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A FUNDAMENTAL STUDY OF THE MECHANISM AND KINETICS OF CELLULOSE HYDROLYSIS BY ACIDS AND ENZYMES

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PREFACE

It is clear that massive quantities of solar energy is conserved on this planet in the forms of cellulose and hemicellulose. It equally obvious that fundamental understanding of cellulose structure and hydrolysis of cellulose is required to utilize and produce useful products. Research in our laboratory reflects the need for understanding of the cellulose hydrolysis processes by enzymes and chemicals. Attempts have been made to use microbial enzymes to hydrolyze cellulose to its constituent sugars (e.g., glucose). Efforts have also been made to study the fundamental problems related to the microbial biosynthesis of cellulase and its regulation in order to design methods to overcome the repression of cellulase biosynthesis to increase enzyme yields.

The final report contains the following significant contribution to our understanding of the structure of cellulose, the kinetics of cellulose hydrolysis by enzymes, and the regulatory control of cellulase biosynthesis.

Four significant area of research have been pursued:

A. Purification of cellulase components by a simple, convenient purification procedure.

B. Kinetics of cellulose hydrolysis by purified cellulase components.

C. Elucidation of the molecular structure of cellulose.

D. Effect of solvents and acids on the structure and reactivity of cellulose.

In addition, we have proposed a mode of cellulase biosynthesis regulation in cellulolytic microorganisms based on the data we have obtained as well as the existing information accumulated by other research laboratories. A description of a novel cellulase enzyme, cellobiosyltransferase, is presented in Section VIII.

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ABSTRACT

There are three basic enzymes [e. g., endoglucanase (C_x), exoglucanase (C_1) and cellobiase] comprising the majority of extracellular cellulase enzymes produced by the cellulolytic mycelial fungi, Trichoderma reesei, and other cellulolytic microorganisms. The enzymes exhibited different mode of actions in respect to the hydrolysis of cellulose and cellulose derived oligosaccharides. In combination, these enzymes complimented each other to hydrolyze cellulose to its basic constituent, glucose.

The kinetics of cellobiase were developed on the basis of applying the pseudo-steady state assumption to hydrolyze cellobiose to glucose. The results indicated that cellobiase was subjected to eng-product inhibition by glucose. The kinetic modeling of exoglucanase (C_1) with respect to cellodextrins was studied. Both glucose and cellobiose were found to be inhibitors of this enzyme with cellobiose being a stronger inhibitor than glucose. Similarly, endo-glucanase (C_x) is subject to end-product inhibition by glucose.

Crystallinity of the cellulose affects the rate of hydrolysis by cellulases. Hence, the changes in crystallinity of cellulose in relation to chemical pretreatment and enzyme hydrolysis was compared.

The study of cellulase biosynthesis resulted in the conclusion that exo- and endo-glucanases are co-induced while cellobiase is synthesized independent of the other two enzymes. The multiplcity of cellulase enzymes are the end results of post-translational modification during and/or after the secretion of enzymes into growth environment.

I. PURIFICATION OF CELLULASE

A. Introduction

Useful information has been accumulated for the study of enzyme hydrolysis of cellulose from various microorganism sources. The detailed study of cellulase function requires significant quantities of highly purified enzyme. A number of methods for cellulase purification have been published, some of these purification methods have been shown to yield enzymes free from measurable contaminating enzymes. Since many cellulase components act in a complimentary fashion toward each other in hydrolyzing cellulose, even a trace contamination of other enzymes could have significant effects on certain features of the specific enzyme action. Without a well defined technique for the purification of individual enzyme components and without an adequate recovery of enzymes, it is impossible to assess the precise function and the actual amount of a given cellulase enzyme component in a cellulase preparation. Often, the impurity of supposedly purified enzymes causes confusion as to the precise mode of action of specific enzymes, thus terms such as an exolike endocellulase, novel type endocellulase, different randomness of endocellulase, etc., are flourishing.

Traditionally, the purification techniques reported in literature include the use of amberlite ion-exchange resins, hydroxylapatite chromatography, Avicel and cellulose derivative columns, DEAE-Sephardex and other ion-exchange columns, gel-filtration and electrophoresis, or a combination of the above (for reviews see 1,2). Most of the procedures employed to purify some specific cellulase enzymes make use of the combination of ion-exchange chromatography and extensive isoelectric focusing techniques. These purification procedures

are rather involved and only very small amounts of enzymes can be employed at one time. The amberlite fractionation techniques are ill-defined, and cross contamination of enzymes is always present. This is probably due to the large quantities of starting materials employed cause over-loading of the column with proteins, thus leading to the incomplete separation of enzymes. Other fractionation methods, such as the use of hydroxylapatite or Avicel columns, are difficult to reproduce and the recovery of enzymes is extremely low due to the unspecific adsorption of proteins by the gels. Selby and Maitland (3), and Wood (2), using a combination of gel filtration and ion-exchange chromatography techniques, achieved the separation, not only of high molecular weight enzymes from low molecular weight enzymes, but also the separation of endocellulase from exocellulase. However, the amounts of enzymes which could be applied were very limited, and due to the sensitivity of Sephadex to a change of salt and pH conditions, it was difficult to separate various cellulase enzymes satisfactorily.

The three major enzymes in the "cellulase complex" have been identified as:

(1) endo-β-1,4-glucanase or β-1,4-glucano glucanohydrolase (EC. 3.2.1.4) or commonly known as CMCase or C_x cellulase.

(2) exo-β-1,4-glucanase or β-1,4-glucan cellobiohydrolase (EC. 3.2.1.91) or commonly known as Avicelase or C_1 cellulase.

(3) β-1,4-glucosidase (EC. 3.2.1.21).

For convenience, the names, endocellulase, cellobiohydrolase and cellobiase have often been used to refer to β-1,4-glucan glucanase, β-1,4-cellobiohydrolase and β-1,4-glucosidase, respectively. In some instances, the name, exocellulase

is also used. Exocellulase could include β-1,4-glucose hydrolase (glucohydrolase) and cellobiohydrolase. However, the existence of glucohydrolase in the cellulase complex is questionable, since the contamination of glucosidase could contribute to the hydrolysis of cellobiose to glucose.

The modes of action of the three major Trichoderma cellulase components have been studied extensively. These enzymes, which act both individually and together to break down cellulose and cellodextrins, appear to have a complex interdependence which has, to date, been only partially quantified. Many different cellulosic substrates as well as modes of action have been reported for these enzymes. While cellobiohydrolase produces cellobiose from cellulose through an end-wise cleavage of cellulose chains, endoglucanase appears to act randomly to hydrolyze cellulose. Cellobiase hydrolyzes cellobiose and perhaps other cellodextrins as well to give glucose. The picture is somewhat complicated by the inhibition of cellobiohydrolase by the produce cellobiose. While cellobiase relieves this inhibition by hydrolysis of cellobiose to glucose, cellobiase itself is inhibited by the product glucose.

B. Sequential Purification of Cellulases

The methods of purifying enzymes are always tedious and the recovery of enzymes is very low. We have developed special procedures to purify various cellulase components from young culture broth of T. reesei. The procedures used are simple, well-defined, and insure a maximum recovery of three major enzyme components.

DEAE-Sepharose and DEAE-cellulose were used exclusively to separate cellulase components by applying linear salt gradients to elute enzymes from an ion-exchange column. The sequential elution of cellulases made

it possible to separate cellulases cleanly and completely. The procedures used to purify cellulases by Gong et al. is outlined in Fig. I-1. Crude enzyme was first concentrated by lyophilization to reduce the volume of the culture filtrate. This was followed by a 75% saturation of ammonium sulfate to precipitate proteins, and the salts were removed by coarse Sephadex G-25 gel-filtration. The removal of salts is essential since the technique employed is the chromatography of the enzymes, endo-cellulase and cellobiase, under very low salt conditions.

The enzyme cellobiase and low-molecular-weight (LMW) endoglucanase were first eluted from DEAE-Sepharose column by water. These fractions were then subjected to DEAE-cellulose column chromatography. The linear salt elutions resulted in the separation of cellobiase and LMW endoglucanase. The LMW endoglucanase obtained from DEAE-cellulose chromatography is almost pure, with only a trace of contaminants (Figure I-2). Cellobiase can be purified further by subjecting it again to DEAE-cellulose chromatography, with enzyme being eluted out of the column by salt elution at low concentration.

High-molecular-weight (HMW) endoglucanase and cellobiohydrolase are retained on the DEAE-Sepharose after the cellobiase and LMW endoglucanase have been eluted with water. The fractionation of cellobiohydrolase and HMW endoglucanase is accomplished by sequential linear salt gradient elution using sodium phosphate for the HMW endoglucanase (Figure I-3) and sodium chloride for cellobiohydrolase (Figure I-5).

A total of four endoglucanases was obtained. Three fractions of endoglucanase activity eluted between 0-25 mM sodium phosphate and one fraction between 25-50 mM phosphate (Figure I-3). The first enzyme peak has the

major endoglucanase activity and is homogeneous as was indicated from SDS-gel electrophoresis (see Figure I-8d). The other three peaks having significant, but lower, endoglucanase activity showed molecular weights that were similar to that of the first peak, as indicated by chromatography of all four fractions on Sephadex G-75 (Figure I-4). The molecular weights obtained from the G-75 runs were all about 52,000. On the basis of these data, we concluded that T. reesei possesses only one major endoglucanase (Peak A in Figure I-3 and Figure I-4), while the other endoglucanases (peaks B, C, D) are post-translational modification products.

After the endoglucanase activity had been washed form the column, the cellobiohydrolase fraction was subsequently recovered. In this case however, sodium chloride, rather than sodium phosphate, was used to perform the linear salt gradient (0-200 mM NaCl). Only one cellobiohydrolase activity peak was obtained (see Figure I-5). Even higher salt concentrations (above 200 mM) failed to wash any other enzyme activity from the column. Thus it is clear that only one enzyme remains after the endoglucanase fractionation step, this single enzyme being cellobiohydrolase. The homogeneity of this enzyme is also verified by the SDS-gel picture shown in Figure I-8(g). The homogeneity of the enzyme is also verified by gel filtration on Sephadex G-100 (Figure I-6) and also indicated by SDS-gel electrophoresis.

Gel filtration of crude cellulase on Sephadex G-75 gave one cellobiase, a major HMW endoglucanase, a LMW endoglucanase, and one cellobiohydrolase (Figure I-7). The purified enzyme and the crude enzyme protein was analyzed by SDS-gel electrophoresis and resulted in the gels shown in Figure I-9.

The product specificity of purified cellobiohydrolase and endoglucanase after incubating enzymes with Avicel was analyzed by liquid chromatography. The chromatograms are Figure I-9 and I-10.

For the purification of large quantities of the cellobiohydrolase, a one-step purification technique can be easily applied. The crude cellulase preparation is applied to a DEAE-Sepharose column and all the endocellulase and cellobiase enzyme are removed from the column by washing the column with 0.07 M sodium phosphate. Cellobiohydrolase enzyme then can be eluted out of column by a proper concentration of a linear salt gradient. With this purification technique cellulase proteins can be recovered without appreciable loss of enzymes since the manipulations of proteins are minimal. More than a few hundred milligrams of physically pure cellobiohydrolase enzymes can be obtained by using as little as 30 ml of DEAE-Sepharose as an ion-exchange resin.

With this sequential purification method, it is possible to measure the amounts of various cellulase components quantitatively. This is an important achievement which other purification methods have not been able to attend. It is not known whether this purification method can be applied to cellulase enzymes from organisms other than T. reesei. However, we have used this method to purify cellulases from Thermoactinomyces with equally satisfactory results (unpublished observation).

Figure Captions

Fig. I-1 Summary of fractionation and purification scheme for cellulases.

Fig. I-2 Low molecular weight (LMW) endoglucanase from Sephadex G-75 column chromatography.

(-) protein (measured at $A_{280\ nm}$)
(-x-) reducing sugar formed from CMC

Fig. I-3 DEAE Sepharose column chromatography of high molecular weight (HMW) endoglucanase

(A) Peak having major endoglucanase activity
(B), (C), (D) Peaks having lower amount of endoglucanase activity.

Fig. I-4 Sephadex G-75 column chromatography of HMW endoglucanase (A), (B), (C), and (D) fractions shown in Fig. I-3.

(-O-) Relative Protein Concentration
(-X-) Relative activity with respect of CMC

Fig. I-5 DEAE Sepharose column chromatography of cellobiohydrolase.

(-O-) Relative Protein Concentration
(-●-) Relative activity with respect to Avicel

Fig. I-6 Sephades G-100 gel filtration of cellobiohydrolase.

(-O-) protein (A_{280nm})
(-●-) activity with respect to avicel.

Fig. I-7 Sephadex G-75 column chromatography of crude cellulases.

(-O-) protein (A_{280nm})
(-X-) activity with respect to CMC (endoglucanase)
(-●-) activity with respect to Avicel (cellobiohydrolase)

Inset, top right:
(-O-) cellobiase activity

Fig. I-8 SDS-gel electrophoresis of purified cellulases from T. viride grown in Avicel and lactose.

(a) crude cellulases (Avicel as growth medium)
(b) crude cellulases (Lactose as growth medium)
(c) purified LMW endoglucanase (Avicel)
(d) purified HMW endoglucanase (Avicel)
(e) purified HMW endoglucanase (Lactose)
(f) mixture of (d) and (e)
(g) purified cellobiohydrolase (Avicel)
(h) purified cellobiohydrolase (Lactose)
(i) mixture of (g) and (h)
(j) purified cellobiase

Fig. I-9 Liquid chromatogram of hydrolysate from action of cellobiohydrolase on Avicel.

Fig. I-10 Liquid chromatogram of hydrolysate from action of endoglucanase on Avicel.

