase" Adsorbant and Substrate

A. Avicel as an Adsorbant of "Hydrocellulase"

Avicel/ Column	Non-adsorbed Units	Removed by Buffer Wash Units	Removed by Water Wash Units	Total Recovered Units
1 mg	24.5	9.0	2.0	35.5
2 mg	11.0	15.0	8.5	34.5
5 mg	1.0	10.0	22.0	33.0

B. Avicel as a Substrate Compared with the Assay Hydrocellulose

Substrate	Amount/ Tube	Turbidity (Klett-Summerson Units)		
		Initial	1 Hour	Δ Klett
Avicel	2 mg	52	47	5
Avicel "fines"	--	83	50	33
Assay hydrocellulose	2 mg	81	52	29

cellobiose, and dilute ethanol solutions, or with distilled water. Although the mechanism of desorption is not known it would appear that a lowering of the ionic-strength is necessary, and that by so doing the affinity of the "hydrocellulase" for the column packing is reduced. The observation that the activity was decreased 50 to 70% when assays were performed in water (the pH of such reaction mixtures being well within the assay range for good activity so that pH was not the factor responsible for such a large apparent decrease in activity) lends support to the idea that ionic strength is a factor affecting the affinity of "hydrocellulase" for hydrocellulose, and thus an effect on the catalytic activity.

Since urea, cellobiose, and ethanol were somewhat inhibitory in the hydrocellulose assay, distilled water was used to elute. The behavior and elution pattern of a typical Avicel column are shown in Fig. 7. On such columns better than 90% of the protein, CMCase activity, and aryl-β-glucosidase activity passed through the column apparently unadsorbed. Buffer washing continued until the column was flushed 10 to 20 times, and then water washing was begun. The early fractions containing the major portion of the CMCase activity were saved and used for further studies on the CMCase (22). The later fractions having the "hydrocellulase" were pooled and concentrated by saturating to 70% with ammonium sulfate and redissolving the precipitated protein in dilute buffer.

Other attempts at purification of crude enzyme solutions, ammonium sulfate and ethanol precipitations, and iso-electric precipitation at 30% saturation of ammonium sulfate, were not very successful giving at

Fig. 7. Behavior and typical elution pattern of crude enzyme chromatographed on an Avicel adsorption column. The experiment shown was performed in the following manner. A column packed with Avicel (4 x 10 cm) was equilibrated with 0.05 M citrate buffer, pH 4.8, and 100 ml of 6% crude enzyme solution was run onto the column followed by extensive buffer washing until the effluent was nearly colorless (about 400 ml). Then integration of distilled water into 100 ml of buffer eluted the "hydrocellulase." Twenty ml fractions were collected and their appearance was as follows:

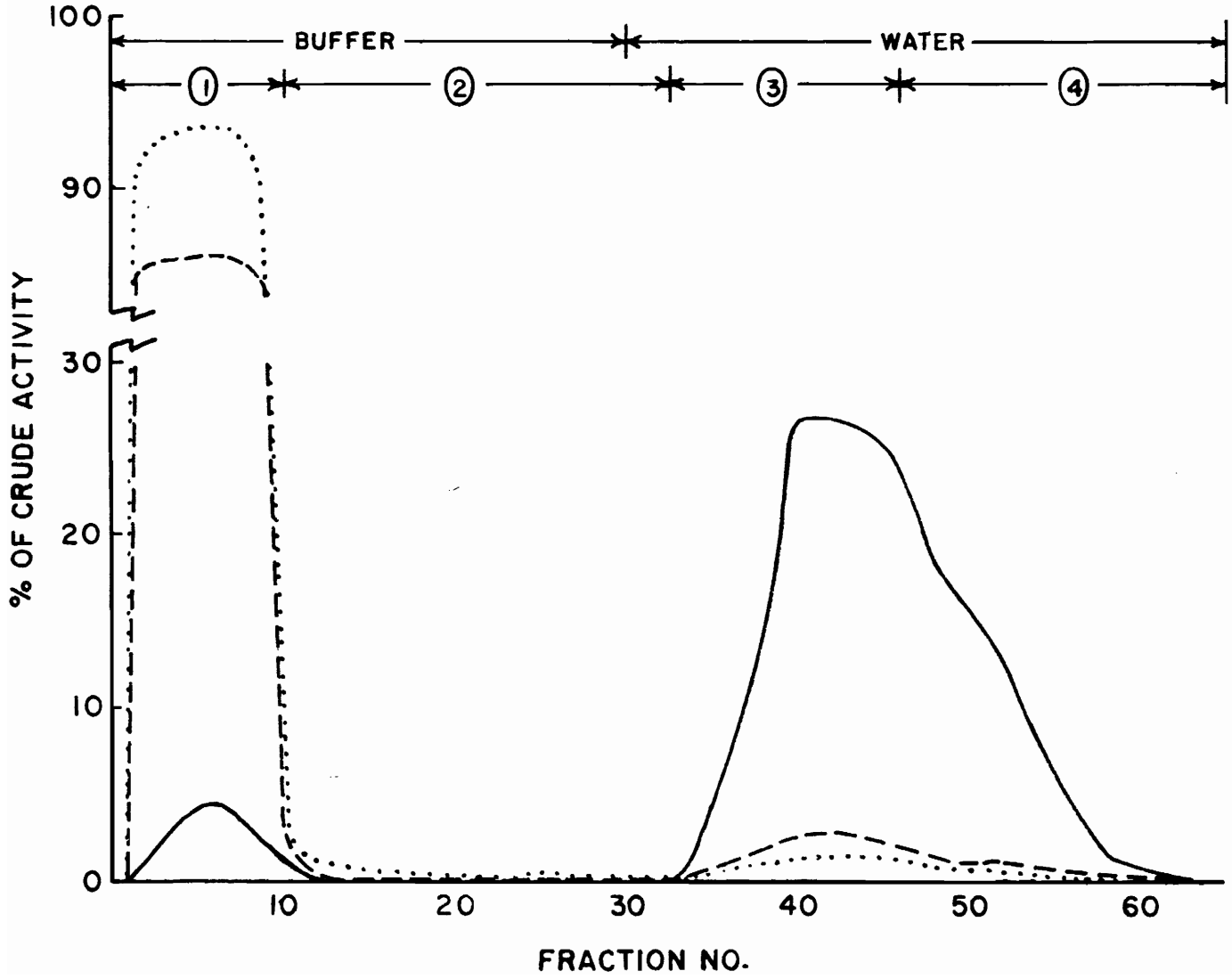
(1) Very dark fractions having the color of the crude enzyme solution

(2) Fractions of decreasing color

(3) Light yellow fractions

(4) Turbid fractions

"Hydrocellulase" (———), CMCase (· · · · ·), and protein (- - - -) are expressed as per cent of the amount present in the crude solution (on a per ml basis).


100
90
30
20
10
0
BUFFER
WATER
①
②
③
④
% OF CRUDE ACTIVITY
10
20
30
40
50
60
FRACTION NO.

most only a three-fold purification. Later it was shown that with acetone precipitation of crude enzyme the 50 to 60% cut yielded about a three-fold purification. It was felt that cellulose columns gave the best initial resolution of the crude enzyme because of the marked affinity of "hydrocellulase" for the column packing.

Further attempts to purify the "hydrocellulase" eluted from cellulose columns included calcium phosphate gel adsorption, ion-exchange chromatography, electrophoresis on glass fiber strips, and differential solubilization by means of ammonium sulfate-Celite and acetone-Celite columns, all with little success. The columns using Celite as a packing material were run in the following manner. Concentrated, partially purified enzyme eluted from Avicel columns was mixed with dry Celite 535, and the protein was precipitated by saturating to 70% with either ammonium sulfate or acetone. The Celite-protein slurry was used to pack a small column (1 x 5 cm), and a gradient integration of 40% precipitant into 70% precipitant was used to wash the columns. Enzymatic activity was eluted as it became solubilized in the washing process. On such columns there was a partial separation of CMCase and "hydrocellulase"; however, there was considerable over-lapping of the activities and recoveries were low.

Ion-exchange appeared to be the most promising as some protein without activity was separated from the "hydrocellulase" activity. The ion-exchange resins used were Amberlite CG-50 Type II (carboxylic acid resin) and Amberlite CG-45 Type II (polyamine resin). The elution patterns from such columns are shown in Figs. 8 and 9. "Hydrocellulase"

Fig. 8. Typical elution pattern of "hydrocellulase" from an Amberlite CG-50 ion-exchange column. Columns of this type were of the following general procedure. The column was packed with the carboxylic acid resin in the hydrogen form and washed well with water. A concentrated solution of "hydrocellulase" in 0.1 M sodium acetate was applied on the column, and 0.1 M sodium acetate was used to elute. "Hydrocellulase" (———), CMCase (·····), and protein (- - - -) eluted from a typical column are shown on a relative scale.

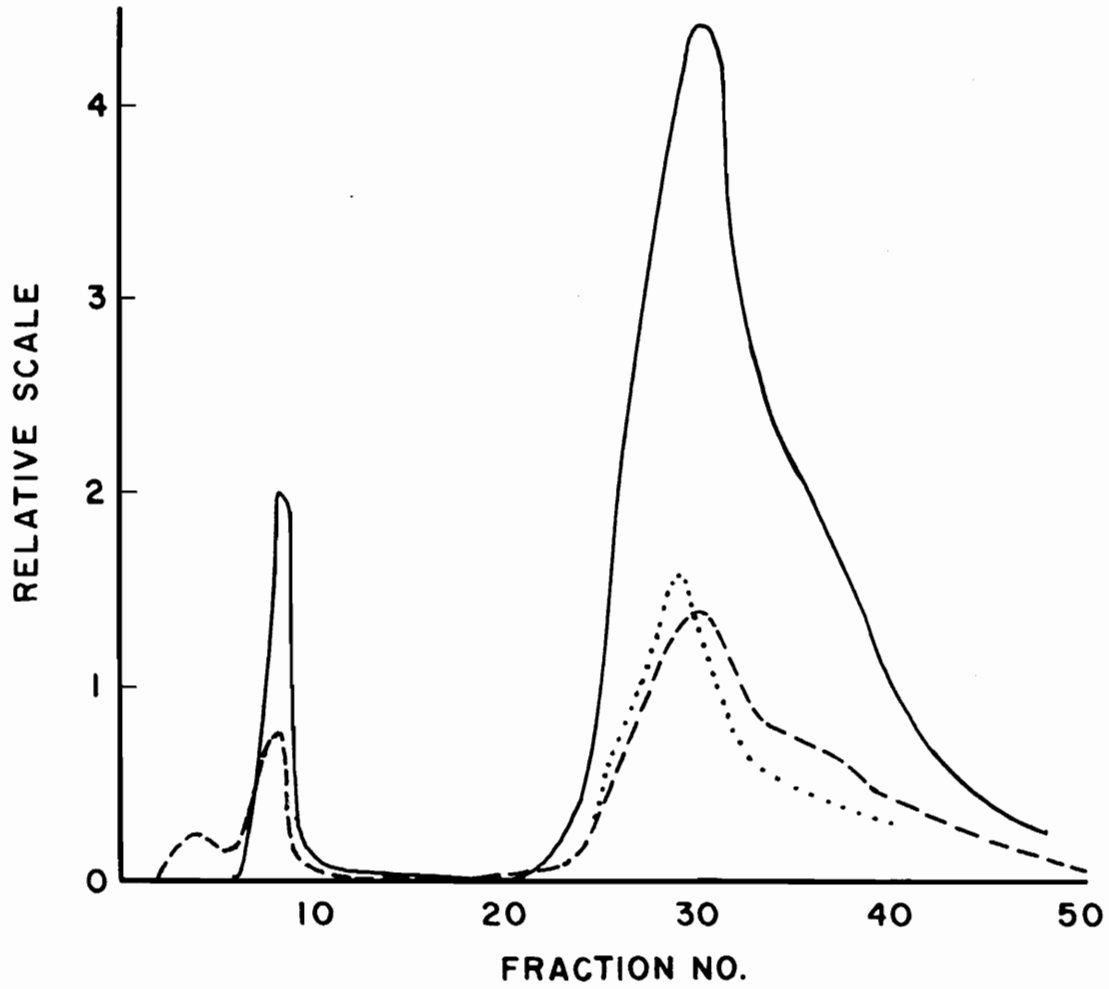
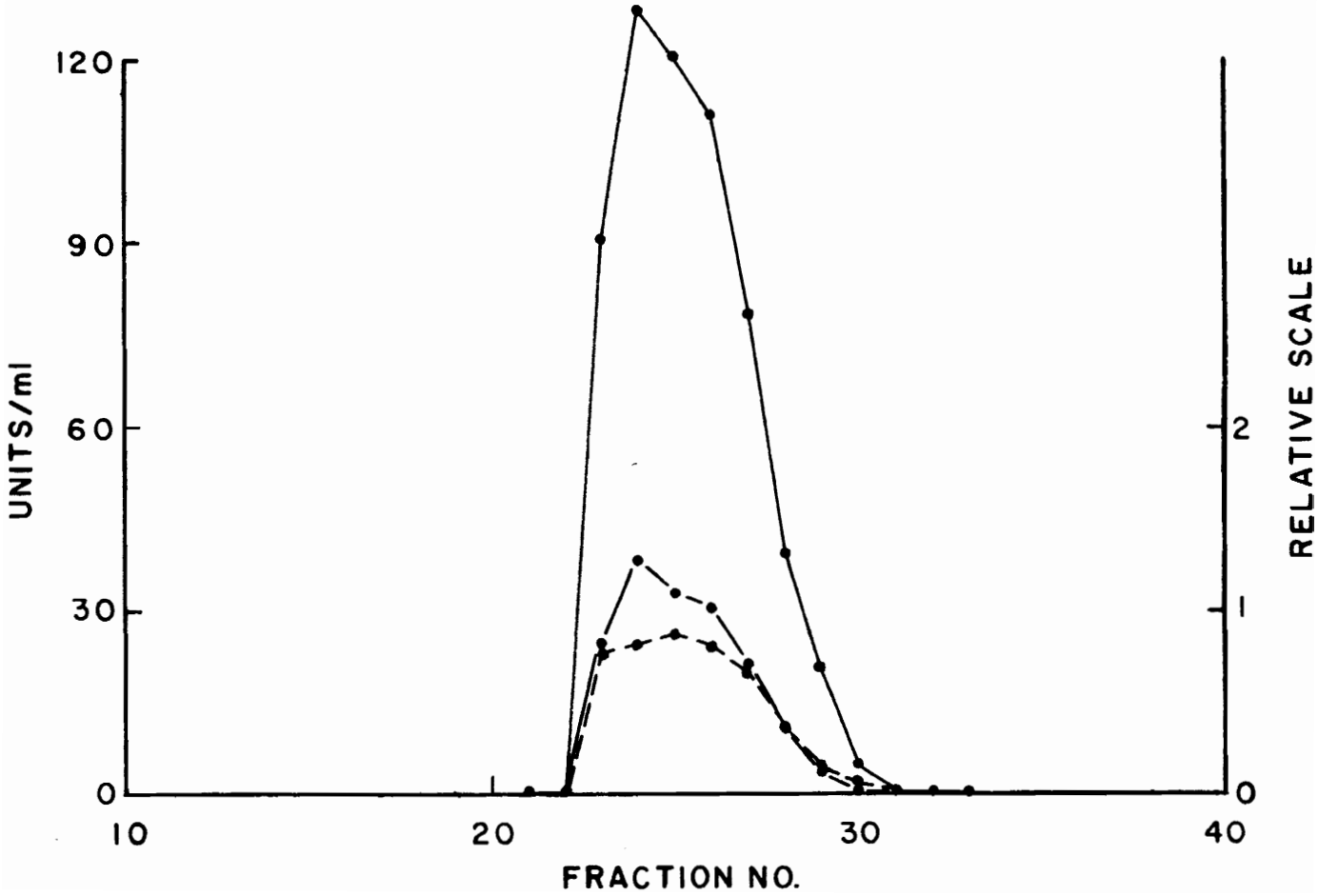

RELATIVE SCALE
0
1
2
3
4
10
20
30
40
50
FRACTION NO.

Fig. 9. Ion-exchange chromatography of "hydrocellulase" on Amberlite CG-45. The polyamine resin was equilibrated with 0.2 M phosphate buffer, pH 6.6, and a column 2 x 20 cm was packed. Concentrated "hydrocellulase" from a cellulose adsorption column was applied to the column. Elution was with the same phosphate buffer used to equilibrate the column. Sixty-five fractions of 3 ml each were collected. "Hydrocellulase" (•—•—•) is shown on a unit/ml basis. CMCase (•-•-•) and protein (•-•-•) are shown on a relative scale.


120
90
60
30
0
UNITS/ml
2
1
0
RELATIVE SCALE
10
20
30
40
FRACTION NO.

migrated essentially as a single peak of protein, but it should be noted that the "hydrocellulase":CMCase ratios were never constant across the protein peak. Ion-exchange chromatography resulted in only little purification but essentially complete removal of aryl-β-glucosidase activity from the "hydrocellulase" (Table 3). Fractions from ion-exchange columns were concentrated by precipitation with ammonium sulfate.

It was thought at the time that the elution pattern of protein and activity from ion-exchange columns represented a high degree of protein homogeneity, and this was borne out by ultracentrifugation studies of the "hydrocellulase" eluted from Amberlite columns. As can be seen from Plate 1 the bulk of the protein moved as a single peak with a sedimentation constant of 4.1 S. Although no estimation was made of the molecular weight, the sedimentation constant would indicate that the molecular weight of the 4.1 S protein is on the order of 50,000 to 65,000.

After the cellulose column and ion-exchange procedures a purification on the order of 10-fold had been obtained over the starting crude material. The stimulatory effect of cysteine had diminished (Table 1), and its use was discontinued. The earlier study of lead acetate inhibition and reversal by cysteine may indicate that the effect of cysteine was to compete for heavy metals which might be present in the crude but removed on purification rather than a direct stimulation of enzyme activity. Cellobiose and Methocel were still potent inhibitors, and CMC exerted an inhibitory effect on "hydrocellulase" activity; however, gluconolactone, a known potent inhibitor of β-glucosidase

Table 3

Representative Purifications of "Hydrocellulase"

Preparative Procedure 1

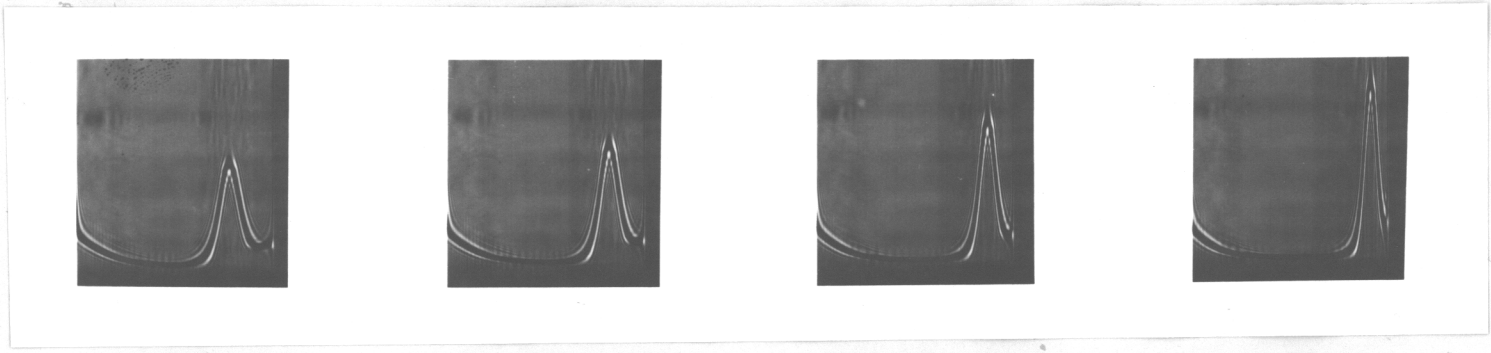
	S.A. Units/mg	Recovery	Ratio of "Hydrocellulase" Activity to: CMCase[1]	$p\text{-}NO_2\text{-}ase$[2]	CMCase[3]
Crude Enzyme	1.38	100%	1:1	1:1	1:1
Avicel Effluent	15.1	60%	13:1	302:1	11:1
Amberlite Effluent	17.9	--	15:1	15300:1	12:1

Preparative Procedure 2

	S.A. Units/mg	Recovery	Ratio of "Hydrocellulase" Activity to: CMCase[1]	$p\text{-}NO_2\text{-}ase$[2]	Amylase	Cellobiase
Crude Enzyme	3.80	100%	1:1	1:1	1:1	1:1
Avicel Effluent	20.7	36%	5.6:1	98:1	>650:1	29:1
Amberlite Effluent	23.5	15%	6.1:1	28800:1	>2500:1	98:1
Sephadex Effluent						
Fraction 34[4]	34.0	--	24:1	--	--	--
Fraction 31	50.0	--	30:1	--	--	--

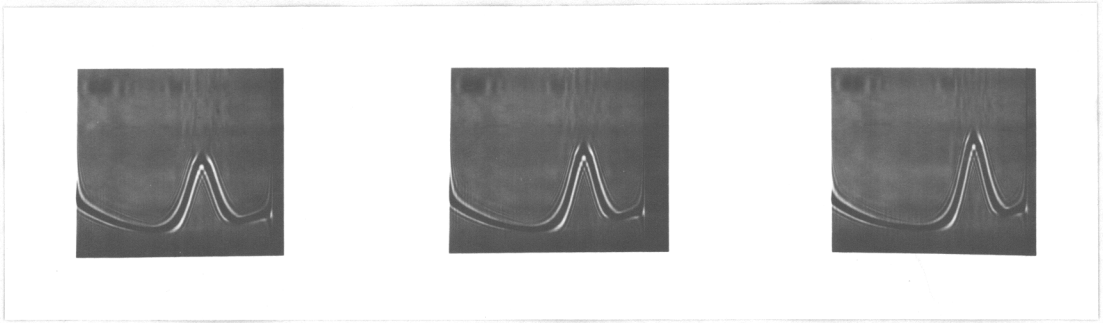
[1]Viscosimetric CMC assay
[2]Aryl-β-glucosidase assay
[3]Reducing sugar CMC assay
[4]See Fig. 10-A, p. 51

Plate 1. Analytical ultracentrifuge schlieren patterns of partially purified "hydrocellulase" (S.A. = 23.5). Run in a Beckman Spinco model E instrument at an approximate 1% protein concentration in 0.1 M sodium acetate buffer, pH 5.5, and at a rotor speed of 56,100 r.p.m. Photographs were taken at 16 minute intervals after the designated speed was attained.



64 48 32 16

MINUTES



112 96 80

MINUTES

activity at 0.001 M, was without effect on the "hydrocellulase" activity (Table 1).

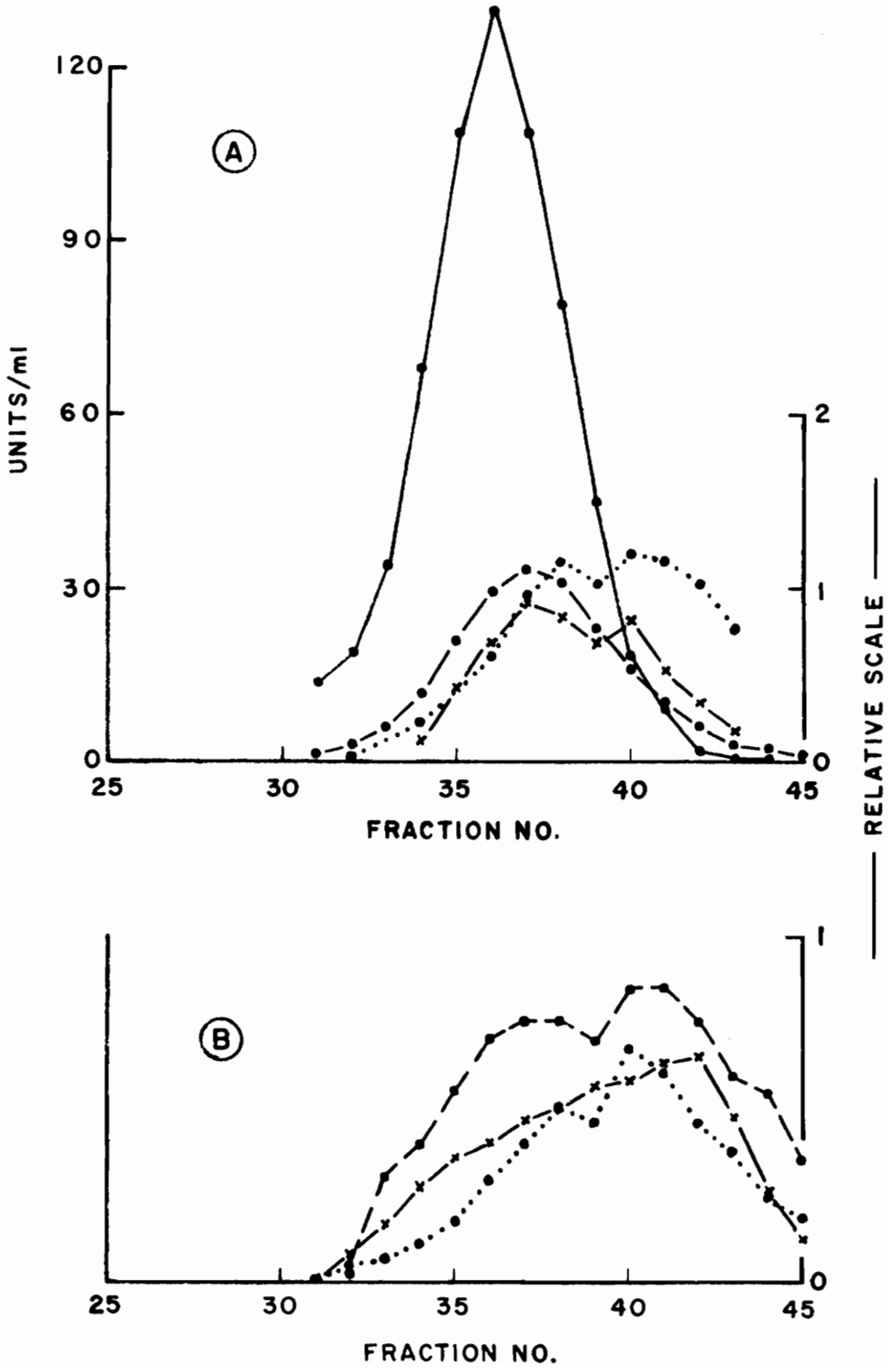
It was observed that "hydrocellulase" was excluded by the gel, Sephadex G-50, but was retarded by Sephadex G-100. The first direct evidence of heterogeneity of activity was obtained by gel filtration on long columns (2 x 75 cm) of Sephadex G-75. The behavior of such a column is shown in Fig. 10-A. As can be seen the maximum activity towards different substrates occurred in different fractions. Those fractions having predominately "hydrocellulase" activity and little of the CMCase were pooled, concentrated with ammonium sulfate, and subjected to a second pass through the Sephadex column. Since individual fractions were used in subsequent experiments, depending upon the desired enzyme activity, recovery and over-all purification data were not obtained for this purification step.