Fractionation and Purification of Cellulases

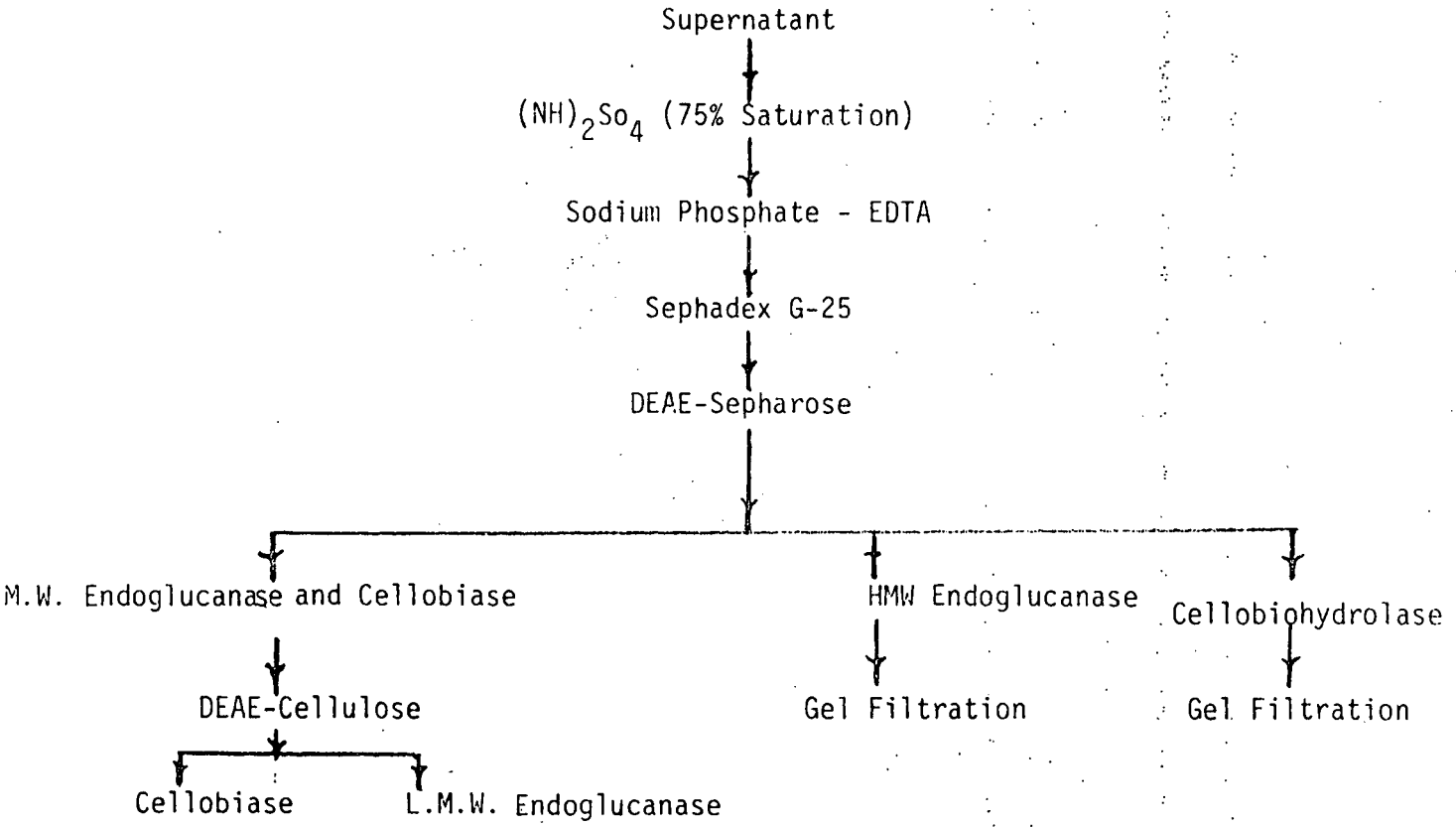

Figure I-1

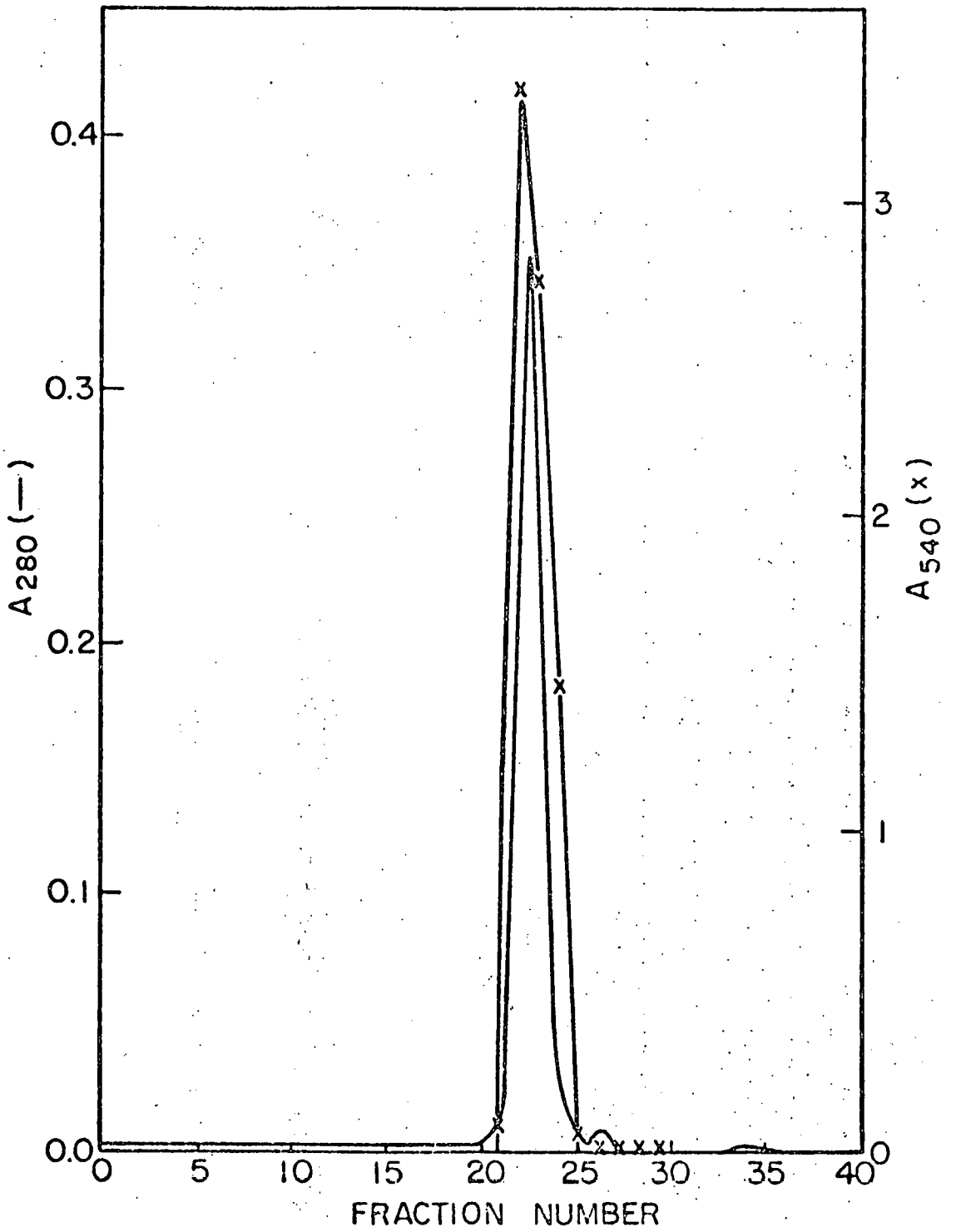
0.4
0.3
0.2
0.1
0.0
A280 (—)
3
2
1
0
A540 (x)
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure I-2

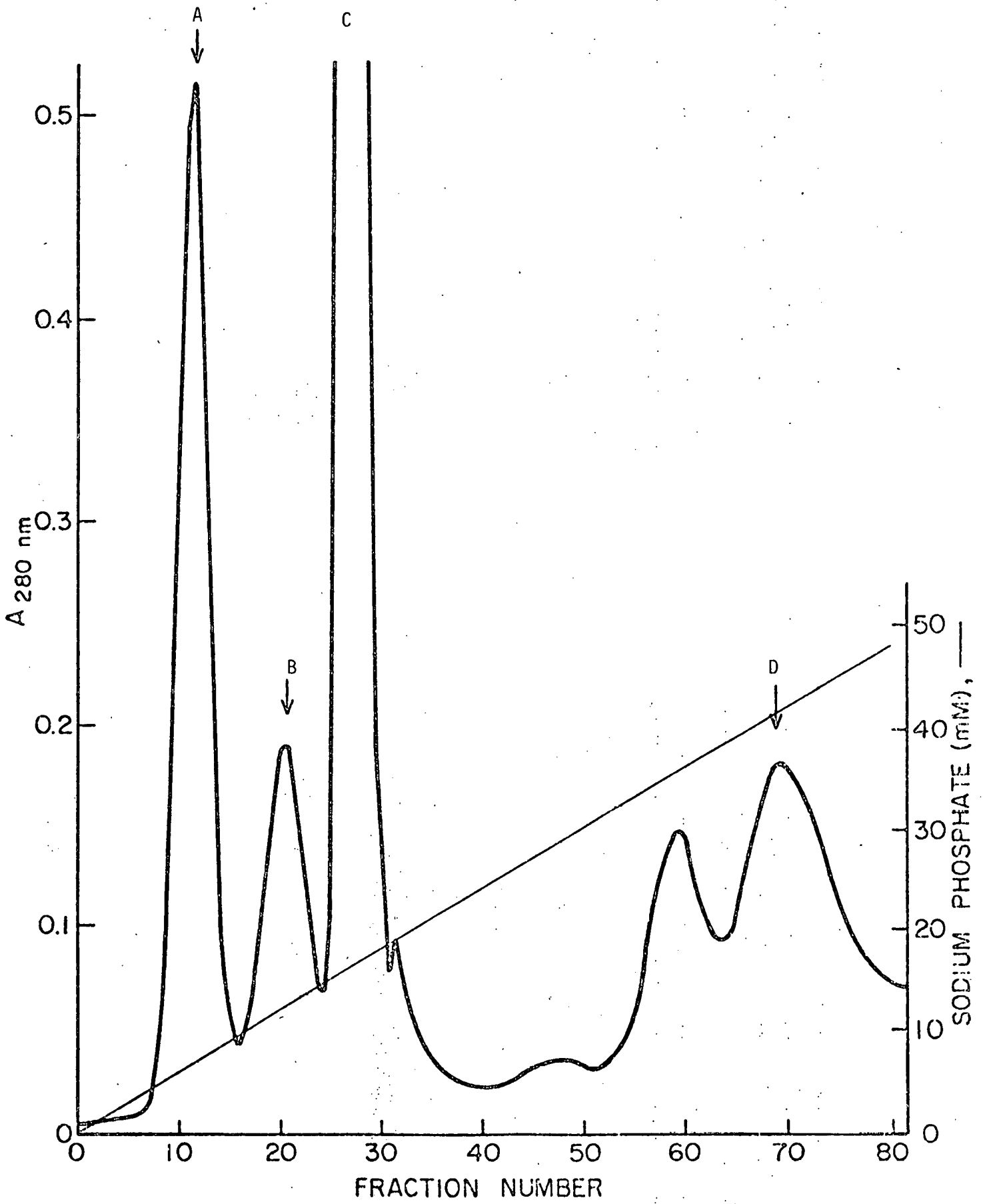
A
C
B
D
0.5
0.4
0.3
0.2
0.1
0
A 280 nm
0
10
20
30
40
50
60
70
80
FRACTION NUMBER
50
40
30
20
10
0
SODIUM PHOSPHATE (mM), —

Figure I-3

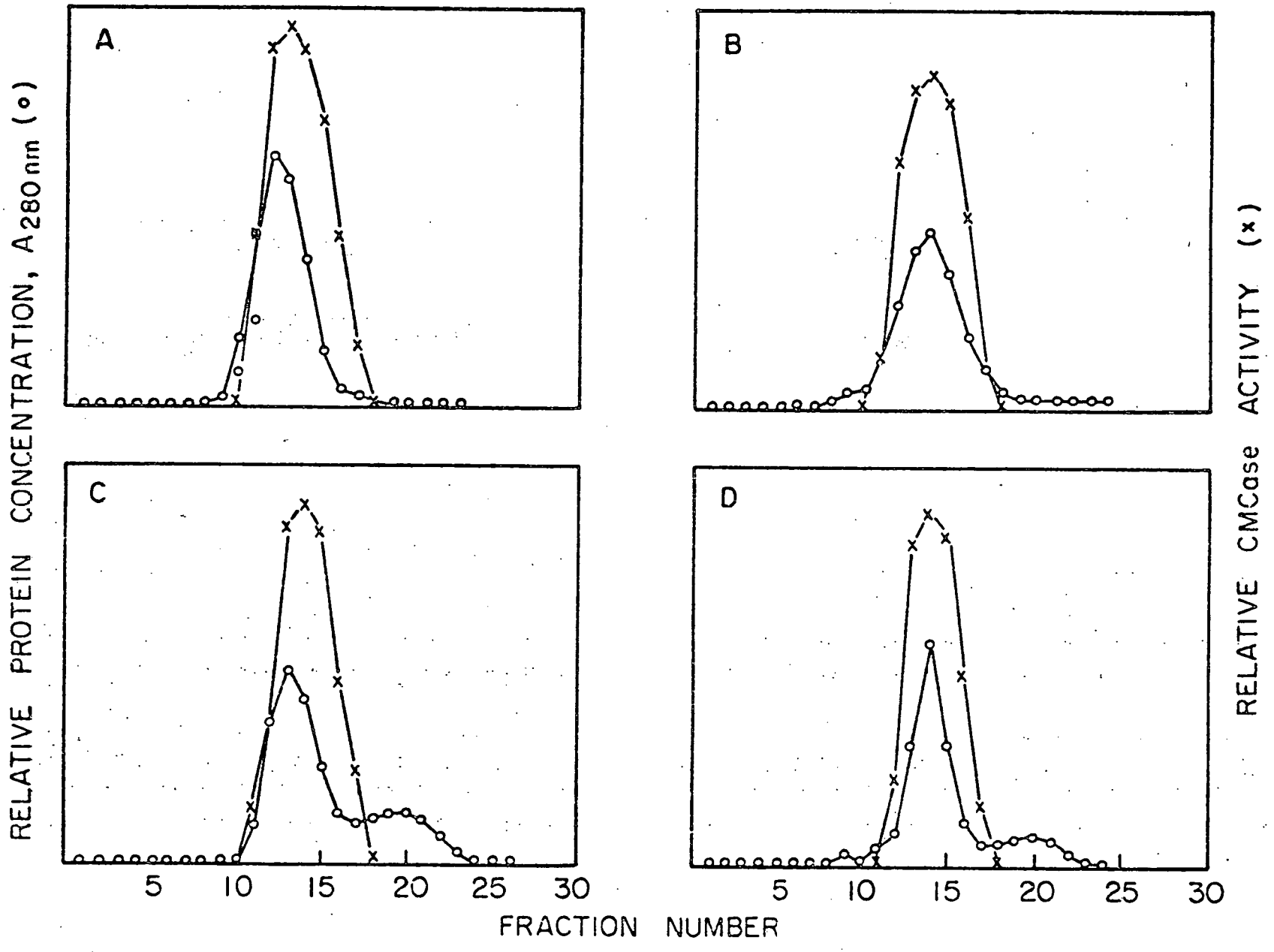
A
B
C
D
RELATIVE PROTEIN CONCENTRATION, $A_{280\,nm}$ (o)
RELATIVE CMCase ACTIVITY (x)
5
10
15
20
25
30
FRACTION NUMBER