The purification obtained in two preparative procedures is presented in Table 3. As indicated previously (Materials and Methods) it is not known what purification of the crude enzyme has been obtained in the processing of the crude enzyme used in these studies. Two different lots of Cellulase Meiji differing in specific activity (S.A.) were used. The earlier work was done with a preparation having a S.A. = 1.38 units/mgm protein and later work with a preparation of higher initial purity, S.A. = 3.80 units/mgm protein. Thus, the relative amount of purification differs with the initial purity of the crude enzyme; however, the specific activities obtained by the various purification steps were roughly comparable.

Fig. 10. Partial resolution of the "hydrocellulase" complex by means of gel filtration.

A. Activity spectrum of fractions washed from a 2 x 75 cm column of Sephadex G-75. A 2 ml sample of concentrated "hydrocellulase" was applied to the column and washed through with 0.1 M acetate buffer, pH 5.0. "Hydrocellulase" (•—•—•) was assayed in the presence of a synergistic CMCase component, and the activity is shown on a unit/ml basis. Eluted CMCase (•···•···•), protein (•-•-•), and cellotriase (×-×-×) are shown on a relative scale.

B. Activity spectrum of the above fractions after heat treatment of aliquots of 70° for six minutes. This treatment inactivates "hydrocellulase" (see Fig. 15-B). CMCase (•···•···•), cellotriase (×-×-×) and amorphous cellulose activity (•-•-•) are shown on a relative scale. Note the slight shifts of CMCase and cellotriase activity between fractions 35 and 40.


A
120
90
60
30
0
UNITS/ml
2
1
0
RELATIVE SCALE
25
30
35
40
45
FRACTION NO.
B
1
0
25
30
35
40
45
FRACTION NO.

The Avicel adsorption column gave the greatest purification of any single step. The Amberlite CG-50 ion-exchange column gave only little additional purification, but was effective in almost complete removal of contaminating activities (e.g. aryl-β-glucosidase and amylase). The gel filtration step gave the best resolution of the "hydrocellulase" complex of several procedures attempted. At this stage of purification a strong synergism of enzyme components was first observed. A maximum S.A. of 50.0 (with synergistic enzymatic activity added) was obtained in an early "hydrocellulase" fraction from the Sephadex G-75 column, which had a "hydrocellulase":CMCase ratio of 30:1.

Although tests for synergistic action between various enzymatic activities were made at several stages of purification this phenomenon was not observed until after Sephadex G-75 gel filtration. The inability to demonstrate synergism earlier may have been the result of either of two factors. First, the tests for synergism were negative because the enzyme fractions tested had a sufficient amount of the synergistic component. This conclusion is warranted by the experimental observation that an excess of a synergistic component will cause no further increase in "hydrocellulase" activity. Second, the proper synergistic component was not used. At present it has been established that although the exo-β-1,4-glucan glucose hydrolase[1] and a CMCase closely associated with the "hydrocellulase" show synergism, the endo-β-1,4-glucan hydrolase[2] does not.

---

[1] Exo-β-1,4-glucan glucose hydrolase, purified extensively by Wu and King in this laboratory from the T. viride crude enzyme system, yields glucose from cellulodextrins with maximum activity toward those having a D.P. of 4 to 5.

[2] Endo-β-1,4-glucan hydrolase, purified extensively by Li in this laboratory from the T. viride crude enzyme system, has potent activity toward CMC and amorphorus cellulose substrates (22).

Considering the above purification attempts in retrospect, which were performed for the most part without knowledge of the synergism effect, there is good reason to believe that some of the low yields of fractionation procedures were the result of unknowingly separating the synergistic component from the "hydrocellulase." Correlated with the preceding idea were observations that fractionation techniques which for one reason or another appeared to be poorly performed often gave better recoveries of "hydrocellulase" activity than did techniques which appeared to have given good resolution.

With the knowledge of the synergism effect, a re-evaluation of several of the potentially useful purification techniques (e.g. electrophoresis, ion-exchange) might serve for further purification of "hydrocellulase."

Enzymatic Characteristics of "Hydrocellulase"

Throughout purification the "hydrocellulase" retained the capacity to solubilize significantly both native cotton and cotton linter as demonstrated by loss in weight of these substrates (Table 4). It is worth noting that as little as 0.2 ml of a concentrated Avicel column "hydrocellulase" (S.A. = 15.8) effected a 47% weight loss of 50 mg of native cotton fiber in 144 hours, and that 0.2 ml of "hydrocellulase" from a Sephadex G-75 gel filtration (S.A. = ca. 25) effected a 34% weight loss of 50 mg of cotton linter in 72 hours. These results obtained with partially purified "hydrocellulase" preparations indicate a greater enzymatic potential to degrade native forms of cellulose than has been observed previously.

Table 4

Solubilization of Cotton Substrates

Enzyme	Substrate	Incubation Time (Hours at 40°)	Weight Loss[1] (Percent)
Crude S.A. = 1.38	Native Cotton	48	21.5%
	Purified Cotton Linters	48	61.7
Avicel Effluent S.A. = 15.1	Purified Cotton Linters	120	43.2
Avicel Effluent S.A. = 15.8	Native Cotton	144	47.2
Sephadex Effluent S.A. ca. 25	Purified Cotton Linters	72	34.4

[1]Determined by the difference of the weighed substrate (50 ± 2.0 mg) and the residue obtained after incubation on a sintered glass filter and dryed at 105°. An average of duplicates corrected for non-enzyme controls.

Extensively purified "hydrocellulase" was capable of complete solubilization of milligram quantities of hydrocellulose in the assay system during extended incubation periods, and similar observations were made a number of times with crude and other partially purified enzyme fractions (Table 5). Thus "hydrocellulase" was capable of completely solubilizing the crystalline form of cellulose as it occurs in hydrocellulose derived from cotton fiber.

Solubilization of hydrocellulose by crude enzyme resulted in better than 80% of the soluble carbohydrate being glucose. "Hydrocellulase" eluted from an Avicel column yielded glucose as 25% of the solubilized carbohydrate. However, after further purification (ion-exchange and gel filtration), glucose could account for no more than 3 to 5% of the total solubilized carbohydrate (Table 6). Purification for "hydrocellulase" activity resulted in a progressive removal of those activities which were present in the crude enzyme yielding glucose as the predominant soluble product.

Cellobiose was identified as the main soluble product resulting from the action of purified "hydrocellulase" on hydrocellulose by means of paper chromatography and comparison with authentic known cellobiose. The trace amount of glucose on paper chromatograms could not be readily detected by the sugar sprays used routinely above (periodate-benzidine, and aniline-diphenylamine-phosphate), but it could be detected by spraying the paper with the enzymatic glucose oxidase (notatin) reagent.

Quantitative analysis (determining the total carbohydrate, the reducing sugar value, and glucose) of the soluble products of hydrocellulose

Table 5

Solubilization of Hydrocellulose[1]

Crude S.A. = 3.7; 7.1 units			Amberlite Effluent S.A. = 16, 2.7 units			Sephadex Effluent S.A. = 26, 15.3 units		
5 mg Substrate			2 mg Substrate			5 mg Substrate		
Hours	Klett	Δ Klett	Hours	Klett	Δ Klett	Hours	Klett	Δ Klett
0	172.0	—	0	77.5	—	0	167.5	—
1	137.0	35.0	0.5	64.5	13.0	1	118.5	49.0
4	103.0	69.0	1.0	58.5	19.0	4	71.0	96.5
12	53.0	119.0	2.0	52.0	25.5	12	21.0	147.5
34	6.5	165.5	5.5	37.0	40.5	34	0	167.5
			24.0	0	77.5			

[1]Determined as turbidity decrease (Δ Klett) according to the hydrocellulose assay procedure. Results are typical of those obtained throughout this study.

Table 6

Changes in Reaction Products During Purification of "Hydrocellulase"

Enzyme	S.A. (Units/mg)	Soluble CH_2O[1] (γ/ml)[4]	Reducing Sugars[2] (γ/ml)[4]	Glucose[3] (γ/ml)	Glucose % of Total	Ave. D.P. of Other Products
Crude	3.67	150	--	122	81.5	--
Avicel Effluent	14.8	174	94	41	23.5	2.51
Amberlite Effluent	16.0	170	88	8.5	5	2.17
Amberlite Effluent	23.5	183	--	6.3	3.4	--
Sephadex Effluent	34.0	84.5	42.0	2.5	3.0	2.08

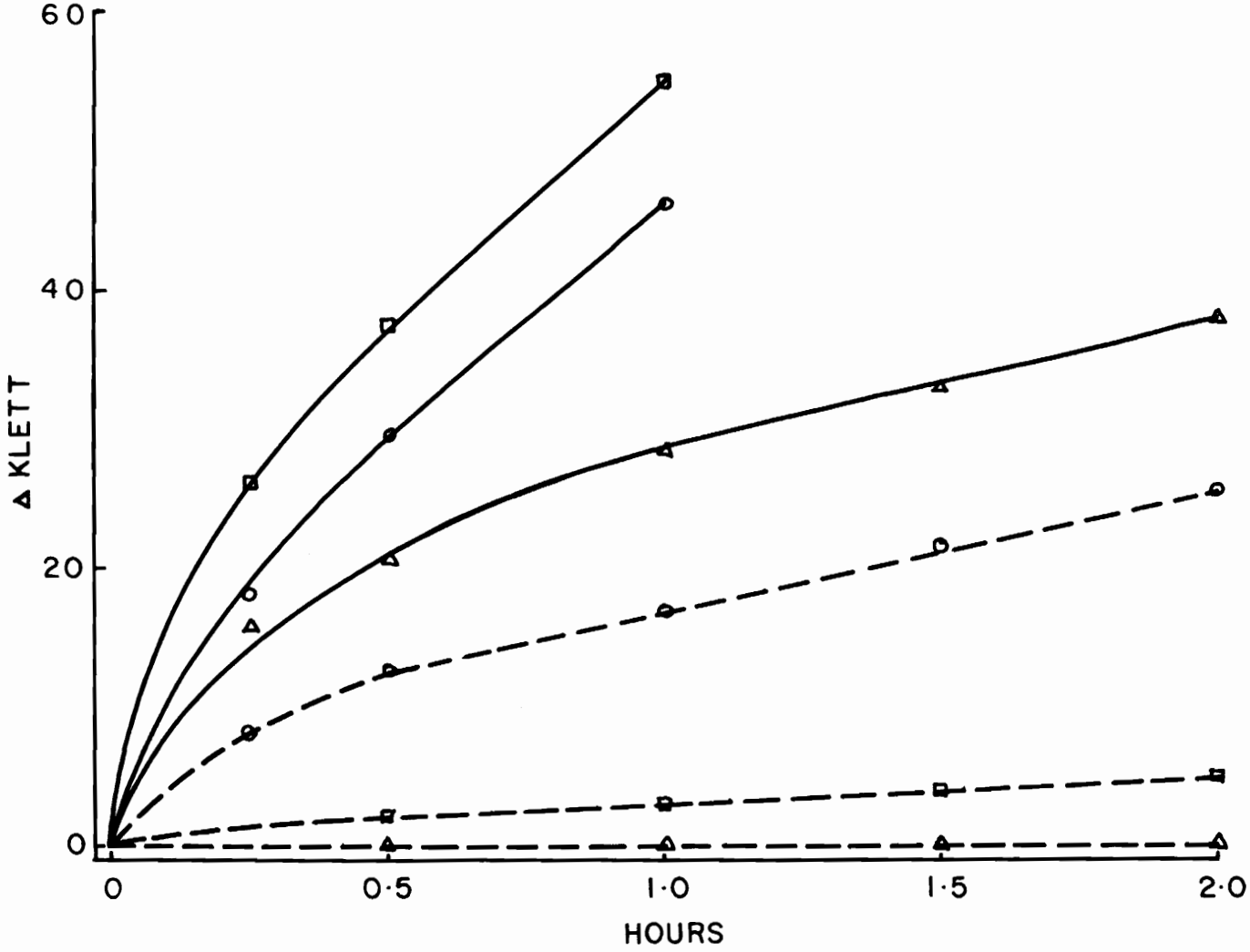
[1] By anthrone or phenol-sulfuric analyses
[2] By Nelson-Somogyi analysis
[3] By glucose oxidase (notatin) analysis
[4] All sugar concentrations in glucose equivalents

reaction mixtures at several stages of "hydrocellulase" purification indicated (Table 6) that the degree of polymerization (D.P.) of the remaining soluble carbohydrate after correcting for glucose present was in the order of D.P. = 2. Timell D.P. analysis of fractions washed from an Avicel cellulose column, subjected to the solubilizing action of "hydrocellulase" (S.A. = 23.5) at 40° gave a D.P. value of 2.08 $\pm$ 0.11 for the soluble products present in several representative fractions. The accumulation of cellobiose would indicate that this was the main product of the hydrolytic action of "hydrocellulase" enzyme system.

Studies have shown that "hydrocellulase" purified on the Avicel column (S.A. = 15.8) has a greater ability to solubilize hydrocellulose and a lesser ability to solubilize amorphous cellulose than did the crude enzyme. The ability of both crude and purified enzyme preparations to solubilize the amorphous substrate was greater at pH 4.0 than at pH 4.8, the pH optimum for hydrocellulose solubilization. A comparison of the activities of three different enzyme preparations, a "hydrocellulase," a CMCase, and a commercial "cellulase," toward amorphous and hydrocellulose is shown in Fig. 11. The relative activities differ markedly, the "hydrocellulase" having greatest activity toward hydrocellulose, the CMCase having greatest activity toward amorphous cellulose, and the commercial "cellulase" having activity only toward the amorphous cellulose.

Purification of "hydrocellulase" resulted in an increased specificity for the crystalline substrate, and an apparent distinction can be made between activities acting on the amorphous and crystalline substrates.

Fig. 11. Relative activity of three different enzyme preparations in solubilizing amorphous cellulose and hydrocellulose. The enzymes used were a "hydrocellulase" (S.A. = 11) and a CMCase both from T. viride and Cellase 1000, a commercial "cellulase." The "hydrocellulase" was purified by a cellulose adsorption column and the CMCase was the unadsorbed activity passing through a cellulose column (see for example Fig. 7, p. 43). The symbols used for the enzymes preparations are; "hydrocellulase" (○), CMCase (□), and Cellase 1000 (△). The same quantity of each enzyme was used for both substrates. Solubilization of amorphous cellulose is shown as a solid line (———), and of hydrocellulose by a broken line (— — —). The points are averages of duplicate determinations.


60
40
20
0
Δ KLETT
0
0·5
1·0
1·5
2·0
HOURS

"Hydrocellulase" eluted from an Amberlite CG-50 column (S.A. = 23.5), although freed of aryl-β-glucosidase activity, retained the ability to produce glucose from cellulodextrins (Table 7). The rate at which glucose was produced from the cellulodextrins of D.P. 3-5 was at least one order of magnitude greater than the rate at which glucose was produced when hydrocellulose was the substrate. Glucose produced from cellobiose, however, was on the same order of magnitude as that produced from hydrocellulose. Such data would indicate that the hydrolytic action of "hydrocellulase" on hydrocellulose proceeds to cellobiose without the production of any significant amount of free cellulodextrins, otherwise much more glucose would be apparent. The small amounts of glucose produced from hydrocellulose can be easily accounted for by the known rate at which cellobiose is cleaved by the best preparations of "hydrocellulase" so far obtained to yield glucose.

These results are very much in accord with Pringsheim's early proposals of cellulose degradation (35,36), that cellulose is degraded to cellobiose and subsequent cleavage of cellobiose yields glucose, if the above observations on hydrocellulose degradation by T. viride enzymes are related to the degradation of the crystalline regions of cellulosic material. Since crystalline regions comprise the bulk of cotton fiber cellulose it seems reasonable to postulate the following degradative scheme.

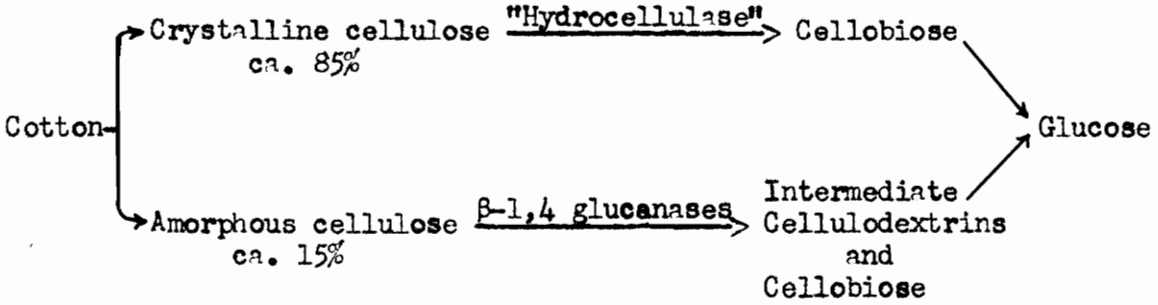


Table 7

Glucose Production from Hydrocellulose and Cellulodextrins

Substrate	Amount (mg)	Enzyme	Incubation Time (Minutes at 40°)	E x T	Soluble CH_2O at Reaction Termination	Glucose Present	Relative Rate of Glucose Production[1]	µmole Glucose / µmole Substrate
Hydrocellulose	ca. 5	25 µl	150	3750	1.1 mg	37.5 µg	0.01	--
Cellobiose	1.0	2 µl	40	80	--	3.5 µg	0.04	.0065
Cellotriose	1.0	2 µl	40	80	--	144.0 µg	1.80	.405
Cellotetraose	1.0	2 µl	40	80	--	78.0 µg	0.98	.286
Cellopentaose	1.0	2 µl	40	80	--	94.5 µg	1.18	.430

[1]Calculated from the glucose present at the termination of the reaction divided by the enzyme x time factor $\left(\frac{\text{Glucose Present}}{E \times T}\right)$. The values obtained are only relative because of the varying substrate and enzyme concentrations used.

The nearly exclusive production of cellobiose may occur for one of the following reasons. Structural models of crystalline cellulose indicate that the linear anhydroglucose chains on the surface of the crystallite have only alternate glucosyl bridge bonds exposed (Plate 2). Attack at the exposed glucosyl bonds would result in release of cellobiose. Energetically cellobiose production would be favored when considering the role of hydrogen bonding in crystalline cellulose. Production of cellulodextrins of relatively high D.P. would involve the simultaneous disruption of many hydrogen bonds. The nature of the hydrolytic enzymes may be such that only an endwise attack mechanism yielding cellobiose is operative. Obviously, a random hydrolytic cleavage yielding odd-membered cellulodextrins from hydrocellulose is not operative although such activity may be present in the "hydrocellulase" complex (see next section).

The rate of solubilization of hydrocellulose in the assay system was found not to follow typical enzyme kinetic rate-constants. It was observed, however, that the rate of solubilization adhered closely to "Schutz's Rule" which states that reaction velocity is proportional to the square root of enzyme concentration, or stated another way, reaction velocity is proportional to the square root of time. Observations of Schutz kinetics were made many times at all stages of enzyme purification and with several different insoluble substrates. In Fig. 12 it can be seen that a plot of extent of solubilization versus the square root of incubation time gave a linear relationship in accordance with Schutz's Rule when hydrocellulose and amorphous cellulose were substrates in turbidmetric assays and when cotton linter was substrate in a weight

Plate 2. Structural models of cellulose fragments.

Upper row: Glucose in the C1 (chair) conformation at left and cellobiose with the glucosidic bond facing up at right.

Middle row: A cellulodextrin or anhydroglucose chain arranged as in the lattice I structure of crystalline cellulose with its reducing group to the right, facing up.

Lower row: A longer anhydroglucose chain presenting the opposite side since its reducing group is to the right, facing down. Note that the two chains present a different arrangement of the oxygen atoms about the exposed glucosidic bonds. The arrows indicate possible sites of attack at glucosidic linkages if such a surface is projecting out from a crystal face. Note that alternate glucosidic bonds are not exposed.

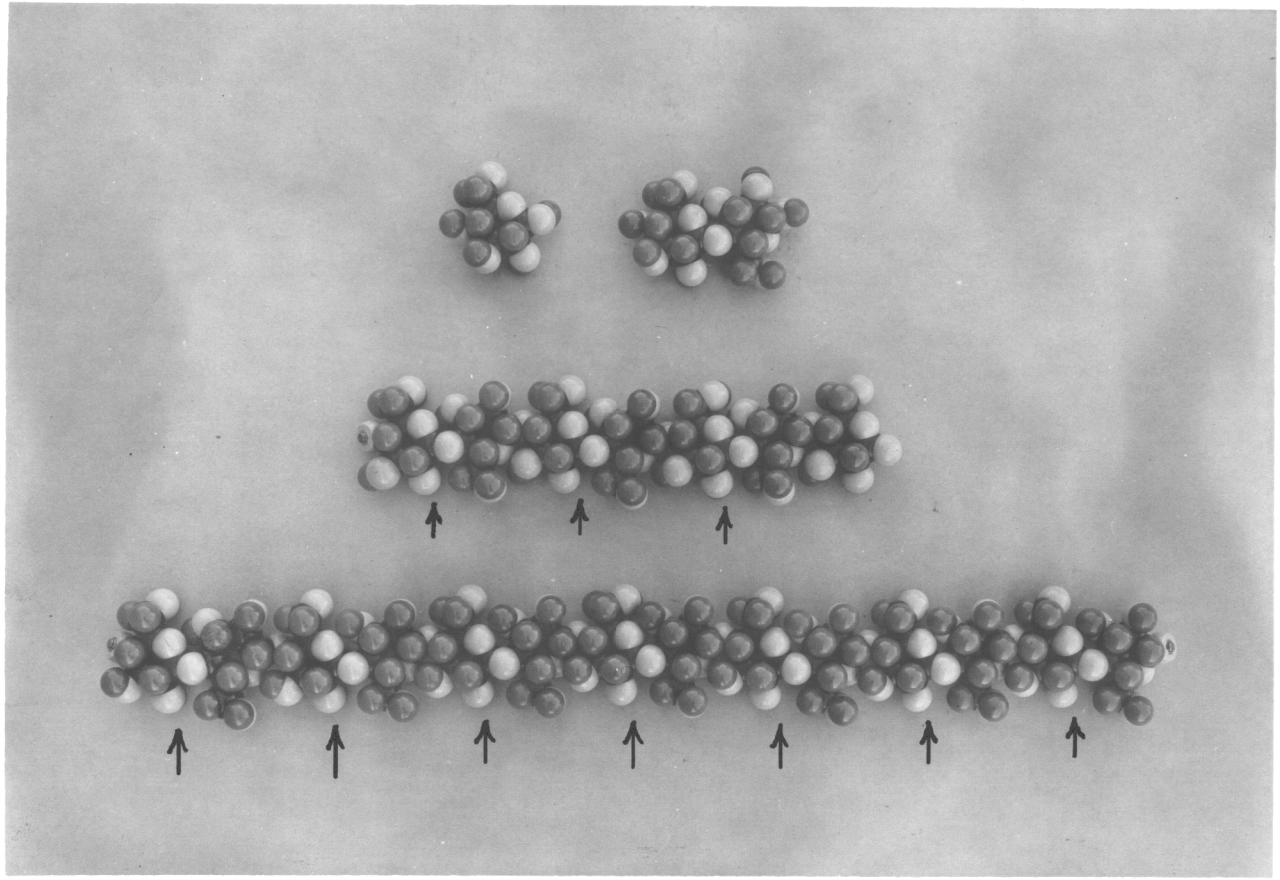
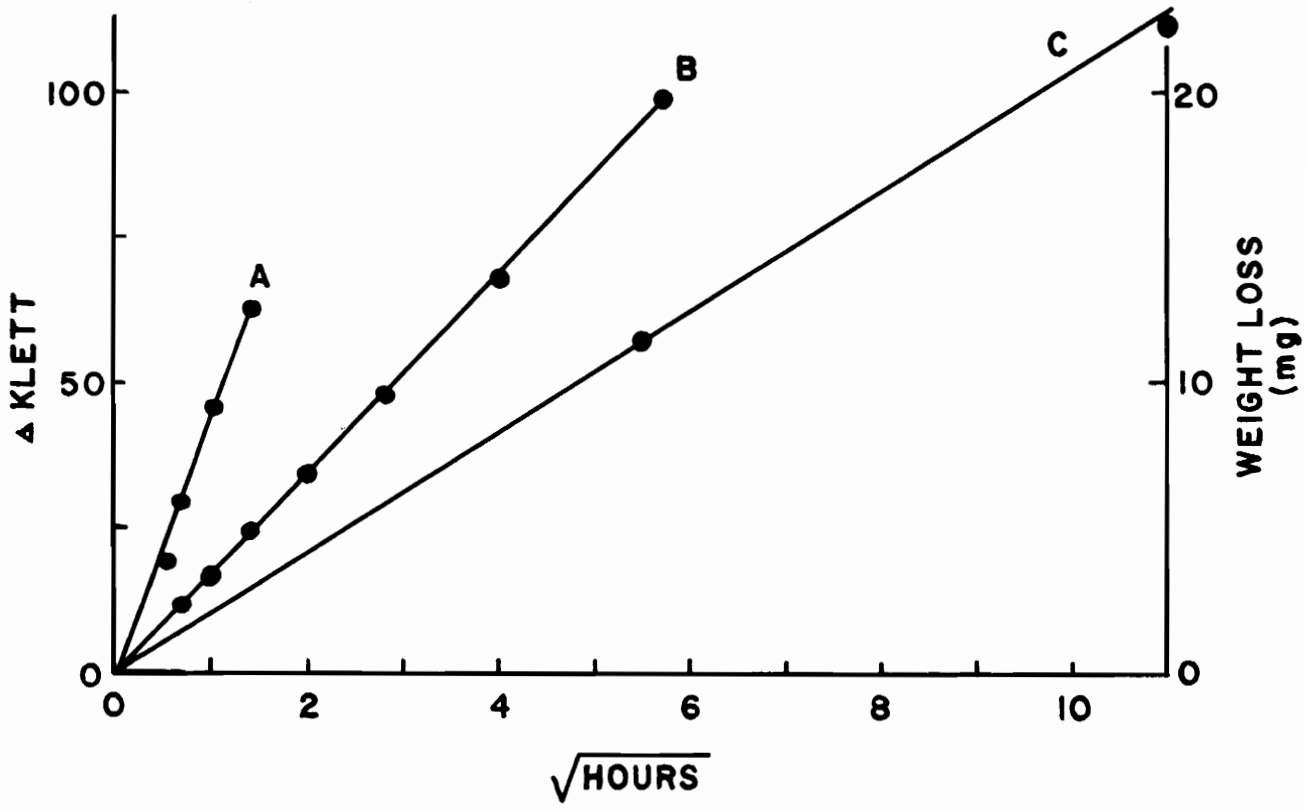