Figure I-4

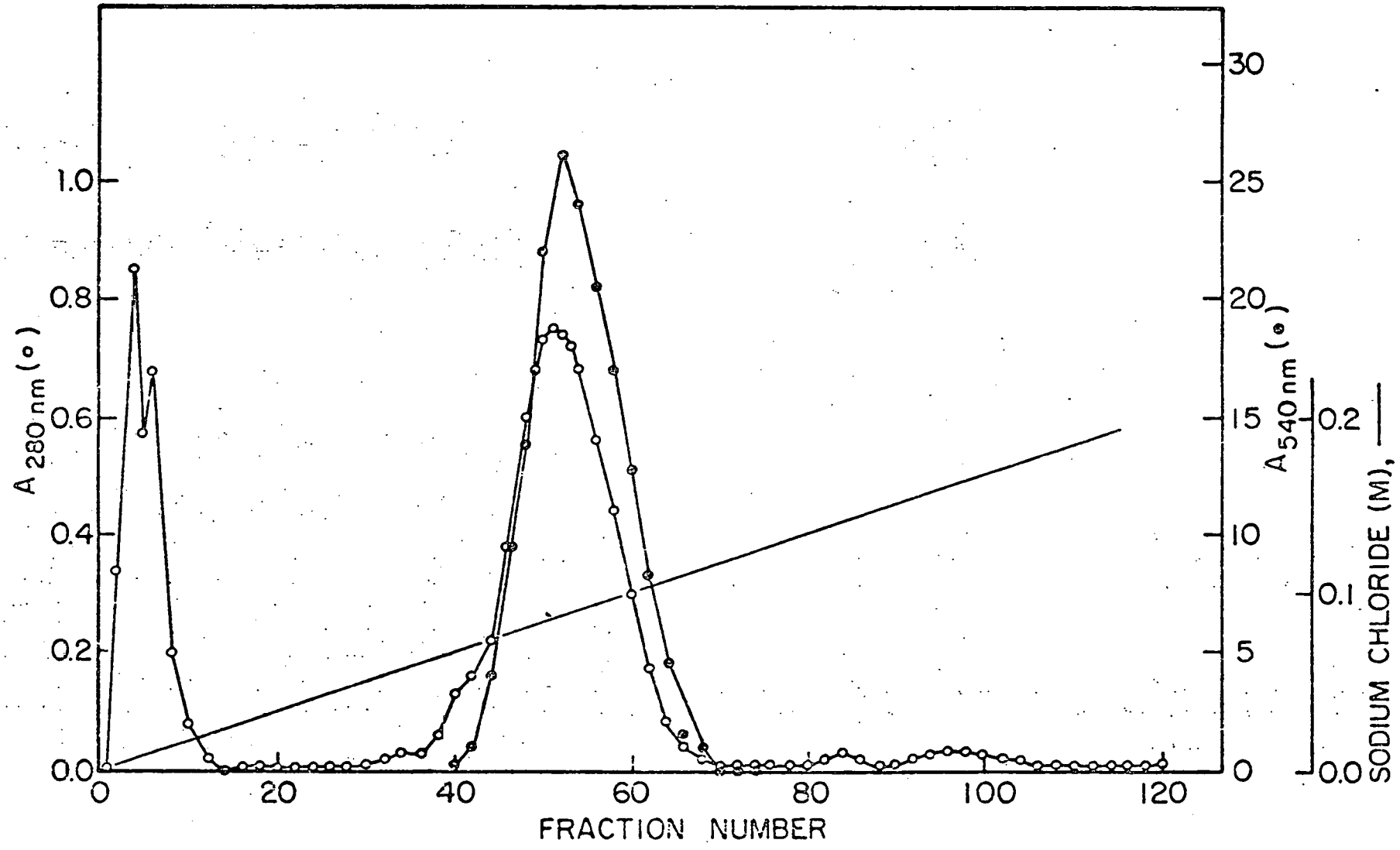
1.0
0.8
0.6
0.4
0.2
0.0
A 280 nm (•)
0
20
40
60
80
100
120
FRACTION NUMBER
30
25
20
15
10
5
0
A 540 nm (•)
0.2
0.1
0.0
SODIUM CHLORIDE (M), ——

Figure I-5

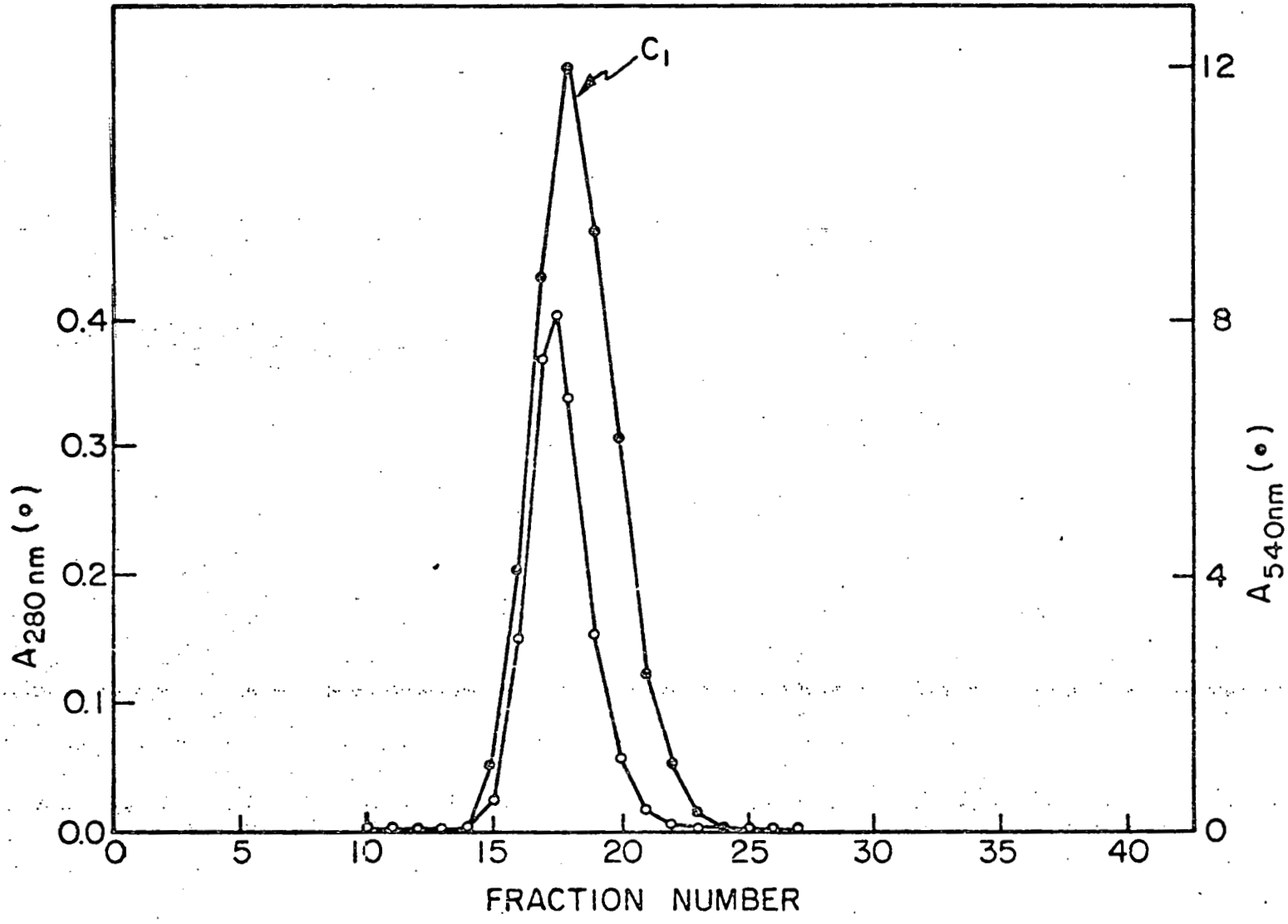
C1
0.4
0.3
0.2
0.1
0.0
A280 nm (•)
0
5
10
15
20
25
30
35
40
FRACTION NUMBER
12
8
4
0
A540nm (•)

Figure I-6

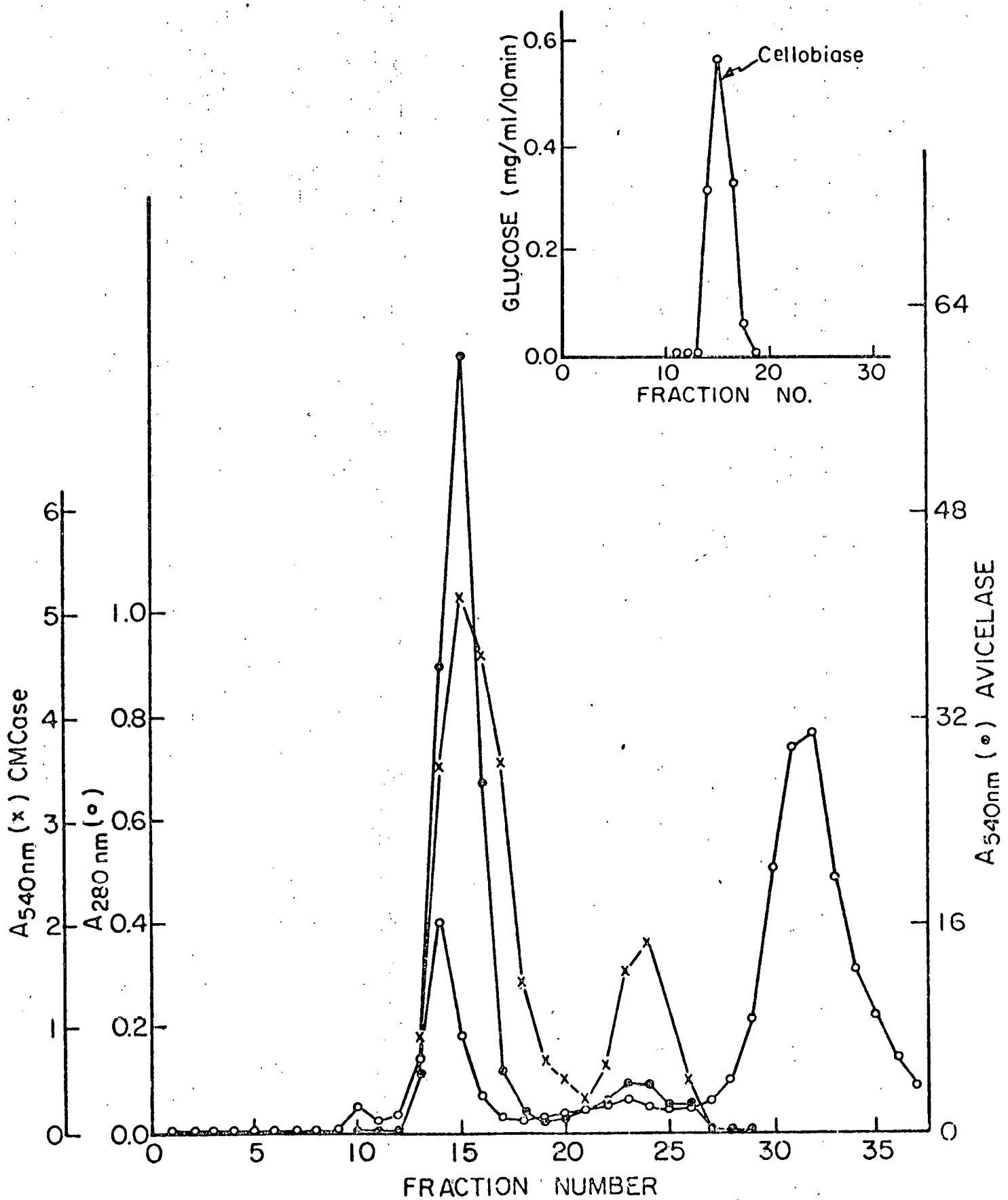
Cellobiase
GLUCOSE (mg/ml/10min)
0.0
0.2
0.4
0.6
0
10
20
30
FRACTION NO.
A540nm (x) CMCase
A280 nm (o)
A540nm (•) AVICELASE
0
1
2
3
4
5
6
0.0
0.2
0.4
0.6
0.8
1.0
0
16
32
48
64
0
5
10
15
20
25
30
35
FRACTION NUMBER

Figure I-7

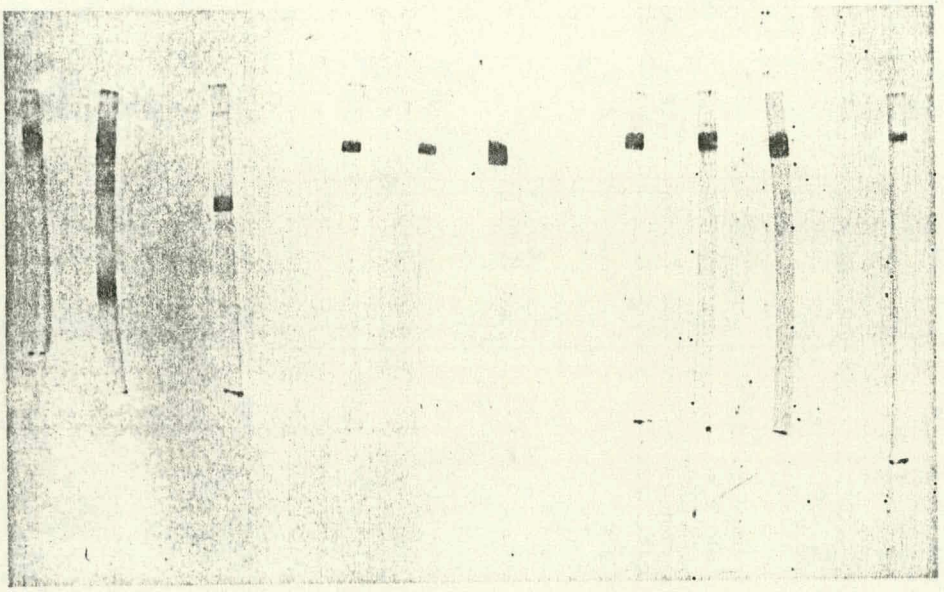

Figure I-8

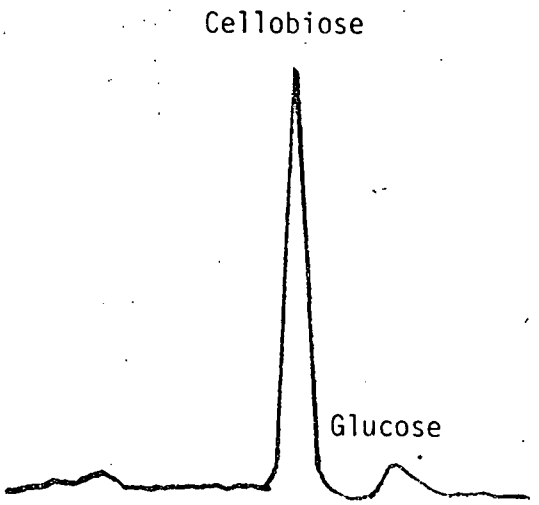

Figure I-9

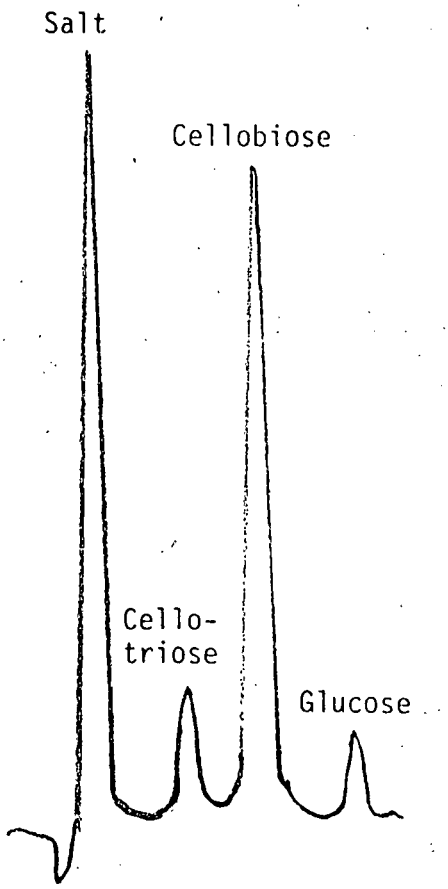

Figure I-10

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2. Wood, T.M., Biotechnol. Bioeng. Symp. 5, 111 (1975).