Fig. 12. Solubilization of three substrates in accordance with Schutz's Rule. The enzyme used was from various preparations of "hydrocellulase" purified to the extent of cellulose adsorption columns (S.A. > 10). The solubilization of the amorphous cellulose (A) and the hydrocellulose (B) was determined by the decrease in turbidity (Δ Klett) of these substrates. The solubilization of cotton linter (C) was determined by weight loss. The extent of solubilization is plotted versus the square-root of incubation time. Each point represents the average of duplicate determinations.


100
50
0
Δ KLETT
A
B
C
20
10
0
WEIGHT LOSS (mg)
0
2
4
6
8
10
√HOURS

loss determination. A plot of log v versus log t (Fig. 13) gave a straight line as expected when Schutz's Rule $v = kt^{1/2}$ is expressed in logarithmic form $\log v = 1/2 \log t + \log k$. In fact, the enzyme-time standard curve (Fig. 1) used to determine "hydrocellulase" activity approximates quite well Schutz's Rule.

It is not possible to give a satisfactory explanation as to why Schutz kinetics are observed. The possibility that it is an artifact of the turbidmetric measurement is ruled out by data which demonstrates that both soluble carbohydrate and reducing groups are also produced in accordance with Schutz's Rule (Fig. 14). The loss in weight of cotton linter also adheres to Schutz's Rule. Eliminating the possibility of an artifact inherent in the assay method a number of other factors have been considered in relation to Schutz kinetics. Among these are the involvement of two or more enzymatic activities participating in the solubilization process acting either sequentially or simultaneously, a progressive inhibition as a result of soluble end-product accumulation, and substrate availability considered in respect to surface area which may or may not decrease at greater than a linear rate during the solubilization process. Other factors that may be involved are the limited rate at which substrate diffuses contrasted to the free diffusibility of enzyme and soluble products, the phenomenon of surface adsorption involving enzyme and/or products, either intermediate or final, the possibility of an enzyme excess or limited substrate surface area, and the possibility of a change in enzymatic specificity during solubilization due to the inherent nature of the enzymes involved and/or polysaccharide complexing. The involvement

Fig. 13. Demonstration of Schutz kinetics by plotting log v versus log t. The equation used is shown on p. 65. "Hydrocellulase" (S.A. = 11.0) was incubated with 4 mg of hydrocellulose, which gave an initial Klett value of 137.0. Each point is an average of duplicate determinations. Note that the axes are log scale.

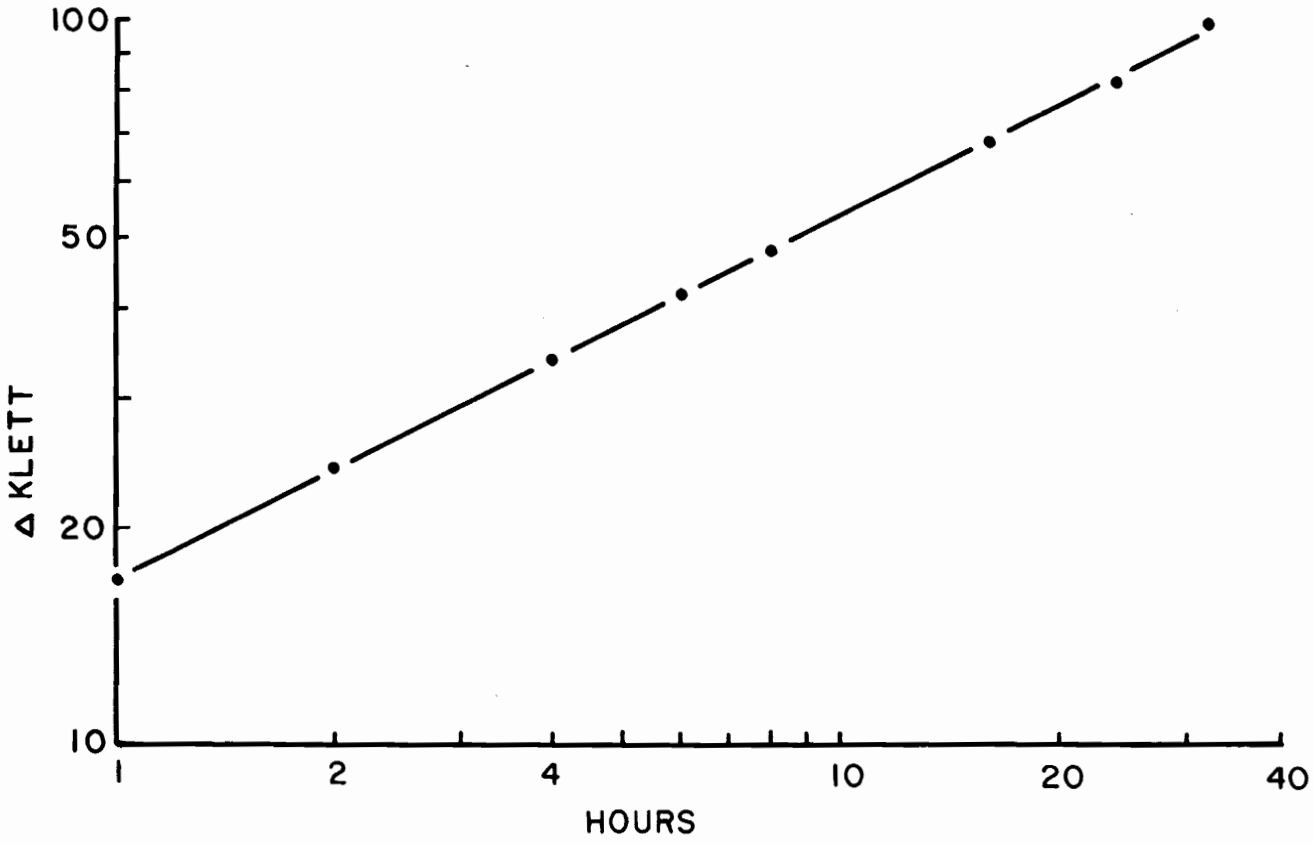
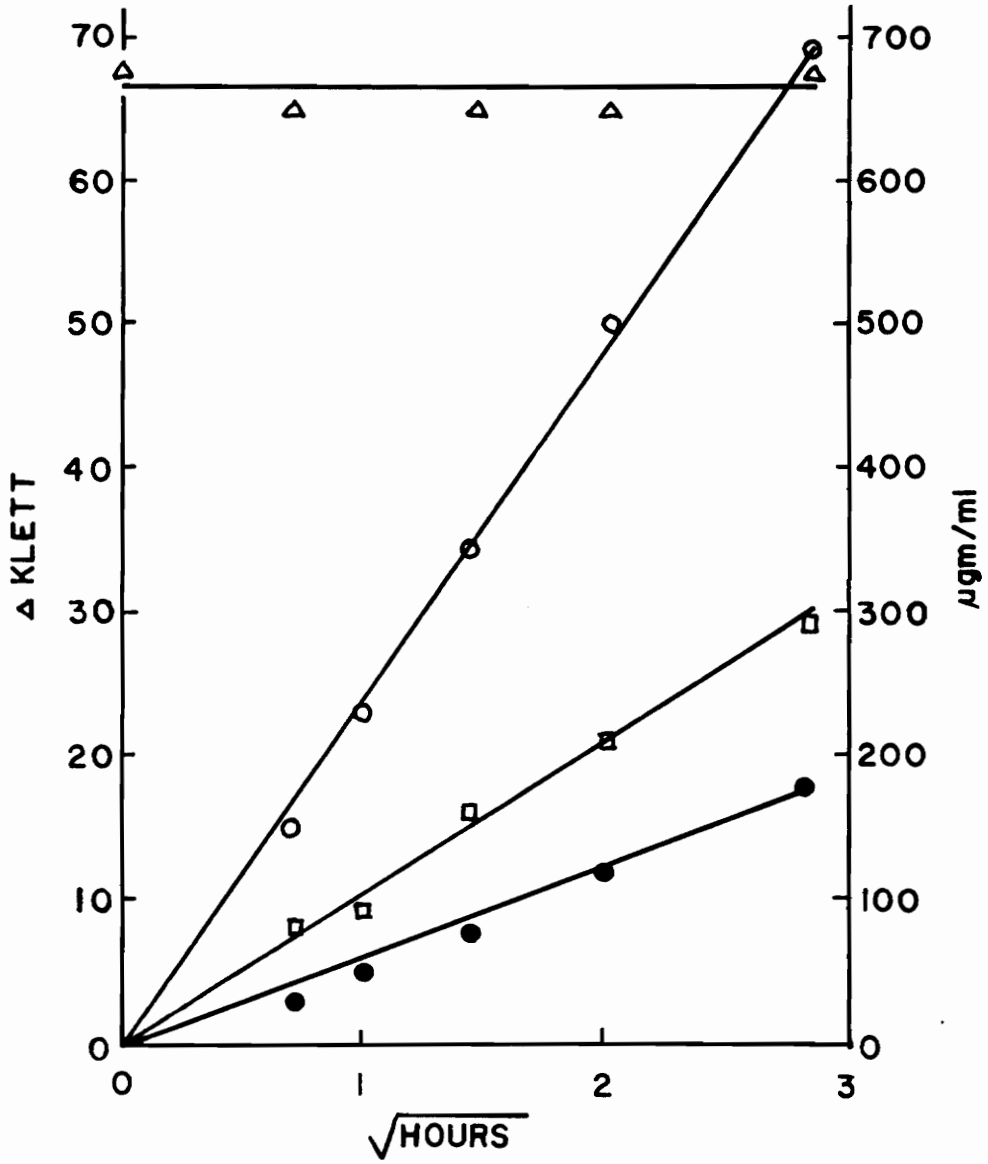

100
50
20
10
Δ KLETT
1
2
4
10
20
40
HOURS

Fig. 14. Substrate-product proportionality in accordance with Schutz's Rule. Total carbohydrate (△) determined by the anthrone method is shown as μg/ml. Soluble carbohydrate (□) also determined by the anthrone method, and reducing sugars (●) are shown as μg/ml. Extent of solubilization (○) is shown as turbidity decrease (△ Klett). Analyses were made on aliquots taken from reaction mixtures at the time of the turbidmetric measurements. The data are plotted on a square-root of time basis to illustrate adherence to Schutz's Rule.


70
60
50
40
30
20
10
0
Δ KLETT
700
600
500
400
300
200
100
0
μgm/ml
0
1
2
3
√HOURS

of two different modes of attack, lateral and endwise on particulate substrate, and non-uniformity of particle size of the substrate initially and during substrate decay would also enter into consideration of solubilization rate. Also consideration should be given to substrate structural heterogeneity and the possible interference of non-cellulosic components present in the substrate material.

Schutz kinetics have been observed in a number of instances, primarily in relation to enzymes acting on insoluble or colloidal substrates. The observation that solubilization of insoluble, relatively crystalline cellulose adheres to or closely approximates Schutz kinetics has been experimentally demonstrated. In respect to the law of causality--that a given cause must always be followed by a given effect--this observation remains incomplete; the effect has been shown, the cause is unknown. Since the same enzyme activities which demonstrated Schutz kinetics when acting on insoluble cellulose have been observed to approximate either zero or first order rate constants when acting on soluble substrates (CMC and cellotriose) the phenomenon of Schutz kinetics should not be overlooked when considering the degradation of natural products in a heterogeneous reaction system (soluble and insoluble phases).

Enzyme Activities of the "Hydrocellulase" Complex and Enzymatic Synergism

The first acceptable although indirect evidence that "hydrocellulase" activity was not a single activity but was composed of several component activities was obtained by thermal inactivation of partially

purified "hydrocellulase." Treatment of "hydrocellulase" at 60° and 70° for extended periods gave different rates of inactivation when assayed with different substrates (Fig. 15-A and 15-B). The factor apparently responsible for hydrocellulose solubilization was most labile to the heat treatment, and the CMCase activity was most resistant. The activity yielding new reducing groups from CMC and the activity yielding glucose from cellotriose were of intermediate stability.

The first direct evidence of heterogeneity was obtained after resolving the "hydrocellulase" into several activities differing in substrate specificity by gel filtration (see, Purification of "Hydrocellulase"). It was found that shifts in the spectrum of substrates attacked occurred after heating aliquots of each fraction at 70°, again demonstrating the difference in thermal sensitivity of the various activities of the "hydrocellulase" complex (Fig. 10-B).

After partial resolution of the "hydrocellulase" complex by gel filtration, it was observed that hydrocellulose solubilization could be markedly stimulated by the addition of certain enzymatically active components which did not themselves solubilize hydrocellulose. The activities which stimulated hydrocellulose solubilization possessed potent CMCase activity, and/or potent β-glucosidase activity and, in general, had the ability to act on amorphous cellulose and cellulodextrins. These activities acted in a synergistic manner with, or coupled to, the action of the "hydrocellulase" factor to promote the solubilization of hydrocellulose. The "hydrocellulase" factor will refer to that activity of the "hydrocellulase" complex which upon partial resolution of the complex

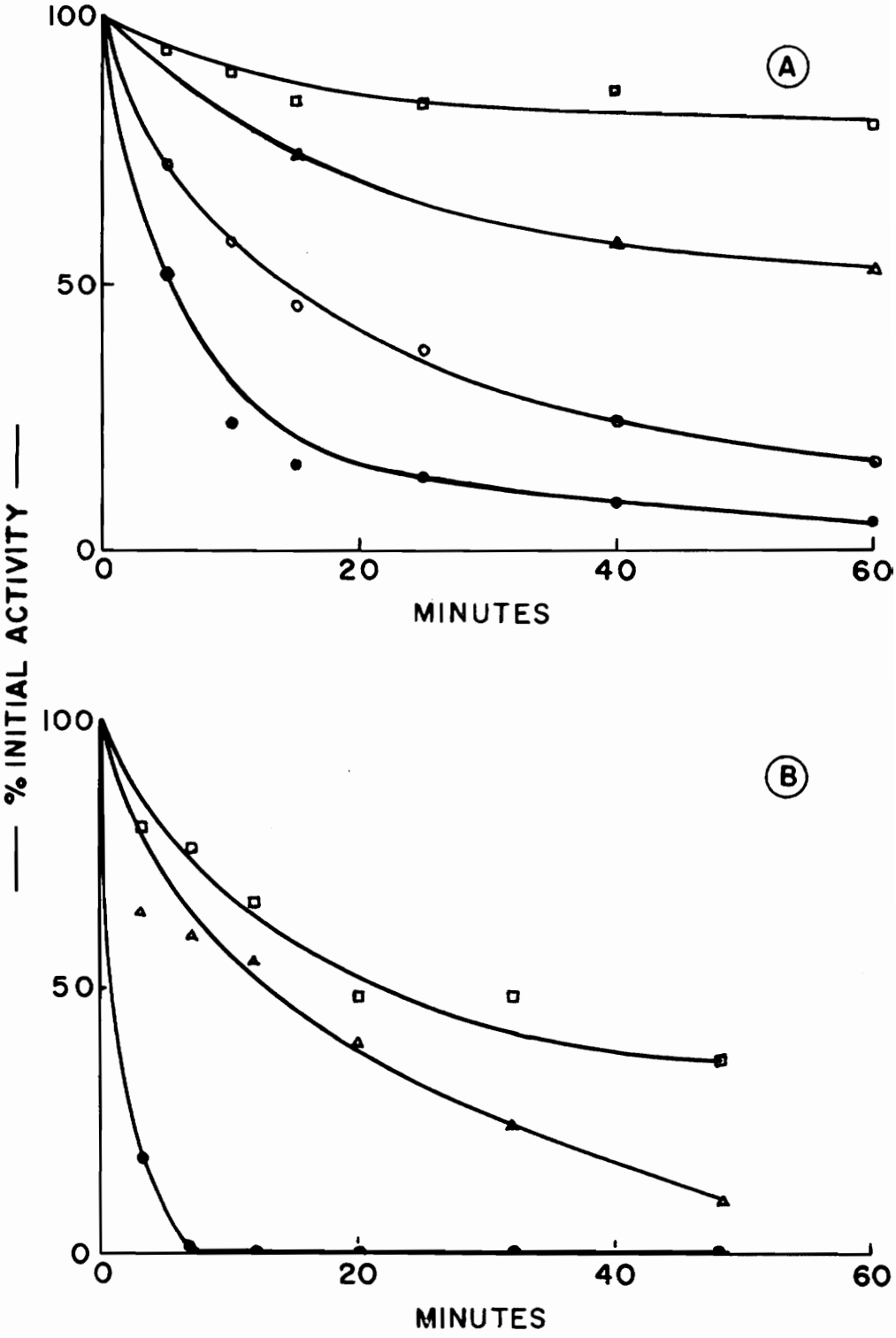
Fig. 15. Thermal inactivation of the "hydrocellulase" complex. "Hydrocellulase" (S.A. = 23.5) was subjected to temperatures of 60° (A) and 70° (B) using a constant temperature oil bath. Aliquots were taken for assay at the intervals indicated. Activity is expressed at per cent of the activity at 0 time, the time at which the stated temperature was attained. Activities were determined by the following assays:

CMC viscosity assay (▫)

CMC reducing sugar assay (▵)

Cellotriose assay (○)

Hydrocellulose assay (●)


100
50
0
0
20
40
60
MINUTES
A
— % INITIAL ACTIVITY —
100
50
0
0
20
40
60
MINUTES
B

retained the capacity to promote solubilization of hydrocellulose, was most closely associated with the bulk of the protein throughout purification, and which had relatively less capacity to act on CMC, amorphous cellulose, and cellotriose than other activities resolved from the complex. The latter activities will continue to be referred to as CMCase.

Enzymatic synergism was obtained when crude enzyme, freed of "hydrocellulase" by Avicel adsorption, but retaining strong CMCase and β-glucosidase activities, was added to the "hydrocellulase" factor, or when the CMCase resolved from the "hydrocellulase" by gel filtration (Fig. 10-A) was added back to the "hydrocellulase" factor. Partially heat inactivated "hydrocellulase" which still retained much of the CMCase activity also acted synergistically with the "hydrocellulase" factor. The maximum synergistic action obtained resulted in better than a three-fold stimulation of the "hydrocellulase" factor; however, extensive experimentation was not done to determine the optimal reconstitution of various activities nor the optimal ratios for reconstitution.

The results of an experiment, reconstituting several enzymatic activities with the "hydrocellulase" factor, are presented in Table 8. The CMCase resolved from "hydrocellulase" factor by gel filtration acted in a synergistic manner. Glucose was produced as the major soluble product only when the partially purified exo-β-1,4-glucan glucose hydrolase[1] was present and there was an apparent synergistic action. The soluble product analyses indicated that the CMCase and the exo-β-1,4-glucan glucose hydrolase had different modes of synergistic action. It should be noted that a partially purified endo-β-1,4-glucan hydrolase[1] did not

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[1] See footnotes, page 52.

Table 8

Reconstitution of Enzyme Fractions and Synergistic Action[1]

Enzyme Fractions				Observed	Soluble Products		
"Hydrocellulase"[2] Factor	CMCase[3]	Endo-Glucanase[4]	Exo-Glucanase[4]	"Hydrocellulase" Units	Soluble CH_2O	Reducing Sugars	Glucose
50 µl	--	--	--	5.1	84.5	42.0	2.5
50	50 µl	--	--	8.1	102.5	45.0	3.8
50	--	50 µl	--	5.4	90.5	42.0	5.5
50	--	--	25 µl	9.0	104.5	80.5	74.0
50	50	50	--	8.1	102.5	52.5	7.0
50	50	--	25	11.4	118.5	99.0	90.0
50	--	50	25	8.4	101.5	88.0	73.0
50	50	50	25	12.0	118.5	99.0	91.0
--	50	--	--	0.7	21.5	3.0	1.2
--	--	50	--	0	6.0	0	3.5
--	--	--	25	0	0	0	1.0
--	--	--	--	0	0	0	0

[1]The experiment was performed as typical hydrocellulose assays. Enzyme fractions were reconstituted in approximate proportion to their relative activities in the crude.
[2]Fraction no. 34 from gel filtration column (see Fig. 10-A).
[3]Pooled CMCase fractions resolved from "hydrocellulase" by gel filtration.
[4]See footnotes, p. 52.

cause significant stimulation of the "hydrocellulase" activity. Maximum synergism in this experiment was obtained when the CMCase and the exo-β-1,4-glucan glucose hydrolase were both present.

Based on the observations of this and the preceding section it is possible to formulate a sequence of hydrocellulose solubilization.

$$\text{Hydrocellulose} \xrightarrow[\text{("Hydrocellulase" factor)}]{\text{"Hydrocellulase"}} \text{"Reactive Cellulose"}$$

$$\text{"Reactive Cellulose"} \xrightarrow[\text{(CMCase activity)}]{\text{"Hydrocellulase"}} \text{Cellobiose}$$

$$\begin{matrix}\text{"Reactive Cellulose"} \\ \text{and/or} \\ \text{Cellobiose}\end{matrix} \xrightarrow{\text{exo-β-1,4-glucan glucose hydrolase}} \text{Glucose}$$

Extensively purified "hydrocellulase" action on hydrocellulose results in cellobiose as the main soluble product. If significant amounts of free cellulodextrins (of D.P. 3-5) are formed in the process, much more glucose would be expected than is observed. The action of the "hydrocellulase" factor can be stimulated by addition of enzyme components which are known to act hydrolytically. The presence of exo-β-1,4-glucan glucose hydrolase activity yields considerable amounts of glucose, but it is not known whether the exo-β-1,4-glucan glucose hydrolase is acting on the "reactive cellulose" or the cellobiose to yield glucose.

Since native cellulose and the crystalline form of native cellulose have been found to be most resistant to enzymatic hydrolysis, the degradation of hydrocellulose must be, at least in part, the result of an enzymatic activity capable of promoting the solubilization of crystalline substrates. From the data obtained there is good indication that more than one enzymatic activity is required for a maximal rate of degradation of

hydrocellulose. The demonstration that enzyme activities which are themselves inactive on hydrocellulose markedly stimulate the ability of the "hydrocellulase" factor to solubilize hydrocellulose supports this conclusion.

It has been observed that hydrocellulose solubilization can depend upon the activity of several enzymatically distinct components provided the "hydrocellulase" factor is present. Reese and coworkers have been among the leading advocators of a multi-enzymatic degradation of cellulose and according to their postulate, for which there is now good evidence (24), the C_1 factor converts native cellulose into a "reactive cellulose" which is subsequently acted upon by C_x enzymes to yield soluble products. It remains questionable as to whether C_1 action is hydrolytic or not, but its action is a prerequisite to the hydrolytic action of the C_x enzymes. Likewise, it is not known whether the action of the "hydrocellulase" factor studied here is hydrolytic. Perhaps it is not appropriate to equate the terminology of the enzyme activities used in this study to those used by Reese and coworkers, but it would appear that the "hydrocellulase" factor has much in common with the C_1 factor in the fact that it can promote the solubilization of hydrocellulose. The CMCase activity represents one or more C_x enzymes which act in a synergistic manner with the "hydrocellulase" factor.