3. Selby, K. and Maitland, C.C., Biochem. J. 104, 716 (1967).

II. HYDROLYSIS OF CELLULOSE BY PURIFIED CELLULASE COMPONENTS

A. Introduction

The primary functions of the three enzymes are described at follows:

1) endoglucanase (C_x)-random scission of cellulose chains yielding glucose, cellobiose, and cellotriose;
2) exoglucanase (C_1)-end-wise attack on the non-reducing end of cellulose with cellobiose as the primary product; and
3) β-glucosidase (cellobiase)-hydrolysis of cellobiose to glucose with high activity.

All three components hydrolyze soluble cellodextrins as well as cellulose. Both endoglucanase and cellobiase hydrolyze cellobiose to glucose. However, cellobiase has a much higher activity with respect to cellobiose than does endoglucanase. Cellobiohydrolase also hydrolyzes soluble cellotriose and cellotetraose to give cellobiose and glucose, or cellobiose, respectively, as products. The soluble products, cellobiose and glucose, have been reported to be inhibitors of the cellulase complex and of the individual enzyme components endoglucanase, cellobiohydrolase, and β-glucosidase. Furthermore, β-glucosidase is also inhibited by its substrate, cellobiose. Thus, the kinetics of cellulase enzymes can be complex since both inhibition and activity with respect to multiple substrates must be considered.

There are several ways in which cellulase kinetics can be studies. A cell-free cellulase enzyme preparation can be combined with cellulose and the disappearance of substrate and/or appearance of sugars can be measured. Alternatively, the endoglucanase, exoglucanase and β-glucosidase components of the cellulase system can be separated and purified and their individual activities quantitated with respect to defined substrates. This approach is perhaps more definitive in obtaining an idea of the mode of action and mechanisms of cellulase enzymes.

B. Cellobiase (β-glucosidase)

Cellobiase is an enzyme which hydrolyzes the β-glucosidic linkage of a cellobiose molecule to give two molecules of β-D-glucose. This enzyme, also named β-glucosidase by virtue of its action on the β-glucosidic bond, is of practical importance as a component of the cellulase system of enzymes, a system which catalyzes the saccharification of cellulose.

Due to the small amounts of cellobiase produced by T. reesei the commercial cellulase enzyme (Enzyme Development Corp., New York, N.Y. Lot, No. WR 1432) was used to obtain pure cellobiase.

Three distinct cellobiase components were purified from a commercial Trichoderma viride cellulase preparation by repeated chromatography on DEAE cellulose eluting by a salt gradient according to the procedures described in Section I. The purified cellobiase preparations were evaluated for physical properties, kinetics, and mechanism. Results include: 1) development of one step enzyme purification procedure using DEAE-cellulose: 2) isolation of three chromatographically distinct, yet kinetically similar, cellobiase fractions of molecular weight of ∿ 76,000: 3) determination of kinetics which shows that cellobiase hydrolyzes cellobiose by noncompetitive mechanism and that the product, glucose inhibits the enyzme, and 4) development of an equation, based on the mechanism of cellobiase action, which accurately predicts the time course

of cellobiose hydrolysis over an eightfold range of substrate concentration and conversions of up to 90%. Based on the data presented in the paper, it is shown that product inhibition of cellobiase significantly retards the rate of cellobiose hydrolysis.

1. Kinetics

Initial rates of cellobiose hydrolysis were measured at substrate concentrations ranging from 2 to 20 mM for all purified cellobiase fractions. To check for product inhibition initial rate studies were performed in the presence of small quantities (5 and 10 mM) glucose.

Peak 1 cellobiase exhibited what appeared to be substrate inhibition at higher (20 mM) cellobiose concentration, while peaks 2 and 3 enzyme showed no such effect (see Figs. II-1(a), II-2(a), and II-3(a), "0mM glucose" lines). The apparent substrate inhibition effect for peak 1 is anomalous relative to the result obtained with peaks 2 and 3 enzyme and possibly reflects the previously mentioned minor contamination of peak 1 enzyme.

When glucose was added and initial rate studies were repeated for peaks 2 and 3 enzyme, patters of straight lines intersecting behind the y axis and above the x axis resulted (Figs. II-3(a) and II-2(a)). Owing to the minor contamination of peak 1 enzyme, an inhibition study for this fraction was not performed.

Product Inhibition

The intersecting patterns obtained from the initial rate data indicated that cellobiase is noncompetitively inhibited by the product glucose.

The observed noncompetitive product inhibition suggests that the reaction sequence for this enzyme might be of the type:

$$E + GG \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^* \xrightarrow{k_3} E + 2G \qquad (1)$$

$$E^* + G \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E^*G \qquad (2)$$

$$E + G \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EG \qquad (3)$$

where E represents free enzyme, GG represents cellobiose (the substrate), G represents glucose (the inhibitor and also the product), and E*, E*G, and EG represent the enzyme-substrate, enzyme-substrate-inhibitor, and enzyme-inhibitor complexes, respectively.

Writing out the differential equations for E*, E*G, and EG and applying the pseudo-steady-state assumption results in the equations:

$$-\frac{d(E^*)}{dt} = 0 = (k_2 + k_3)(E^*) - k_1(E)(GG) + k_5(E^*)(G) - k_6(E^*G) \qquad (4)$$

$$-\frac{d(E^*G)}{dt} = 0 = k_6(E^*G) - k_5(E^*)(G) \qquad (5)$$

$$-\frac{d(EG)}{dt} = 0 = k_8(EG) - k_7(E)(G) \qquad (6)$$

Equations (4)-(6) combined together with eq. (7), an equation representing conservation of enzyme:

$$E_{tot} = E + E^* + E^*G + EG \qquad (7)$$

result in an expression for E*:

$$E^* = k_1 E_{tot}(GG) \Big/ \left[\left(1 + \frac{(G)}{k_8/k_7}\right)(k_2+k_3) + k_1 \left(1 + \frac{(G)}{k_6/k_5}\right)(GG) \right] \qquad (8)$$

The rate of appearance of the product glucose is given by:

$$dG/dt = 2(k_3E^*) \tag{9}$$

Substitution of eq. (8) into eq. (9) gives:

$$v = \frac{dg}{dt} = 2k_3E_{tot}(GG) \Big/ \left[\frac{k_2 + k_3}{k_1} \left(1 + \frac{(G)}{k_8/k_7} \right) + \left(1 + \frac{(G)}{k_6/k_5} \right) (GG) \right] \tag{10}$$

where the constants may be redefined as:

$$K = (k_2 + k_3)/k_1 \tag{11}$$

$$K_{i,1} = k_6/k_5 \tag{12}$$

$$k_{i,2} = k_8/k_7 \tag{13}$$

$$V = 2k_3E_{tot} \tag{14}$$

In eqs. (11)-(14), K is the Michaelis constant; $K_{i,1}$ and $K_{i,2}$ are dissociation constants for the E*G and EG complexes, respectively; and V is the reaction velocity at saturating substrate concentration. Equation (10), expressed in terms of "lumped" constants (11)-(14), takes the form

$$v = V(GG) \Big/ \left[\left(1 + \frac{(G)}{K_{i,1}} \right) (GG) + \left(1 + \frac{(G)}{K_{i,2}} \right) \right] K \tag{15}$$

At zero inhibitor (i.e., glucose, G=0), eq. (15) reduces to:

$$v = V(GG)/[(GG) + K] \tag{16}$$

which is the standard form of the Michaelis-Menten equation. The inverted form of eq. (15):

$$\frac{1}{v} = \frac{K[1 + (G)/K_{i,2}]}{V} \times \frac{1}{(GG)} + \frac{1 + (G)/K_{i,1}}{V} \tag{17}$$

predicts that a Lineweaver-Burk plot of the data should be linear. Replots of the slope

$$(K/V)\ [1 + (G)/K_{i,2}]$$

and intercept

$$(1/V)\ [1 + (G)/K_{i,1}]$$

terms in eq. (17) as a function of (G) should also be linear if the proposed reaction sequence given in eqs. (1)-(3) is valid. By these criteria, then, Figures 6-8 indicate that the mechanism does fit the data.

Since the plots and replots are linear, values of the kinetic constants, K, $K_{i,1}$, and $K_{i,2}$ can be determined directly from them. The x intercept of the Lineveaver-Burk plot (Figs. II-3(a), II-2(a), and II-1(a) gives - k_m and the y intercept, E_{tot}/V. The x intercept of the replots (Figs. II-1(b), 2(b), and 3(b)) give - $K_{i,1}$ and - $K_{i,2}$, respectively. The kinetic constants may also be computed directly from a linear least-squares fit of the data. The kinetic constants in Table II were obtained by a linear least-squares calculatation. The lines shown in Figures 6-8 are "best-fit" lines to the data by linear least squares. Note that the reaction velocities are shown in terms of specific activities in both the table and the plots to facilitate comparison of activities of the three fractions. This is equivalent to multiplying eq. (17) through by the constant E_{tot}.

The V/E_{tot} values in Table II-1, show the specific activity of the enzyme with respect to cellobiose to be quite high. Not surprisingly, magnitudes of these specific activities are not directly comparable to the specific activities obtained with the artificial substrate p-nitrophenyl-β-D-glucoside.

The three cellobiase fractions have similar affinities for cellobiose as shown by the almost equivalent values of their respective Michaelis constants.

TABLE II-1

SUMMARY OF KINETIC CONSTANTS FOR PEAKS 1, 2, and 3
CELLOBIASE FRACTION

Kinetic constant		Cellobiase enzyme fraction		
		Peak 1	Peak 2	Peak 3
$\frac{V}{E_{tot}}$	$\frac{\mu mol\ glucose}{min \cdot mg\ protein}$	66.2	116	44.6
K	(mM)	2.65	2.5	2.74
$K_{i,1}$	(mM)	---	16.4	14.7
$K_{i,2}$	(mM)	---	1.22	4.26

The kinetic similarity of the cellobiase fractions is further supported by the similar values of the inhibition constants of peaks 2 and 3 cellobiase. The values of these inhibition constants indicate that the free enzyme has about the same affinity for substrate as it does for inhibitor (the product in this case) while the enzyme-substrate complex appears to have a lower affinity for inhibitor than free enzyme.

The mechanism of action of cellobiase on cellobiose is of the non-competitive type as is shown by the initial rate data. This knowledge allows derivation of an integrated rate expression from which the time course of the enzyme catalyzed hydrolysis of cellobiose may be predicted. Recalling that $v = dG/dt$, eq. (15) may be rearranged to give:

$$V\int_0^t dt = \int_0^G \left[\left(1 + \frac{(G)}{K_{i,1}}\right)\right] + \left(1 + \frac{(G)}{K_{i,2}}\right)\frac{K}{(GG)}\Bigg] dG \qquad (18)$$

The concentration of substrate, (GG), at any time may be expressed in terms of the initial substrate concentration, $(GG)_o$, and the product concentration, G:

$$(GG) = (GG)_o - (G)/2 \qquad (19)$$

Substitution of eq. (19) into eq. (18) and integration results in

$$Vt = \frac{(G)^2}{2K_{i,1}} + \left(1 - \frac{2K}{K_{i,2}}\right)(G) - K\left(2 + \frac{4(GG)_o}{K_{i,2}}\right) \ln\left(1 - \frac{(G)}{2(GG)_o}\right) \qquad (20)$$

Given values of the kinetic constants, V, K, $K_{i,1}$, and $K_{i,2}$ and the initial substrate concentration, $(GG)_o$, the time, t, required to attain a certain product concentration, G, may be calculated from eq. (20).

A study using the most active cellobiase fraction, i.e., peak 2, showed eq. (20) to accurately predict the time course of cellobiose hydrolysis (Fig.

II-4). The experiment was carried out at pH 4.75, 50°C, and initial substrate concentrations of 40, 20, and 5.25 mM cellobiose with reaction times not exceeding 60 min. The short reaction time avoided complications with enzyme deactivation, which has previously been shown to be negligible over the indicated time span. The predicted curves (solid lines in Fig. II-4), obtained from kinetic parameters from Table II-1 and the enzyme concentrations indicated in Figure II-4, agree with the experiment data (circles in Fig. II-4).

If all the product were removed as it was formed, there would be no inhibition. The time course of the reaction for such a situation can be predicted using the integrated form of eq. (16), shown below:

$$Vt = (G) - 2K \ln [1 - G/2(GG)_0] \qquad (21)$$

Computation using eq. (21) shows that the reaction would proceed much faster if the product were removed as it was formed (indicated by dotted lines in Fig. II-4).

The strong inhibitory effect glucose exerts on cellobiase would appear to be an important factor to consider in the design of a large scale process. For example, eq. (20) can be used to calculate the time required for a cellobiose hydrolysis, where the product, glucose, is not removed, to go to 99% completion. Using eq. (21) the same calculation can be made assuming that all the product is removed as it is formed. This comparison, presented in Figure II-5, where reaction time is shown as a function of substrate concentration, shows a significant difference between the two cases, a difference which increases with increasing substrate concentration.

The inhibition of cellobiase by glucose is strongly competitive as shown by the value of $K_{i,2}$ which is 1.22 mM for peak 2 enzyme and 4.26 mM for peak 3. It has been reported in the literature that cellobiase from Trichoderma is inhibited by the substrate cellobiose. Although this effect was not observed

for peak 2 and 3 cellobiase at the substrate concentration (up to 40 mM) used in this study, it is possible substrate inhibition might be observed at higher cellobiose concentrations. However, inhibition by glucose and would still predominate in view of the value of the substrate (cellobiose) inhibiton constant $K_{i,5}$, which is reported to be 31.5 ± 7 a value 10 to 40 times greater than that of $K_{i,2}$. (Note: The magnitude of the inhibition effect is inversely proportional to the value of the inhibition constant).

The most interesting result of this study is the ability to predict the time course of cellobiose hydrolysis by cellobiase over a range of substrate concentrations and conversions. The mechanism used to derive the necessary kinetic equations is based directly on the results of initial rate studies and makes no assumptions. In the derivation, the only assumption made is that of pseudo-steady state. On this basis it is possible to model the reaction and to accurately predict the time course of cellobiose hydrolysis. This is the first time, to our knowledge, that the kinetics of cellobiase from Trichoderma has been quantitated in this manner.

NOMENCLATURE

E	free enzyme
E*	enzyme-substrate complex
EG	enzyme-inhibitor complex
E*G	enzyme-substrate-inhibitor complex
E_{tot}	total enzyme present in both free and complexed forms (mg)
G	glucose concentration (mM)
GG	cellobiose concentration (mM)
GG_o	initial cellobiose concentration(mM)
K	Michaelis constant (mM)
$K_{i,1}$	dissociation (inhibition) constant for E*G complex (mM)
$K_{i,2}$	dissocation (inhibition) constant for EG complex (mM)
t	time, min
v	reaction velocity, μmol glucose/min
V	reaction velocity at saturating concentration, μmole glucose/min

C. Glucanohydrolase [Endoglucanase or C_x Cellulase (CMCase)]

Glucanohydrolase from Trichoderma reesei, having a molecular weight of 52,000, was evaluated for kinetic properties with respect to cellobiose. Results from this work include: 1) initial rate studies that show that glucanohydrolase hydrolyzes cellobiose by a competitive mechanism and that the product, glucose, inhibits the enzyme; 2) low-pressure aqueous liquid chromatography that shows that formation of a reversion product, cellotriose,

is minor and occurs in detectable amounts only at very high (90 mM) cellobiose concentrations; 3) development of an equation based on the mechanism of glucanohydrolase action as determined by initial rate kinetics, which accurately predicts the time course of cellobiose hydrolysis; 4) derivation of an initial rate expression for the combined activity of cellobiase and glucanohydrolase on cellobiose. Based on data in this paper it is shown that the difference in inhibition patterns of the two enzymes could be used for determining the contamination of one enzyme by small quantities of the other.

1. Optimum pH and Temperature

The optimum pH was determined using cellotriose as a substrate. Cellotriose was chosen since glucanohydrolase is known to react rapidly with cellotriose and higher oligosaccharides while gaving a relatively low affinity for cellobiose. Thus, the optimum pH was obtained for enzyme activity. This is anticipated to reflect the condition at which the enzyme would most likely be used in a practical situation. The cellotriose for these experiments was prepared by aqueous, preparative scale, low-pressure chromatography.

To measure the optimum pH, the enzyme was incubated at 40°C in a mixture that contained 7 mM cellotriose and 2 to 4 mM sodium acetate buffer. The pH of the buffer ranged from 3 to 7. Enzyme activity was computed from the initial rate of appearance of cellobiose which was measured by LPLC (see Figure II-6 for sample chromatograms).

The optimum temperature was determined by incubating the enzyme at either 40 to 50°C for various lengths of time and then assaying for residual activity at 40°C, again using cellotriose as substrate.

The pH profile for glucanohydrolase (Figure II-7) shows the optimum pH to be 4.8. The lack of reversion products (Figure II-6) indicates that pH has little, if any, effect on the formation of these products.

The optimum temperature for kinetic studies is 40°C. As shown in Figure II-5, the enzyme rapidly loses activity at 50°C while it is very stable at 40°C. At 50°C the enzyme half-life was on the order of 1 hr. At 40°C the enzyme activity was found to be stable for at least one day.

All kinetic data reported in this paper were obtained from enzyme assays performed at 40°C and pH 4.8.

2. Kinetics

Using the enzyme assay procedure described above, initial rates of cellobiose hydrolysis were measured at substrate concentrations ranging from 1 to 10 mM. In a typical assay, enzyme, sodium acetate buffer (pH 4.8), and cellobiose were combined to give a total volume of 0.7 ml and incubated at 40°C for 30 to 90 min. At the end of the incubation period the glucose formed was measured by injecting a 10 to 50 µl sample of the mixture into the Beckman glucose analyzer. When small quantities of glucose were added to the initial incubation mixtures, inhibition of the cellobiose hydrolysis reaction occurred. Hence, a series of initial rate studies were performed where both initial cellobiose and glucose concentrations were varied.

The data, consisting of measured initial rates of glucose appearance as a function of substrate concentration, were plotted in the form of inverse rate versus inverse substrate concentration. The use of this plot together with the appropriate derivation of Michaelis-Menten kinetics allows development of a kinetic model of enzyme action.

Data for glucanohydrolase activity with respect to cellobiose hydrolysis are plotted in Figure II-8. The straight line (open circles, no glucose added) shown in Figure II-8(a) resulted. This line simply indicates that as substrate concentration increases, the rate also increases. When small amounts of glucose are added and initial rate studies are repeated, the lines indicated by the squares and triangles result. In these cases, due to the inhibitory effect of glucose, the rate is smaller at a given substrate concentration when glucose is initially added. The resulting pattern of lines, giving what is known as a Lineweaver-Burk plot, indicated that the enzyme is product inhibited by a competitive mechanism.

The pattern of lines in Figure II-8 intersecting on the y axis suggests competitive product inhibition for which the Michael-Menten equation is

$$v = \frac{V(GG)}{K(1 + G/K_{i,2}) \_ GG} \qquad (1)$$

A detailed derivation is given in the Appendix. At zero inhibitor (i.e., glucose, G = 0), Eq. (1) reduces to

$$v - V(GG)/(K + GG) \qquad (2)$$

which is the standard form of the Michaelis-Menten equation.

The inverted form of Eq. (1):

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{G}{K_{i,2}}\right)\left(\frac{1}{GG}\right) + \frac{1}{V} \qquad (3)$$

is of the form

$$y - m \cdot x + b \qquad (4)$$

i.e., the form of a straight line. Hence, Eq. (4) predicts that a Lineweaver-Burk plot of the data should be linear. A replot of the slope

$$(K/V)(1 + G/K_{i,2})$$

as a function of (G) should also be linear if the reaction sequence reflects competitive inhibition. By these criteria, then, Figure II-6 indicates that the mechanism is consistent with the data.

Since the plot and replot (see Figure II-8) are linear, values of the kinetic constants K and $K_{i,2}$ can be determined directly from them. The x intercept of the replot gives $-K_{i,2}$. The x intercept of the Lineweaver-Burk plot gives K and y intercept, E_{tot}/V. The values of these kinetic constants, obtained from lines fit to the data in Figure II-6 by linear least squares, are summarized in Table II-2. Note that the reaction velocity is shown in terms of specific activity. This is equivalent to multiplying Eq. (1) through by the constant $1/E_{tot}$.

The mechanism of action on cellobiose by glucanohydrolase is competitive as is shown by the initial rate data. Thus, integration of the rate expression [Eq. (3)] should give an equation that will accurately predict the hydrolysis time course of cellobiose. Recalling that v = dG/dt, Eq. (1) may be rearranged to give:

$$V\int_0^t dt = \int_0^G \left[K\left(1 + \frac{G}{K_{i,2}}\right) + GG\right]\frac{dG}{GG} \qquad (5)$$

TABLE II-2

SUMMARY OF KINETIC CONSTANTS FOR GLUCANOHYDROLASE

Constant	Value
V/E_{tot}(μmol glucose/min·mg protein)	0.58
K(mM cellobiose)	1.6
$K_{i,2}$(mM glucose)	0.98

where

$$GG = (GG_o) - G/2 \tag{6}$$

Substitution of Eq. (6) into Eq. (5) and integration results in:

$$Vt = \left(1 - \frac{2K}{K_{i,2}}\right)(G) + K\left(2 + \frac{4(GG_o)}{K_{i,2}}\right)\ln\left(\frac{GG_o}{GG_o - G/2}\right) \tag{7}$$

Given values of the kinetic constants V, K, and $K_{i,2}$ and the initial substrate concentration $(GG)_o$, the time t required to attain a certain product concentration G may be calculated from Eq. (7).

A time-course study at two starting concentrations of cellobiose (5.85 and 38.8 mM) showed Eq. (7) to accurately predict the rate of hydrolysis of cellobiose (Figure II-9). The experiment was carried out at pH 4.8, 40°C, and with reaction times not exceeding 25 hr. The predicted curves (solid lines in Figure II-9), computed from Eq. (7) using the values of kinetic parameters determined from the initial rate study agree with the experiment data (squares in Figure II-9).

If all the product were removed as it was formed, there would be no inhibition. The time course of the reaction for such a situation can be predicted by using Eq. (1) where G = 0 and integrating:

$$Vt = (G) - 2\,K \ln [1 - G/2(GG_0)] \tag{8}$$

Computation using eq. (8) shows that the reaction would proceed much faster if the product were removed as it was formed (indicated by dotted lines in Fig. II-9.

The competitive pattern of glucanohydrolase action (Fig. II-8) implies that the noncompetitive reaction step:

$$E^* + G \rightleftharpoons E^*G \tag{9}$$

is at most very small. Low-pressure liquid chromatography analysis (Fig. II-10) of the hydrolysate done at the end of the run shown in Figure 7(b), shows no detectable cellotroise (G_3). This is further indication that the reaction given by eq. (9) is small since G_3 formation would require the presence of the enzyme comples E*G.

At high enough cellobiose concentrations, however, some G_3 is formed. The chromatograms in Figure II-11 resulted from incubation of glucanohydrolase with approximately 90 mM cellobiose. At time zero there is essentially pure cellobiose [Fig. II-11(a)]. After 72 min, a small amount of G_3 is formed. This level stays almost constant even after 19.5 hr of incubation [Fig. II-11(c)]. On a mole basis, G_3 accounts for about 3.5% of the cellobiose that was hydrolyzed.

These data indicate that at high enough substrate concentrations,the formation of an E*G [or EG(GG)] type complex will occur and some reversion product, i.e., cellotriose, will form. This result is not surprising since it is well known that at high enough substrate concentrations most enzymes will exhibit substrate inhibition. On a practical note, however, the cellobiose concentrations commonly encountered from the hydrolysis of cellulose by the cellulase complex from T. reesei in our laboratory have typically been 10 mM or less. This range corresponds to the concentration used in generating the hydrolysate for the LPLC chromatogram in Figure II-10.

These results show it is possible to predict the time course of cellobiose hydrolysis by glucanohydrolase using equations derived by making the pseudo-steady-state assumption. Again, this approach is different from ordinary curve fitting since the model as well as kinetic parameters were derived from initial rate data. This information was then taken to predict the time course for up to 90% conversion of cellobiose. This is the first time, to our knowledge, that theory and experiment have been combined in this particular manner to quantitate glucanhydrolase activity with respect to cellobiose.

Thus, glucan glucanohydrolase from T. reesei has cellobiose-hydrolyzing activity. However, this activity is one to two magnitudes of order less than that observed for cellobiase. Thus, it appears that the enzyme component in the cellulase complex of T. reesei having the major cellobiose-hydrolyzing activity is cellobiase. Although both glucanohydrolase and cellobiase are both subject to glucose (product) inhibition, the mode of inhibition of glucanohydrolase is competitive, while the mode of cellobiase in noncompetitive.

D. CELLOBIOHYDROLASE

The kinetics of the hydrolyses of cellotriose and of cellotetraose by cellobiohydrolase were studied using a convenient integral technique. Reaction mechanisms and mathematical models were postulated to describe the reactions. The end-products of the reaction were found to be inhibitory toward hydrolysis in a competitive mode. Hydrolysis of cellotetraose produces cellobiose and hydrolysis of cellotriose produces cellobiose and glucose. Both sugars inhibit the enzyme with cellobiose being stronger inhibitor.

MODEL DESCRIPTION

The reaction mechanism and the rate equation for the hydrolysis of cellotetraose, G_4, assuming that the reaction follows the irreversible Michaelis-Menten kinetics Model and is subject to a competitive inhibition by its product G_2 are as follows:

$$G_4 + E \rightleftharpoons EG_4 \xrightarrow{k} E + 2G_2$$

$$G_2 + E \rightleftharpoons EG_2 \qquad (1)$$

$$v = -\frac{dG_4}{dt} = \frac{V_m G_4}{K_m\left(1 + \frac{G_2}{K_{G_2}}\right) + G_4}$$

where E = enzyme

K_m = Michaelis-Menten constant

$V_m = kE_T$ = maximum reaction rate for given enzyme concentration

E_T = total enzyme concentration

K_{G_2} = competitive inhibition constant associated with G_2

Equation (1) can be applied to the entire time course of the hydrolysis data. By a simple material balance, $G_2 = 2\ (G_{4,0} - G_4)$ where $G_{4,0}$ = initial G_4 concentration. If V_m is held constant, Equation (1) can be rearranged and integrated to give Eq. (2),

$$\int_o^t dt = -\int_{G_{4,0}}^{G_4} \frac{K_m\left(1 + \frac{2(G_{4,0} - G_4)}{K_{G_2}}\right) + G_4}{V_m G_4}\, d\,G_4$$

$$t = \frac{1}{V_m}\left(\frac{2K_m}{K_{G_2}} - 1\right)(G_4 - G_{4,0}) + \frac{K_m}{V_m}\left(1 + \frac{2G_{4,0}}{K_{G_2}}\right) \ln \frac{G_{4,0}}{G_4} \qquad (2)$$

Defining c, the G_4 conversion,

$$c = \frac{G_{4,0} - G_4}{G_{4,0}} \qquad (3)$$

and dividing both sides of Eq. (2) by c,

$$\frac{t}{c} = \frac{1}{V_m}\left(1 - \frac{2K_m}{K_{G_2}}\right)G_{4,0} + \frac{K_m}{V_m}\left(1 + \frac{2G_{4,0}}{K_{G_2}}\right)\frac{1}{c} \ln \frac{1}{1-c} \qquad (4)$$

According to Eq. (4), when t/c is plotted against $\frac{1}{c} \ln \frac{1}{1-c}$, a straight line results with a slope and intercept of

$$\text{slope} = \frac{K_m}{V_m}\left(1 + \frac{2G_{4,0}}{K_{G_2}}\right) \text{ or slope } xE_T = \frac{K_m}{k}\left(1 + \frac{2G_{4,0}}{K_{G_2}}\right) \tag{5}$$

and

$$\text{intercept} = \frac{1}{V_m}\left(1 - \frac{2K_m}{K_{G_2}}\right) G_{4,0} \text{ or intercept } xE_T = \frac{1}{k}\left(1 - \frac{2K_m}{K_{G_2}}\right) G_{4,0} \tag{6}$$

A family of straight lines can be obtained from several sets of time course data with varying initial substrate concentrations, $G_{4,0}$. A "replot" of the slopes of the straight lines against $G_{4,0}$ should also yield a straight line according to Eq. (5). Similarly, a replot of the intercepts against $G_{4,0}$ should yield another straight line, according to Eq. (6). From the linear plots and replots, the kinetic constants K_m, V_m, and K_{G_2} can be determined.

With substrate G_3, both G_2 and G_1 will be the reaction products of the cellobiohydrolase. If it is assumed that both G_1 and G_2 are competitive inhibitors and that there is no reverse reaction, the reaction mechanism and the rate equation will be as follows:

$$G_3 + E \rightleftharpoons E\,G_3 \xrightarrow{k} E + G_1 + G_2$$

$$G_2 + E \rightleftharpoons E\,G_2$$

$$G_1 + E \rightleftharpoons E\,G_1$$

$$v = -\frac{dG_3}{dt} = \frac{V_m G_3}{K_m\left(1 + \frac{G_1}{K_{G_1}} + \frac{G_2}{K_{G_2}}\right) + G_3} \tag{7}$$

where K_{G_1} = competitive inhibition constant associated with G_1 with the other parameters remaining the same as previously defined.

By a material balance, $G_1 = G_2 = G_{3,0} - G_3$ where $G_{3,0}$ = initial G_3 concentration. Equation (7) can be rearranged and integrated to give Eq. (8), assuming constant V_m

$$t = \frac{1}{V_m}\left(\frac{K_m}{K_{G_1}} + \frac{K_m}{K_{G_2}} - 1\right)(G_3 - G_{3,0}) + \frac{K_m}{V_m}\left(1 + \frac{G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right)\ln\frac{G_{3,0}}{G_3} \qquad (8)$$

substituting,

$$c = \frac{G_{3,0} - G_3}{G_{3,0}} \qquad (9)$$

into Eq. (8) and rearranging

$$\frac{t}{c} = \frac{1}{V_m}\left(1 - \frac{K_m}{K_{G_1}} - \frac{K_m}{K_{G_2}}\right) G_{3,0} + \frac{K_m}{V_m}\left(1 + \frac{G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right)\frac{1}{c}\ln\frac{1}{1-c} \qquad (10)$$

Again, a plot of $\frac{t}{c}$ vs $\frac{1}{c}\ln\frac{1}{1-c}$ yields a straight line with its slope and intercept being

$$\text{slope} = \frac{K_m}{V_m}\left(1 + \frac{G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right) \text{ or slope } xE_T = \frac{K_m}{k}\left(1 + \frac{G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right) \qquad (11)$$

and

$$\text{intercept} = \frac{1}{V_m}\left(1 = \frac{K_m}{K_{G_1}} - \frac{K_m}{K_{G_2}}\right) G_{3,0} \text{ or intercept } xE_T = \frac{1}{k}\left(1 - \frac{K_m}{K_{G_1}} - \frac{K_m}{K_{G_2}}\right) \qquad (12)$$

Equations (10) to (12) are similar to Eqs. (4) to (6) but include one more kinetic parameter, K_{G_1}. From replots of the slope and intercept vs $G_{3,0}$, K_m, V_m and $\frac{1}{K_{G_1}} + \frac{1}{K_{G_2}}$ can be determined, but not the individual K_{G_1} and K_{G_2}.