Other Observations

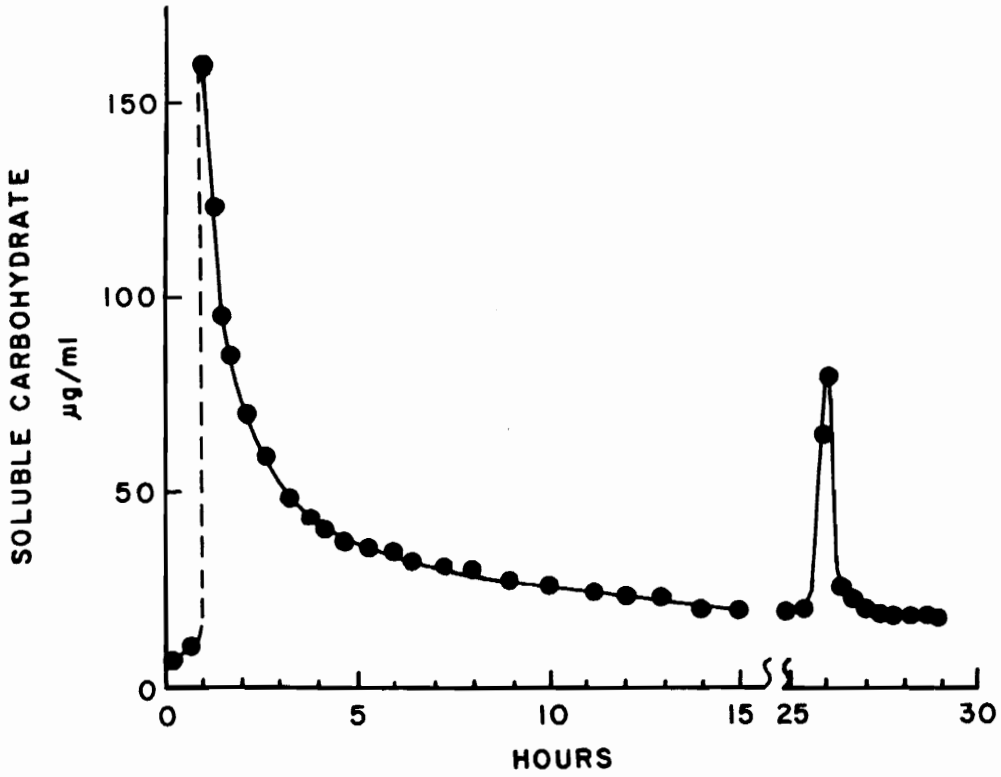
At several stages of purification protein (bovine serum albumin) was tested for a stimulatory effect on "hydrocellulase" activity.

No stimulation was observed when the protein was present at a level of 50 µg/ml in assay reaction mixtures. The presence of Cellase 1000 did not stimulate "hydrocellulase" activity when tested at several stages of purification; however, it was not tested for its effect as a synergistic component for the gel-filtered "hydrocellulase" factor preparation. Hemicellulase (Nutritional Biochemicals Corporation) had no stimulatory effect on "hydrocellulase" activity purified to the extent of adsorption chromatography on Avicel.

A small cellulose column, packed with 2.5 g of Avicel was equilibrated with 0.05 M acetate buffer, pH 4.7, and kept at a constant temperature of 39-40° by circulating water through an outer jacket. The column was washed well with buffer prior to placing 10 µl of "hydrocellulase" (S.A. 23.5; 300 units/ml) on the surface of the cellulose bed. Buffer washing continued and fractions were collected over an initial 15 hour period at a constant buffer flow rate of 10 ml/hour. Fractions were analyzed for total carbohydrate by the phenol- H_2SO_4 method, and the results are shown in Fig. 16. As was indicated earlier, Timell D.P. analysis of soluble products in fractions from this column gave D.P. values of 2.08 ± 0.11.

The amount of soluble product eluted from the column steadily decreased, and it was observed that the rate of soluble product elution approximated well the exponential behavior expected according to Schutz's Rule. A log-log plot of soluble carbohydrate produced versus time gave an approximately linear relationship. These data tend to rule out the likelihood that product accumulation or insufficient substrate causes

Fig. 16. "Hydrocellulase" degradation of a cellulose column. Details of this experiment appear in the text, p. 75.


SOLUBLE CARBOHYDRATE
µg/ml
150
100
50
0
0
5
10
15
25
30
HOURS

Schutz kinetics. When the buffer washing was suspended for 10 hours and then restarted, a large quantity of soluble product was rapidly eluted; however, the rate of soluble product elution appeared to continue at about the same rate as that observed prior to stopping the column. The peak of soluble carbohydrate recovered after resuming elution represented the zone of column liquid surrounding the adsorbed enzyme during the period of stagnation.

Selby's work with culture filtrates of Myrothecium verrucaria is degrading cotton led him to postulate the existence of an "exhaustible" cellulase (47,48). He found that the activity responsible for the loss in weight of cotton (A-enzyme) was labile and must be repeatedly replenished for extensive degradation to occur. The activity towards swollen or soluble cellulosic substrates (B-enzyme) is distinct from the labile activity. Since A-enzyme appears in the filtrates of growing cultures before B-enzyme he suggested that B-enzyme may represent partially inactivated A-enzyme--in other words that A is a precursor of B. Mandels and Reese (24) working with T. viride culture filtrates have found that frequent changes of enzyme solution were unnecessary for extensive weight loss of dewaxed cotton.

Two experiments were performed to test whether "hydrocellulase" while acting on its substrate lost the ability for continued attack on hydrocellulose (i.e. changed its substrate specificity). In the first experiment three cycles of degradation with substrate renewal were performed over a 21 hour period. No loss of "hydrocellulase" activity was found other than that attributable to thermal losses and cellobiose

accumulation (end-product inhibition) as determined by the appropriate controls. In the second experiment, after nearly complete solubilization of a hydrocellulose sample, no increase in the CMCase activity was found. Consequently, it has been concluded that Selby's hypothesis of an "exhaustible" enzyme is not pertinent to the system of T. viride.

An interesting observation was made when deuterium oxide (D_2O) replaced normal water in reaction mixtures containing hydrocellulose and amorphous cellulose substrates. The experiment was so designed that better than 90% of the liquid was D_2O, buffered to the same pH as the water controls. A "hydrocellulase"-rich fraction and a CMCase-rich fraction from a Sephadex G-75 column were compared for their ability to solubilize hydrocellulose and amorphous cellulose respectively in the presence and absence of D_2O. The results are presented in Table 9. "Hydrocellulase" activity was only slightly affected by the presence of D_2O, but there was a pronounced effect on the CMCase activity. The most conservative interpretation of these results would be that different factors are involved in the solubilization of the substrates.

Comments Regarding the Enzymatic Degradation of Cellulose

There are apparently misconceptions and notions of questionable validity, some more widely proclaimed than others, regarding certain aspects of the enzymatic degradation of cellulose. A large part of the vagueness of the current knowledge has arisen through the ill-defined use of the terms cellulose and "cellulase," but it is also true that some of the current concepts are based upon no more than extrapolation of the implications of very limited experimental data. Too often an insignificant

Table 9

Effect of Substitution of D_2O for H_2O on Cellulose Solubilization

Substrate	Hydrocellulose		Amorphous Cellulose	
Enzymes[1]	"Hydrocellulase"		CMCase	
Time (minutes)	Δ Klett (ave.)	Ratio $\frac{H_2O}{D_2O}$	Δ Klett (ave.)	Ratio $\frac{H_2O}{D_2O}$
30_{H_2O}	17.5	1.09	18.0	1.38
30_{D_2O}	16.0		13.0	
60_{H_2O}	24.0	1.09	27.0	1.39
60_{D_2O}	22.0		19.5	
120_{H_2O}	33.0	1.08	41.5	1.45
120_{D_2O}	30.5		28.5	
180_{H_2O}	46.0	1.12	64.0	1.36
180_{D_2O}	41.0		47.0	
Average $\frac{H_2O}{D_2O}$		1.095		1.395

[1] Enzymes were fraction numbers 35 and 41 from a gel filtration column (see Fig. 10-A), rich in "hydrocellulase" and CMCase activity respectively.

solubilization of a natural cellulose (i.e. a pure cellulose material retaining its native crystalline character) has been interpreted as evidence of a cellulose decomposing enzyme system. The following comments are made in relation to the results, observations, and indications of this study.

The term "cellulase" has been applied to some crude commercial enzymes and to some "pure" enzyme preparations which have the demonstrated ability to hydrolyze β-1,4 glucan bonds of CMC and certain highly amorphous substrates (swollen cellulose, reprecipitated cellulose, and filter paper). It has been inferred that such "cellulases" will also solubilize native cellulose which, for the most part, consists of a dense lattice type I crystalline structure. The fact remains that significant solubilization of native cellulosic materials by "cellulases" of the type referred to above has not been demonstrated and cannot be in most instances.

Cellulose can be considered as having the empirical formula $(C_6H_{10}O_5)_x$ and on complete hydrolysis will yield x glucoses. In considering a single anhydroglucose chain the only bonds present between the monomeric glucose units are of the covalent β-1,4 glucosidic type. But cellulose occurs naturally as a supermolecular material consisting of many individual anhydroglucose chains. The very nature of these chains (linear, planer) favors an orientation with respect to each other through interaction by the mechanisms of complementarity and self-recognition. In crystalline regions this orientation is rigid and fixed as the result of numerous so-called "weak bonds" (hydrogen bonds,

van der Waals forces, etc.). The cumulative effect of these "weak bonds" is quite great, so great that it requires chemical treatments more harsh than many biological materials will tolerate to disrupt them. All too often these "weak bonds" and the role they play in cellulose structure have been neglected. The "weak bonds" of cellulose are in fact just as much a part of the structure of cellulose as the covalent bonds.

Cellulose has bonds that individual anhydroglucose chains and highly hydrated amorphous chains do not have. The solubilization of anhydroglucose chains involves a hydrolytic attack at only one type of bond. But what about the solubilization and hydrolysis of crystalline cellulose? What disrupts the "weak bonds" of cellulose? Does a non-hydrolytic enzyme act in a manner to break hydrogen bonds or do hydrolytic enzymes wait around until the cellulose mysteriously falls apart into anhydroglucose chains before they act? More likely, it would appear that certain enzymes have the biological specificity that will permit them to act at the surface of crystallite cellulose, perhaps in a manner to chip out small fragments or form new chain ends, thus allowing thermal motions to break a few "weak bonds," and the resulting fragment or loose chain end diffuses away from the crystal surface becoming hydrated in the process.

Native crystalline cellulose can be appreciably solubilized if subjected to the action of the proper enzymes. It has been indicated that modified insoluble celluloses (alkali and acid swollen, and reprecipitated) are more or less crystalline, but from the standpoint of enzymatic

degradation it is the amorphous "hydrated" condition of such substrates that makes them readily susceptible to attack. Obviously cellulose derivatives in solution have little crystalline character. It is a general opinion that accessibility of a given cellulosic substrate and therefore its intrinsic susceptibility to enzymatic degradation is on the order of soluble cellulodextrins > soluble cellulose derivatives (CMC) > reprecipitated cellulose sol > swollen cellulose >> native cellulose or hydrocellulose. The fallacy of this concept can perhaps best be illustrated by considering the partial resolution of the potent Cellulase Meiji crude enzyme system. There was an enzyme activity ("hydrocellulase" complex) which was relatively much more active on crystalline cellulose than amorphous cellulose, another enzyme activity which was active on amorphous but not crystalline substrate, and a third enzyme activity which was more active on cellulodextrins than either of the above. The fact that the relative activity toward crystalline substrate can be considerably enriched over the relative activity toward amorphous substrate is difficult to compromise with the accessibility-enzyme susceptibility concept. There is evidence that at least one C_x enzyme, which has a readily demonstrable activity toward a reprecipitated cellulose, has negligible activity toward hydrocellulose (22). Also it has been indicated that some purified enzymes and the cellulolytic enzyme systems of some organisms which do demonstrate the accessibility-enzyme susceptibility relationship do not have the capacity to cause significant degradation of native cellulose. It is reasonable to believe that the rate of hydrolysis of cellulosic materials can be related to their

"accessibility" (degree of amorphous character or degree of hydration), but it seems unreasonable to conclude that native crystalline cellulose can be effectively solubilized by the same enzymes that act on amorphous and soluble cellulose forms in light of the fact that some enzymes are only active on amorphous substrates.

A major distinction between cellulolytic enzymes appears to lie in their ability to handle the crystalline substrates as opposed to the amorphous substrates. The notion that it is necessary to modify cellulose from its native form in order to observe an appreciable enzymatic reaction is unfounded and in many respects unsound. Any time that cellulose is modified in order to obtain an appreciable reaction rate for assay it modifies the assay in a manner such that there is a preferential selection for those activities handling the modified substrate most effectively. Is it little wonder that the use of modified cellulose substrates has frequently led to the study of enzyme preparations which are incapable of causing significant cellulose degradation?