In order to determine K_{G_1} and K_{G_2} independently, the following technique can be used. When a known concentration $G_{1,0}$ of glucose is included in the initial reaction mixture, Eq. (7) becomes

$$v = \frac{dG_3}{dt} = \frac{V_m G_3}{K_m \left(1 + \frac{G_{1,0} + G_1}{K_{G_1}} + \frac{G_2}{K_{G_2}}\right) + G_3} \tag{13}$$

When this equation is integrated and combined with Equation (9),

$$\frac{t}{c} = \frac{1}{V_m}\left(1 - \frac{K_m}{K_{G_1}} - \frac{K_m}{K_{G_2}}\right) G_{3,0} + \frac{K_m}{V_m}\left(1 + \frac{G_{1,0} + G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right) \frac{1}{c} \ln \frac{1}{1 - c} \tag{14}$$

A plot of $\frac{t}{c}$ vs $\frac{1}{c} \ln \frac{1}{1-c}$ gives a straight line with its slope being

$$\text{slope} = \frac{K_m}{V_m}\left(1 + \frac{G_{1,0} + G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right) \text{ or}$$

$$\text{slope } xE_T = \frac{K_m}{k}\left(1 + \frac{G_{1,0} + G_{3,0}}{K_{G_1}} + \frac{G_{3,0}}{K_{G_2}}\right) \tag{15}$$

From a family of $\frac{t}{c}$ vs $\frac{1}{c} \ln \frac{1}{1-c}$ plots with varying $G_{1,0}$ but constant $G_{3,0}$, a linear replot of the slopes against $G_{1,0}$ can be obtained. This replot, with its slope being $\frac{K_m}{K_{G_1} V_m}$, together with the previous replot of the slope of Eq. (10) against $G_{3,0}$ (in the absence of added glucose), allows for the determination of K_{G_1}.

RESULTS

Two prerequisites have to be met in order to apply the method involving a plot of t/c vs. (1/c) ln [1/(1 - c)] in studying the kinetics oc cellobiohydrolase: the enzyme must be stable under the assay conditions, and the reverse reactions must be negligible.

Stability

The enzyme was first incubated at 50°C in a sodium acetate buffer (pH 4.8) for various time periods before the introduction of substrates. Figure II-12 shows that cellobiohydrolase is stable for more than 4 hr of preincubation. This is an indication that the enzyme is stable under assay conditions.

Reverse Reactions

To determine the possible presence of a reverse reaction, a high concentration of G_2 (60 mM) was incubated with the enzyme. Following incubation, samples were taken and analyzed by liquid chromatography. As shown in Figure II-13(a) there were no detectable high-molecular-weight cellodextrins formed. Similarly, when the enzyme was incubated with G_1 alone, or with a mixture of G_1 and G_2, no detectable reverse reaction products [Figs. II-13(b) and II-13(c)] were formed, nor was the reverse reaction observed when cellotriose or cellotetraose was used as substrate (Fig. II-14).

Kinetics

Since the two prerequisites were both fulfilled, the method involving the t/c vs. (1/c) ln [1/(1 - c)] plot can be applied to study the kinetics of cellodextrin hydrolysis by cellobiohydrolase.

1. Cellotetraose hydrolysis

Figure II-15(a) shows two sets of experimental time course data for G_4 hydrolysis. The plots of t/c vs. (1/c) ln [1/(1 - c)] do appear to be linear. This suggests that the reaction might follow Michaelis-Menten kinetics with or without competitive inhibition. From several additional time course experiments, the above-mentioned replots of the slopes and intercepts were established in (Figures II-15(b) and II-15(c)), with the lines determined by the least-squares methods. By manipulating the straight line in Figure II-15(c) to pass through the origin as required by eq. (6), the kinetic parameters k, K_m, and K_{G2} can then be determined by using equs. (5) and (6). The results are listed in Table II-3.

2. Cellotriose hydrolysis

Time course data of G_3, hydrolysis also appear to be linear on the t/c vs. (1/c) ln [1/(1 - c)] plot as shown in Figure II-16(a)). This indicates that G_1 and G_2, the hydrolysis products, both function as competitive inhibitors. The slope and intercept replots shown in Figures II-16(b) and II-16(c) enables k, K_m, and $1/K_{G1} + 1/K_{G2}$ to be determined. In order to determine K_{G1} and K_{G2}, G_3 was incubated in the presence of varying amounts of either G_1 or G_2. The replot of the slope versus $G_{1,0}$ in Figure II-17, togeher with eq. (15) made it possible to determine K_{G1} and K_{G2} values. The kinetic constants so obtained are also listed in Table II-3. It is apparent that the K_{G2} values obtained from the two sets of exerpiments with G_4 and G_3 as the substrates are in reasonable agreement (Table II-3).

DISCUSSION

Since cellobiohydrolase is both stable and has only a negligble reverse reaction under the described experimental conditions, it is possible to study

TABLE II-3

KINETIC PARAMETERS FOR G_3 AND G_4 HYDROLYZED BY CELLOBIOHYDROLASE

	K_m (mM)	k (mol/min mg protein)	K_{G1} (mM)	K_{G2} (mM)
G_3	0.2	0.1	2.1	0.2
G_4	0.08	2.7	-	0.4

enzyme kinetics by using the time course kinetic technique. Data obtained by this method reflect the entire time course rather than just the initial stage of the reactions. The initial rate kinetics method is unreliable in some cases, primarily because of inherent experimental uncertainty and instrumental limitation.

Using the present method of kinetic analysis, we are able to estabilish that both cellobiose and glucose are competitive inhibitors of cellobiohydrolase. The comparison of the enzyme hydrolysis of cellotetraose with and without inhibition is given in Figure 9. It clearly indicates that a continuous removal of the hydrolysis products is important to maintain a high rate of the enzyme hydrolysis.

In the absence of product inhibition, the equation for the hydrolysis of G_3 or G_4 would be [cf. eqs. (4) and (10)],

$$t = \frac{G_{i,0}}{V_m} c + \frac{K_m}{V_m} \ln \frac{1}{1 - c} , \qquad i = 3 \text{ or } 4 \qquad (16)$$

Our finding that glucose is an inhibitor is in contrast to the report by Halliwell and Griffin (1) who claimed that glucose was not an inhibitor of cellobiohydrolase from Trichoderma koningii.

Listed in Table I, K_{G1} and K_{G2} indicate that both glucose and cellobiose, the end-product of cellulose hydrolysis, inhibit cellobiohydrolase. The inhibitory effect of glucose on cellobiohydrolase would become important in a complete cellulase enzyme system. The inhibition by cellobiose could be partially relieved by the presence of cellobiase in the enzyme system. However, glucose, the product of cellobiase, could inhibit not only cellobiase, but cellobiohydrolase as well, which lowers the net effect of the cellobiase action. Nevertheless, since K_{G2}, tha addition of cellobiase would have an enhancing effect on the overall cellulose hydrolysis.

Equations (1) and (7) can be combined into the following general form:

$$v = -\frac{dG_i}{dt}$$

$$= \frac{V_{mi} G_i}{K_{mi}(1 + G_1/K_{G1} + G_2/K_{G2}) + G_i} \quad , \quad i = 3,4 \qquad (17)$$

The integrated form of eq. (17) gives the mathematical description of the hydrolysis of G_3 and G_4 by cellobiohydrolase. When G_4 hydrolysis is performed in the absence of initially added G_1, the G_1 term in eq. (17) simply diminishes and the equation degenerates to eq. (1).

Okazaki and Moo-Young (2) proposed a theoretical model for the degradation of cellulose by assuming a noncompetitive end-product inhibition with K_m and V_m being independent of the chain length of cellulose. If the assumption were correct, then the complete kinetics of cellulose hydrolysis by cellobiohydrolase would have been solved. Unfortunately, this is not the case. As shown in Table II-1 and also reported by Wood and McCrae, (3) the values of K_m and V_m are directly related to the chain length of cellulose. The results in this paper can serve as a basis for studying the hydrolysis kinetics of cellulose and cellodextrins of high chain lengths.

The kinetics of the hydrolyses of cellodextrins of higher polymers are more complicated because of the consecutive reactions involved. For instance, the hydrolysis of G_5 involves the generation of G_3 plus G_2. In turn, G_3 serves as a substrate to product G_1 plus G_2. Therefore, the t/c vs. (1/c) ln [1/(1 - c)] plot technique is not directly applicable. However, with the aid of a computer and some initial rate experiments, the K_m and V_m values of the hydrolysis of long chain length cellulose can be obtained.

The kinetic parameters determined in this study are able to describe G_3 and G_4 hydrolysis reactions of up to 80% substrate conversions. An 80%

conversion is equivalent to a $(1/c)\ \ln\ [1/(1 - c)]$ value of 2.0. (See Figs. II-15 and II-16). For higher conversions, the experimental data tend to deviate somewhat from the predicted values, as shown by the tail portion of the solid curve in Figure II-18.

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2. M. Okazaki and M. Moo-Young, Biotechnol. Bioeng. 20, 637 (1978).

3. T. M. Wood and S. I. McCrae, Biochem. J. 128, 1183 (1972).

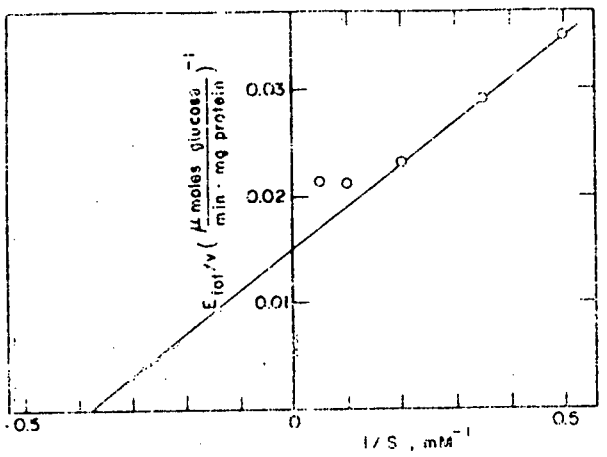


Fig. II-1. Lineweaver-Burk plot for peak 1 cellobiase.

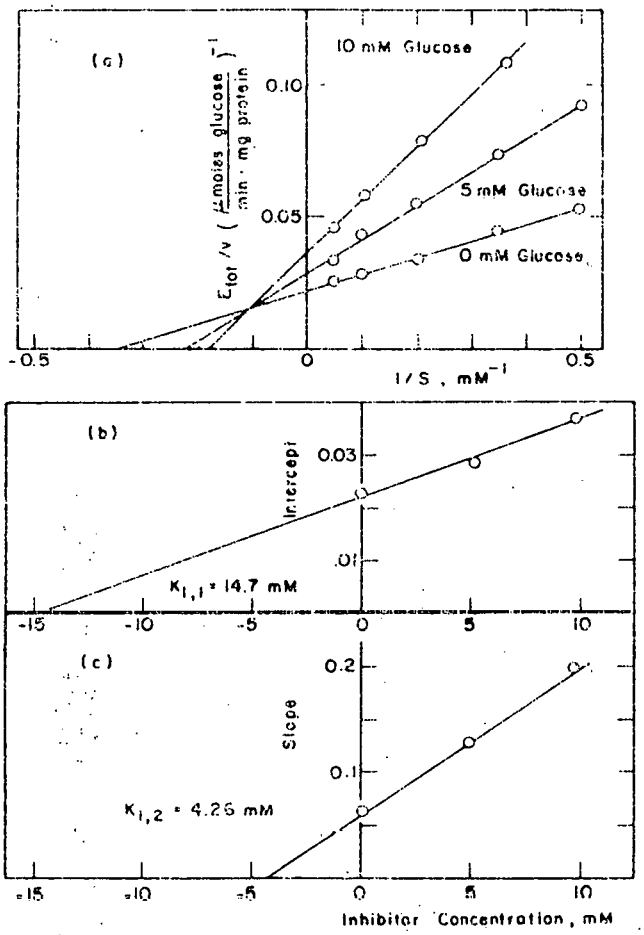


Fig. II-2. Plot of kinetic data for peak 3 cellobiase (See Fig. II-3 for explanation).

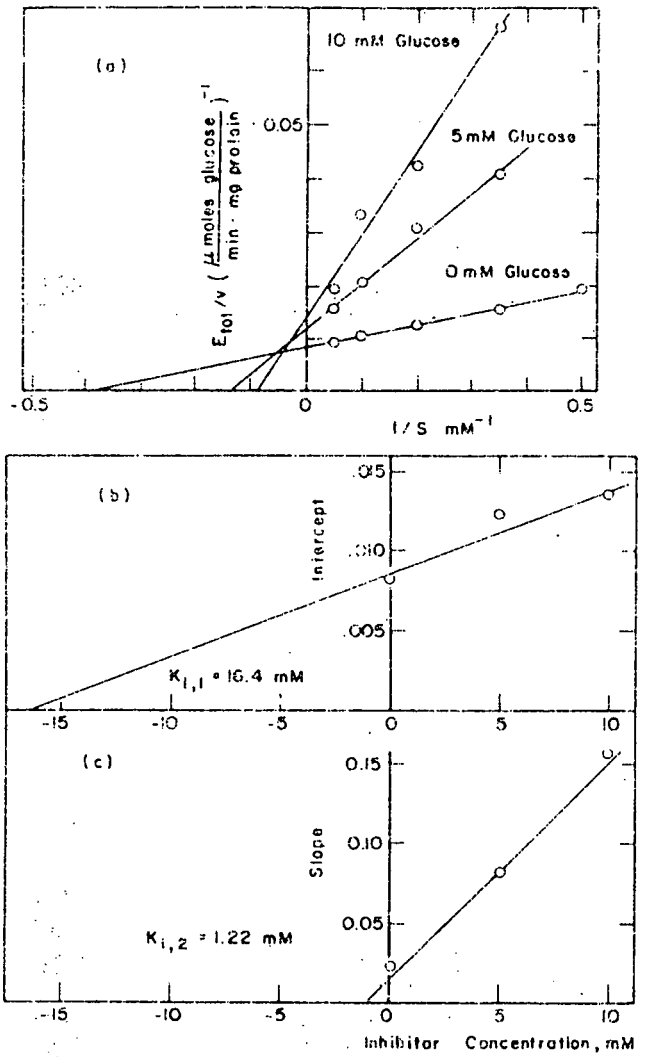


Fig. II-3. Plot of kinetic data, for peak 2 cellobiase: (a) Lineweaver-Burk plot at several product (inhibitor) levels; (b) replot of intercepts in (a) as a function of inhibitor concentration; (c) replot of slopes in (a) as a function of inhibitor concentration.

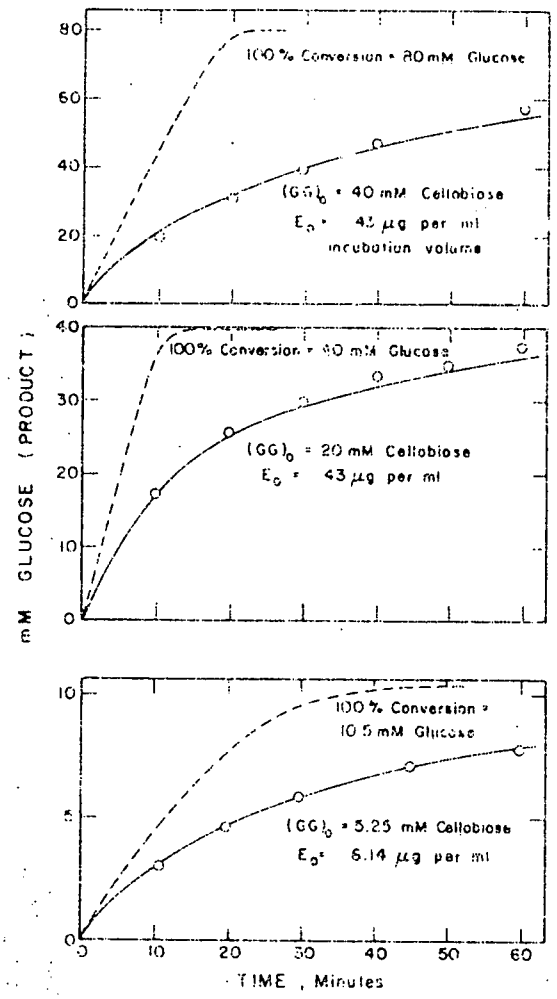


Fig. II-4. Time course of cellobiose hydrolysis: (a) 40mM initial substrate concentration; (b) 20mM; (c) 5.25 mM. (O) Data point; (———) curve predicted by eq. (20); (------) curve predicted by eq. (21) in the absence of inhibitor. All runs conducted at pH 4.75 and 50°C at the enzyme concentration indicated on the figure.

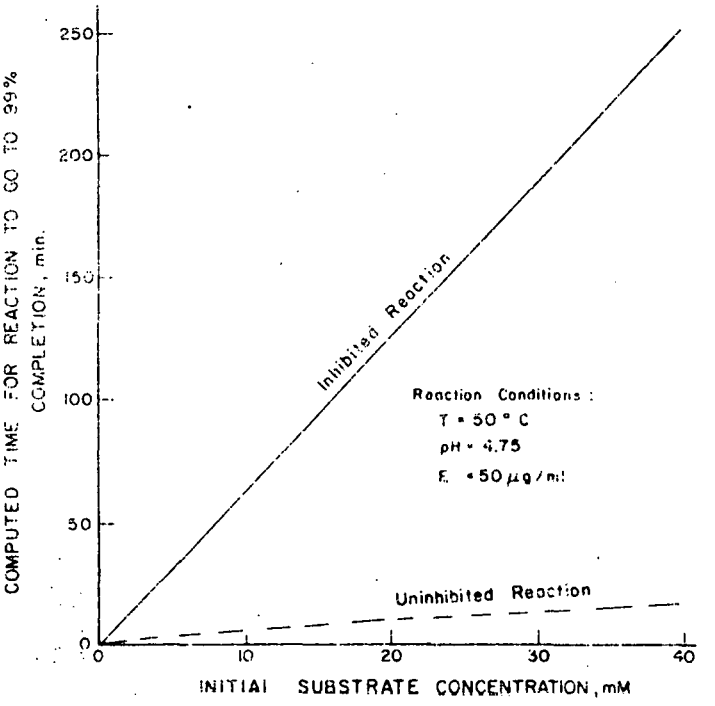


Fig. II-5. Comparison of reaction times required for reactions to go to 99% completion: (———) reaction where product is not removed; (----) reaction with product removed as formed. Data calculated from eqs. (20) and (21) and the kinetic constants for peak 2 enzyme.

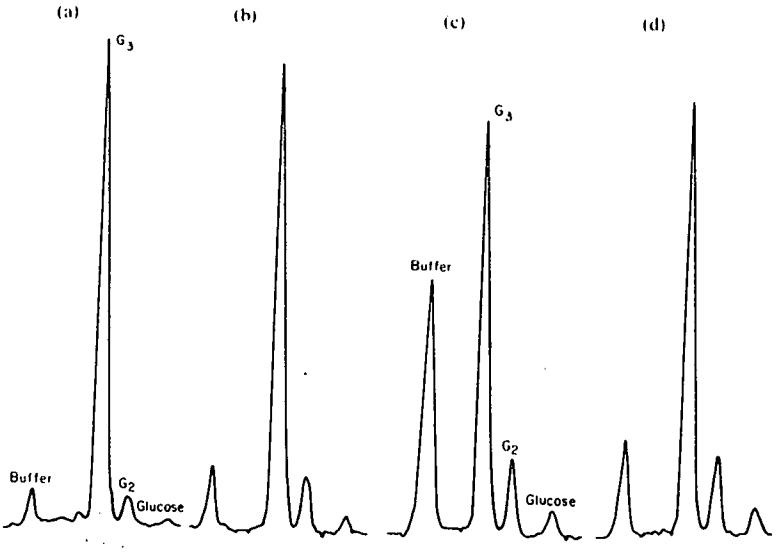


Fig. II-6. LPLC chromatograms of cellotriose hydrolysates at various pH's. (a) pH 4; (b) pH 4.5; (c) pH 4.8; (d) pH 5.

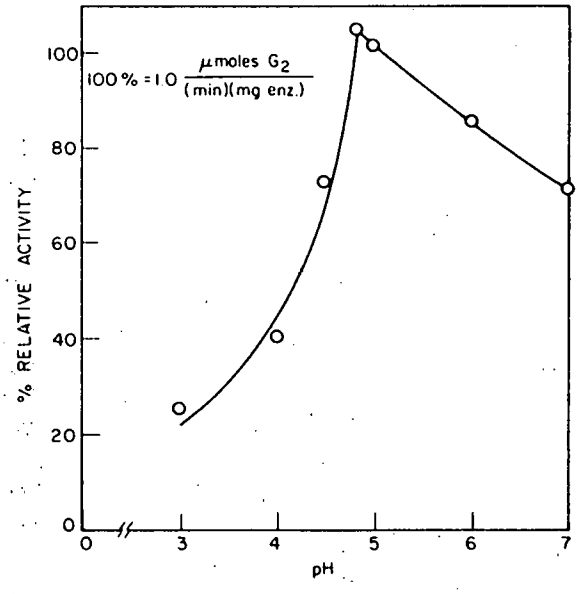


Fig. II-7. PH profile for glucanohydrolase with respect to cellotriose. Temperature is 40°C.

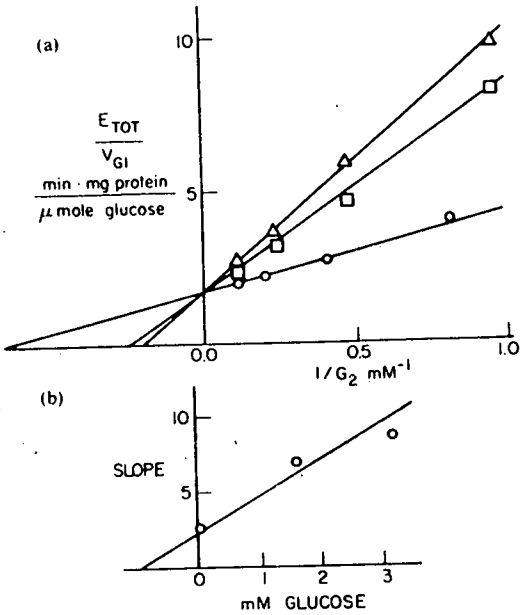


Fig. II-8. Lineweaver-Burk plot for glucanohydrolase with respect to cellobiose. (a) (Δ) 3.17 mM glucose; (□) 1.59 mM glucose; (O) no glucose added; temperture = 40°C; (b) replot of slopes.

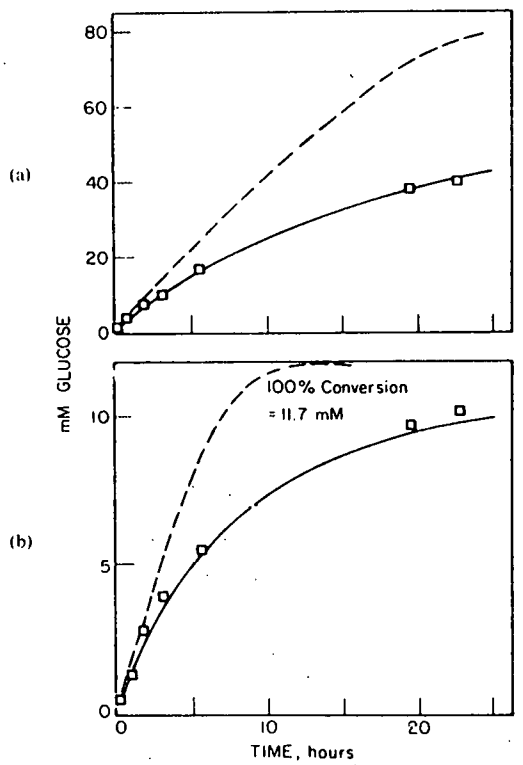


Fig. II-9. Time course of hydrolysis of cellobiose by glucanohydrolase. (———) Date predicted by competitive inhibition model; (----) predicted for case of no glucose inhibition. (a) GG_0 = 38.8 mM; E_{tot} = 0.118 mg/ml; temperature = 40°C. (b) GG_0 = 5.85 mM; E = 0.0672 mg/ml; temperature = 40°C.

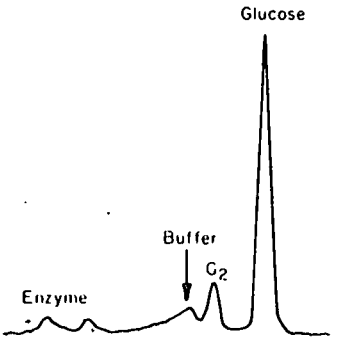


Fig. II-10. LPLC chromatogram of cellobiose hydrolysate.

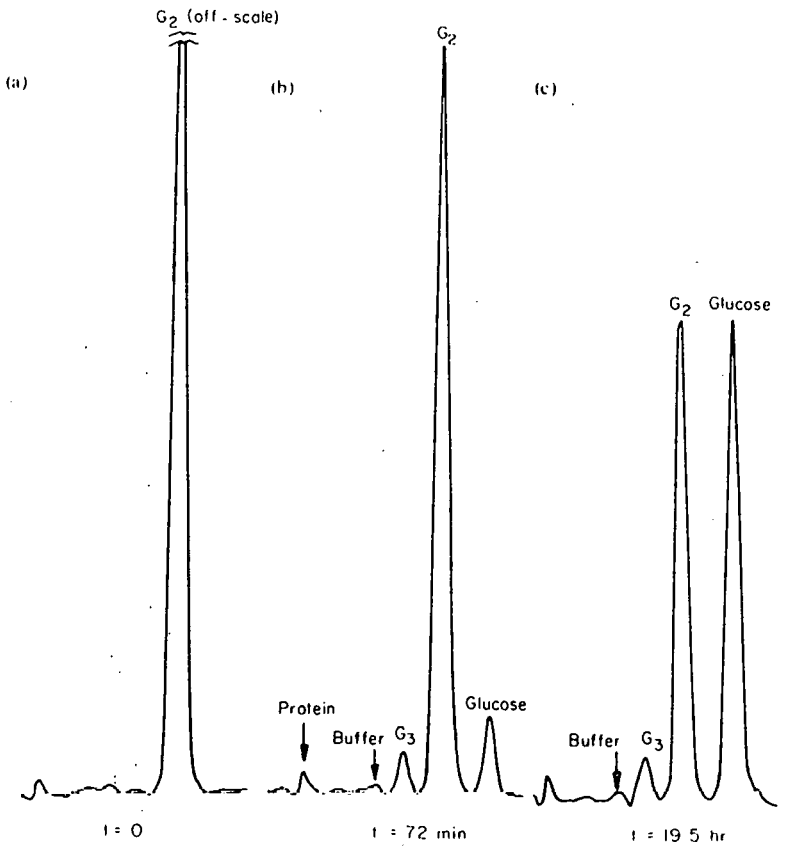


Fig. II-11. Glucanohydrolase action at very high (90mM) cellobiose concentration.

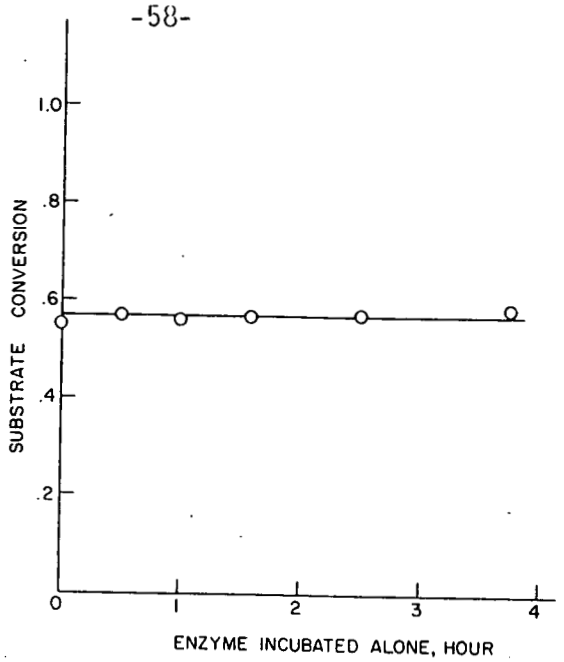


Fig. II-12. Thermal stability of cellobiohydrolase at 50°C. Cellobiohydrolase was preincubated at 50°C for a period of time as indicated. After the preincubation substrate was introduced and incubated for another 20 min. Hydrolysis product was analyzed by LPLC. 0.79 mM G_4 substrate; enzyme = 10.9 μm/ml; temperature - 50°C; reaction time = 20 min.

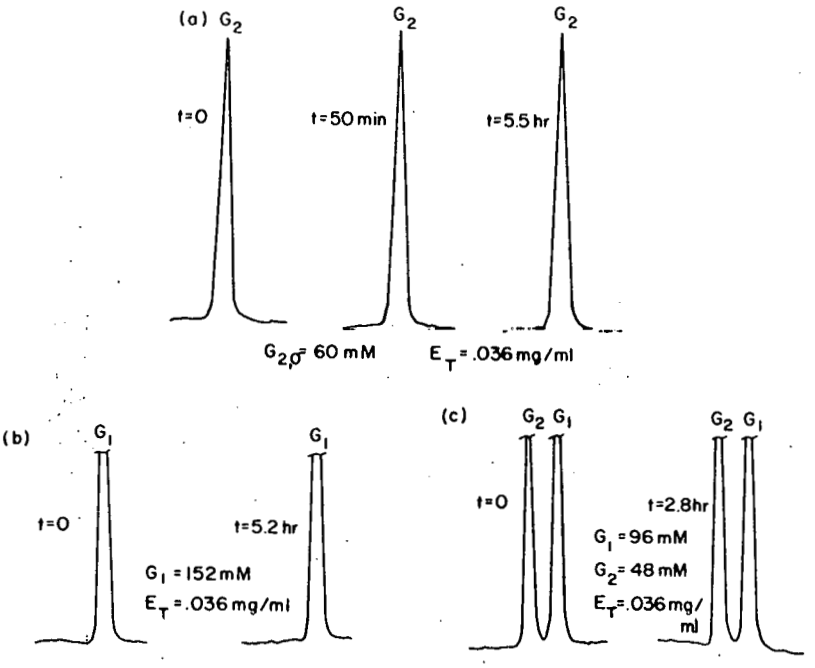


Fig. II-13. LPLC chromatograms of glucose and cellobiose in the presence of cellobiohydrolase. Symbols G_1, glucose G_2 cellobiose; E_T, total enzyme concentration.