Cellulose is inaccessible only in the sense that enzymes cannot penetrate the crystalline matrix. Thus the whole concept of accessibility becomes meaningless when considering the degradation of native cellulose. The predominant form of native cellulose is the lattice type I crystalline structure. True, the amorphous regions may vary somewhat in accessibility, but the fact that amorphous regions by their very nature are more accessible than crystalline regions makes their degradation even less significant. Crystalline cellulose must be and is degraded in nature. Any enzyme system that cannot solubilize the lattice type I crystalline

cellulose is inappropriately referred to as a "cellulase." Perhaps a better name for such preparations would be "amorphocellulohydrolase."

The intent here has not been to belittle the importance of enzymes attacking amorphous cellulose, for no doubt they play a role, probably an important role, in degrading cellulose. The very existence of some organisms may depend on such enzymes. Rather the objective of the preceding discussion was to emphasize that continued use of soluble and amorphous substrates as in the past will only delay recognition and understanding of the most challenging aspects of the overall process of cellulose degradation, namely the attack on crystalline celluloses.

A second point is significant with regard to the mode of cellulase action. If the anhydroglucose chains of cellulose can be considered to have a $\overline{D.P.}$ of 3000 and the crystalline micelles a $\overline{D.P.}$ of 200, then it is reasonable to assume that a single anhydroglucose chain must pass through a number of crystalline and amorphous regions of a microfibril.

To make a very simple analogy let us consider that a passenger train represents a cellulose microfibril. Let us call the coaches crystalline micelles, and the walk-through coupling areas amorphous regions since they are flexible but are necessary to join the coaches. The coaches represent by far the bulk of the train, but it is the coupling areas that preserve the unity of the train. Now assuming that an enzyme can attack and remove coupling areas, it could randomly attack at any number of specifically defined points, the final result being that all

the coaches are separated from one another. The original length of the train has been reduced to the length of the individual coach units. Applying the analogy to cellulose, an enzyme which removes the amorphous regions can readily lower the $\overline{D.P.}$ from 3000 to 200. Many reports have indicated that a drastic reduction in $\overline{D.P.}$ has been accompanied by only small losses in weight, but significant loss in tensile strength. Returning to the train, remove the couplings and the train loses its integrity, but an appreciable loss in weight has not been effected. Likewise, remove the amorphous regions of a fiber and the fiber falls apart, but not much weight is lost. Since the attack may occur at any of the points where there is amorphous material the decrease in D.P. may appear to be the result of a random cleavage, and so it may well be within amorphous regions. Should one infer, however, that cellulose degradation occurs by a completely random cleavage in the absence of evidence that the degradation of crystalline regions (the bulk of cellulose) takes place by random cleavage? Why have some studies shown that the D.P. falls to that expected for crystalline micelles and the weight loss is small? Could it be that only amorphous regions are being attacked? Why is there often a good correlation between weight loss and the amorphous content of cellulose subjected to the action of "cellulase"?

A third point is significant with regard to the multiplicity of cellulolytic enzymes. A notion among some cellulase investigators concerns the idea that the observed physical and enzymatic heterogeneity of the cellulase activity from a single culture may represent a variety of carbohydrate complexes involving a single protein base. To believe

that anything so vague as polysaccharide complexing can account for a multiplicity of cellulolytic activities of a single enzyme is absurd in light of the fact that cellulolytic activities from a single organism have considerably different amino acid composition, different sedimentation constants, different affinities for cellulose, different pH optima, different relative activity towards different substrates and so on. . . Would it not be just as reasonable to say that the different manifestations of activity are the result of different metallic ion complexing instead of polysaccharide complexing? It has been demonstrated that presence or removal of the strongly bound calcium ions of α-amylase exerts a considerable influence over its catalytic activity, but does not change its mode of action. Likewise, the partial proteolysis of a single enzyme could hardly account for differences in substrate specificity and mode of action observed for the enzyme components of a potent cellulolytic enzyme system.

V. SUMMARY

A crude cellulolytic enzyme preparation derived from Trichoderma viride was capable of solubilizing native crystalline forms of cellulose. Enzyme activity ("hydrocellulase") was determined by measuring the decrease in turbidity of the assay reaction mixture which contained a suspended hydrocellulose substrate.

Preliminary studies of "hydrocellulase" showed that under assay conditions maximum activity was obtained at pH 4.7 to 4.8 and at 40° for 3 hours. The activity was relatively stable for a three hour period between pH 4.0 and 7.0 and at temperatures up to 40°. Cellobiose was several times more inhibitory than glucose. Methylcellulose was very inhibitory. Sulfhydryl compounds stimulated activity of the crude preparation. EDTA was without effect.

"Hydrocellulase" activity could be removed from solution by adsorption to hydrocellulose. The activity was stable to $(NH_4)_2SO_4$, ethanol and acetone precipitation treatments, and to freezing.

Purification of "hydrocellulase" was obtained by the following procedures, a selective adsorption on Avicel columns and elution with water, then ion-exchange chromatography on a carboxylic acid resin. "Hydrocellulase" and some CMCase activity were observed to migrate together. There appeared to be protein homogeneity in the ultracentrifuge. Upon purification of "hydrocellulase," contaminating activities such as aryl-β-glucosidase and amylase were extensively removed. The stimulatory effect of sulfhydryl compounds was diminished. Methylcellulose, CMC, and cellobiose were inhibitory.

Evidence for heterogeneity of "hydrocellulase" was obtained by partial thermal inactivation and gel filtration. Resolution by gel filtration yielded components which acted synergistically. Additional purification was obtained by gel filtration.

Glucose was the main soluble product resulting from the action of crude enzyme, but as purification proceeded glucose production decreased to a small percentage of the total soluble product. Cellobiose constituted the bulk of the soluble product, as determined by paper chromatography and D.P. analysis. Partially purified "hydrocellulase" produced glucose in considerable amounts from cellulodextrins, but only slight amounts of glucose arose from the enzyme action on hydrocellulose and cellobiose.

Partially purified "hydrocellulase" was capable of completely solubilizing hydrocellulose and causing extensive weight loss of native fibrous celluloses. The partially purified enzyme compared to the crude was more enriched in activity towards crystalline substrate than towards amorphous substrate.

The kinetics of insoluble cellulose degradation appeared to follow a square-root relationship often referred to as Schutz's Rule.

Thermal inactivation of "hydrocellulase" activity gave different rates of inactivation for enzymatic activity toward several substrates. Partial inactivation of fractions from gel filtration gave shifts in the spectrum of substrates attacked.

Recombination of components which acted synergistically gave a three-fold stimulation of "hydrocellulase" activity. Activities present in the crude enzyme and one of the activities partially

purified by another procedure from the crude acted synergistically. Other activities did not act synergistically. The presence of some synergistic components yielded considerable amounts of glucose from hydrocellulose. It was observed that hydrocellulose solubilization could depend upon the activities of several enzymatically distinct components. The action of "hydrocellulase" is not known, but it is markedly stimulated by the presence of known hydrolytic enzymes.

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VIII. VITA

Robert Montgomery Flora was born October 1, 1938, in Richmond, Virginia, the second of three children of Jacob P. and Ora M. Flora.

His elementary and secondary education was obtained in the public schools of Henrico County, Virginia, and he received the high school diploma from Highland Springs High School in 1956. He entered Bridgewater College, Bridgewater, Virginia and received the B.A. degree cum laude in 1960, with General Science as his field of major concentration. Graduate work was begun in the Department of Biochemistry, Western Reserve University, Cleveland, Ohio, during the school year 1960-1961. He then moved to the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia in the summer of 1961 to continue graduate work toward the Ph.D. degree as a research assistant and a National Science Foundation Cooperative Graduate Fellow.

He has been elected to membership in Sigma Xi and Phi Lambda Upsilon and is a member of the American Chemical Society.

Robert Flora

IX. APPENDIX

Hulcher and King (16) have described the isolation of an aerobic cellulolytic organism, Cellvibrio gilvus, which had a distinct preference for cellobiose over glucose. Prior to the work with the T. viride crude cellulase system reported in this study it was observed that lyophilized culture filtrates and fresh culture supernatants of C. gilvus grown on media containing cellulose were found to have the ability of solubilizing hydrocellulose at a rate discernable by the turbidmetric method. The following information was obtained relating to the solubilization of hydrocellulose by the cell-free enzyme system of this organism. The pH optimum was found to be at 5.8, phosphate buffer, and the activity was relatively stable over the pH range 4.6 to 8.8 during a four hour period. Maximum activity was obtained at 40° during a three hour assay, and activity was found to be stable at 40° for a three hour period. The hydrocellulose solubilizing activity could be removed from enzyme solutions by cellulose adsorption. In contrast to the Trichoderma system cellobiose was found to occur in a greater amount than glucose in the soluble products of a hydrocellulose reaction mixture, concentrated, and subjected to paper chromatography. Cellobiose present at 2% level in reaction mixtures was extremely inhibitory.

The following comments are made in regard to culturing C. gilvus for the purpose of obtaining the hydrocellulose solubilizing activity. There was less activity toward hydrocellulose when cultures were grown on powdered filter paper cellulose (i.e. it is better to use a highly crystalline

ABSTRACT

A crude enzyme preparation derived from Trichoderma viride was studied in relation to its capacity to solubilize hydrocellulose. A hydrocellulose prepared from cotton linter was used as an assay substrate permitting the selective purification of the enzyme activity promoting the solubilization of the native crystalline form of cellulose. Enzyme activity ("hydrocellulase") was determined routinely by measuring the decrease in turbidity of reaction mixtures which contained the suspended hydrocellulose substrate.

The crude enzyme was studied in regard to factors affecting the activity. Among these were conditions of pH and temperature, and activators and inhibitors of "hydrocellulase" activity.

"Hydrocellulase" was purified by selective adsorption on columns of hydrocellulose, ion-exchange chromatography, and gel filtration. The purification procedures resulted in extensive removal of contaminating activities such as aryl-β-glucosidase and amylase which were present in the crude. On a specific activity basis "hydrocellulase" was purified better than 10-fold over the starting material which previously had been purified to an unknown extent by several procedures in the preparation of the crude enzyme. Some cellulolytic activities were more closely associated with the "hydrocellulase" than were others, and those activities which chromatographed with the "hydrocellulase" during cellulose adsorption and ion-exchange column steps comprised the "hydrocellulase" complex.

cellulosic material). Significant activity was not observed in culture supernatants until the majority of the cellulosic substrate had been solubilized. In general, much better yields of activity were obtained from small shake cultures than from bulk cultures using forced aeration. It was important that stock cultures of C. gilvus were well adapted to growth on cellulose in order to obtain good growth and enzyme yields from shake cultures. A medium of the following composition was found to be satisfactory for growth of C. gilvus in shake cultures:

	g/liter
KNO_3	1.2
KCl	0.5
$MgSO_4$	0.5
$CaCO_3$	trace
$Fe_2(SO_4)_3$	trace
Yeast extract	0.5
Casein hydrolyzate	2.0
Hydrocellulose	2.0

Buffered with 0.02 M sodium phosphate buffer to give a pH of 6.7.

Partially purified "hydrocellulase" was relatively more active on hydrocellulose substrate than on amorphous cellulose substrate.

The "hydrocellulase" complex appeared to have a good degree of protein homogeneity as demonstrated by ultracentrifuge studies. Partial thermal inactivation of the complex gave evidence of enzymatic heterogeneity from the rates of inactivation when assayed using several substrates. The "hydrocellulase" complex was partially resolved by the process of gel filtration into several enzymatically distinct components as determined by the assay of fractions using several substrates. Fractions containing the "hydrocellulase" factor were capable of promoting the solubilization of hydrocellulose. Other fractions having relatively more activity toward carboxymethylcellulose and amorphous cellulose had little ability to solubilize hydrocellulose, but were shown to act synergistically with the "hydrocellulase" factor in a manner to increase the apparent activity of the "hydrocellulase" factor. Of two partially purified components obtained from the same crude material, one, an exo-β-1,4-glucan glucose hydrolase, was demonstrated to act in a synergistic manner with the "hydrocellulase" factor yielding glucose as the main soluble product, the other, an endo-β-1,4-glucan hydrocellulase, did not act in a synergistic manner, and neither had the ability to act on hydrocellulose in the absence of the "hydrocellulase" factor.

Glucose was the predominant soluble product obtained from hydrocellulose when subjected to the action of the crude enzyme. Upon purification of the "hydrocellulase" activity glucose contributed

progressively less to the total soluble product obtained by enzyme solubilization. Cellobiose appeared to be the main soluble product resulting from "hydrocellulase" action as indicated by paper chromatography and degree of polymerization analysis of the soluble products. Although activities in the "hydrocellulase" complex readily yielded glucose from cellulodextrins but not from hydrocellulose, it was indicated that free cellulodextrins were not formed in significant amounts during the hydrocellulose solubilization process.

It was observed that the rate of insoluble cellulose degradation appeared to follow Schutz's Rule. Throughout purification, the "hydrocellulase" was capable of completely solubilizing assay quantities of hydrocellulose and causing significant solubilization of raw native cotton and purified cotton linter as demonstrated by weight losses of these materials in the range of fifty percent.

A proposed scheme for hydrocellulose degradation is given, and the degradative process is mentioned in relation to the C_1-C_x enzymatic cellulose degradation concept.