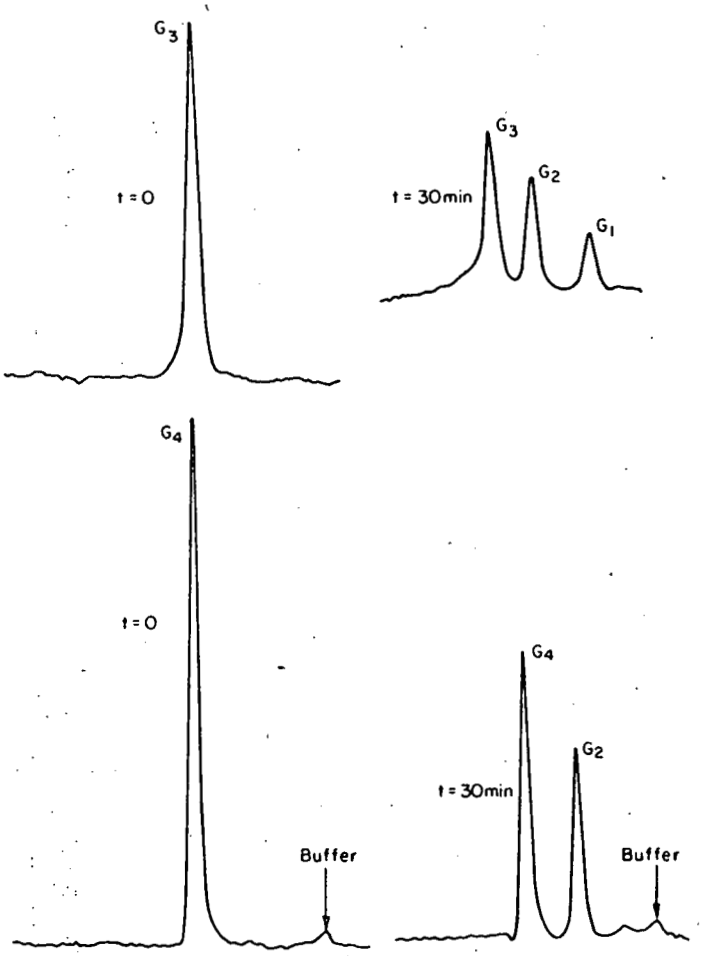


Fig. II-14. Chromatograms of low-pressure liquid chromatography (LPLC) of cellotriose and cellotetraose hydrolysis by cellobiohydrolase.

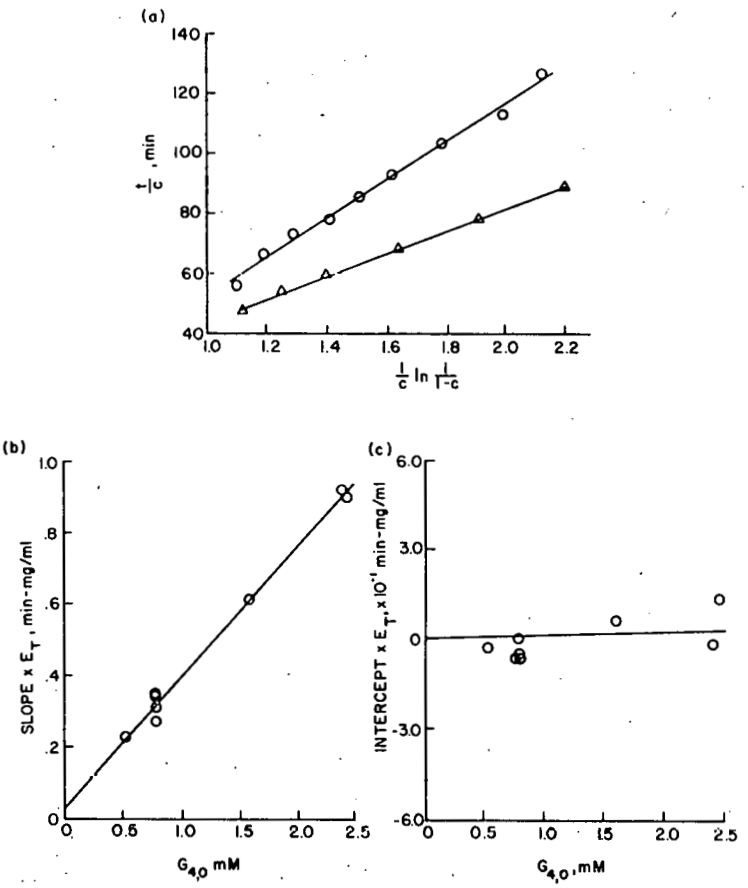


Fig. II-15. Plot of kinetic data of cellotetraose (G_4) hydrolysis by cellobiohydrolase: (a) t/c vs. (1/c) ln [1/(1-c)] plot; (b) replot of slopes in (a) as a function of cellotetraose concentration; (c) replot of intercepts in (a) as a function of cellotetraose concentration (G_4). Symbols: E_T total enzyme concentration; $G_{4,0}$ = 2.5 mM; E_T = 0.024 mg/ml.

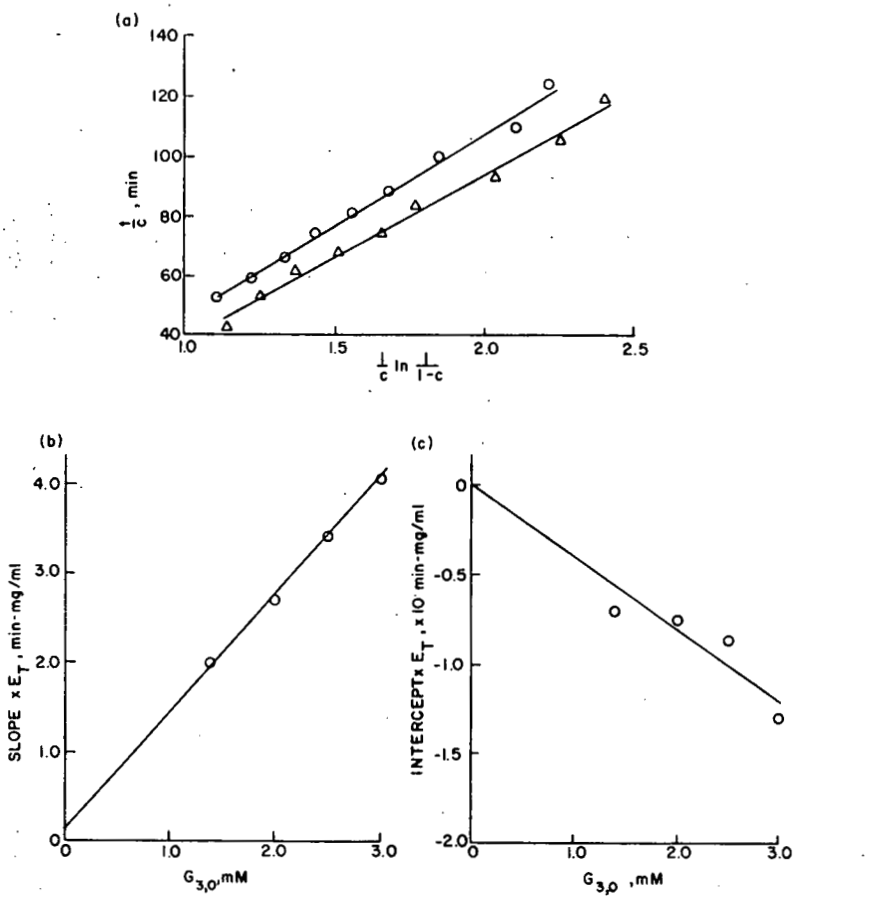


Fig. II-16. Plot of kinetic data of cellotriose (G_3) hydrolysis by cellobiohydrolase; (a) t/c vs. (1/c) ln [1/(1-c)] plot; (b) replot of slopes in (a) as a function of cellotriose concentration; (c) replot of intercepts in (a) as a function of cellotriose concentration. Symbols: E_T total enzyme concentration; $G_{3,0}$, initial cellotriose concentration. (a) (o) $G_{3,0} \cong 2.5$mM; E_T = 0.55 mg/ml. (Δ) $G_{3,0}$ mM; E_T = 0.73 mg/ml/

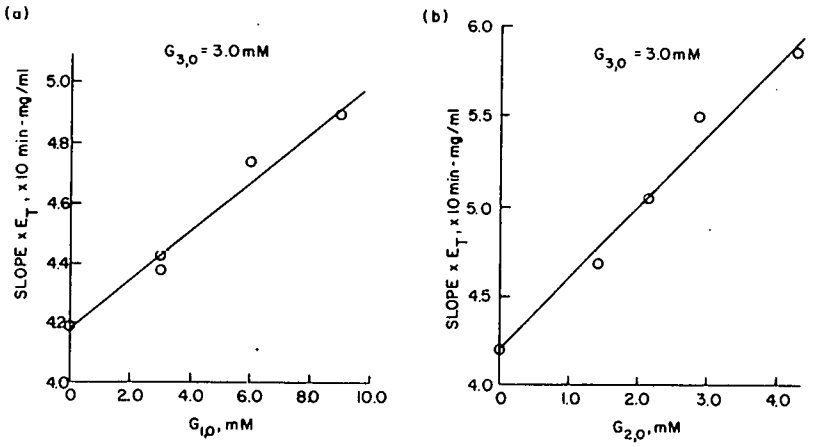


Fig. II-17. Slope vs. initial inhibitor replot of hydrolysis of G_3 by cellobiohydrolase. (a) Replot of slopes as a function of G_1 concentration; (b) replot of slopes as a function of G_2 concentration. Symbols: $G_{1,0}$ initial cellobiose concentration; $G_{3,0}$ initial cellotriose concentration.

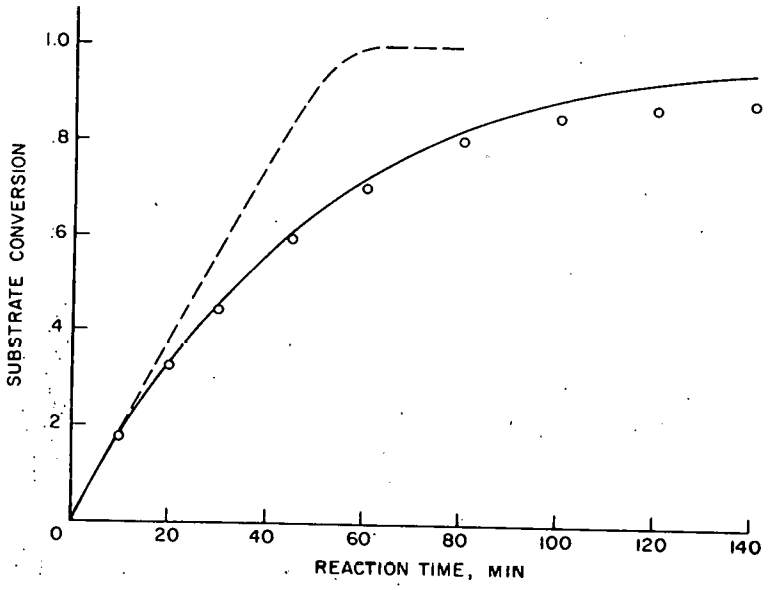


Fig. II-18. Comparison of G_4 hydrolysis by cellobiohydrolase where product is not an inhibitor (---); predicted reaction with product inhibitor (———); and the actual experimental data (O). G_4 = 2.4 mM; E_T = 0.018 mg/ml.

III. THE MODE OF ENZYMATIC HYDROLYSIS OF CELLULOSE

A. Introduction

The hydrolysis of cellulose can be effectively catalyzed both by acid and by the cellulase enzyme. Potentially, the enzymatic catalyst offers the advantages of product specificity and energy conservation. This is to say, the enzymatic reaction can produce a pure, soluble sugar with a relatively low consumption of energy. It also does not pollute the environment and therefore should be a much preferred process over acid hydrolysis. But presently, the efficiency of the cellulosic enzyme is extremely low so that the enzyme cost has been prohibitively high. To imporve the efficiency, in-depth understanding of the function of the enzyme and the mechanism of cellulose degradation are of vital importance.

Due to many recent studies on the mode and kinetics of the enzymatic degradation of cellulose, the phenomena have begun to be understood; yet many controversies remain to be resolved. On the one hand, a bi-modal type of degradation was proposed in which the amorphous regions of cellulose were first degraded leaving the crystalline regions behind (1). On the other hand, a mono-phasic degradation was suggested based on the observed simple first-order kinetics of the reaction (2). A comprehensive analysis of the relevant studies is appropriate prior to presenting new data on the kinetics of the degradation.

1. Apparent First-Order Reaction of Cellulose Degradation.

The simplest form of kinetic behavior for the enzymatic degradation of cellulose was reported by Ghose and Das (3): a pseudo first-order reaction

for the hydrolysis of rice hull cellulose by Aspergilus cellulase was proposed. Thus:

$$t = \frac{1}{k} \ln \frac{S_o}{S_o - P} \qquad [1]$$

where k is the rate constant, S_o is the initial substrate concentration and P is the product concentration. Their experimental data followed the proposed model very closely to about 60% conversion to the sugar product. This was done without introducing any product inhibition factor. Moreover, the traditional weight-loss curves based on their experimental data show simple linear plots extrapolated back directly through the origin (Fig. 1). This behavior is characteristic of a homogeneous reaction as opposed to the bimodal reaction of acid hydrolysis of cellulose (4). Further searches of the literature showed that this phenomenon is not limited to Ghose and Das's study alone. The observations by Shelby (2) and the earlier data by Mandel and Reese (5) on the degradation of cotton cellulose both showed similar reaction kinetics. Their corresponding weight-loss curves are included in Figure 1. As we know, one of the important experimental foundations for the bi-phasic theory--the amorphous and the crystalline phases--is the change in the slope of the weight-loss curve. In the absence of such a change, the reaction can no longer be regarded as bi-phasic but must be considered to be a mono-phasic reaction. This becomes even more apparent when we consider the actual degradation pattern of the long-chain molecule.

Determinations of chain length after enzymatic degradation have also produced controversial results. By far, the majority indicated a very low extent of degradation. Table 1 summarizes the relevant experimental observations in the literature for native celluloses. Except for the case of the Penicillum cellulase, these data all show quite clearly that

with enzymatic degradation cellulose molecules remain essentially intact. Neither the chain length nor the molecular weight distribution has been appreciably altered by enzyme. There is an average of less than one cut for every five molecules in these residues (the extent of weight loss ranged from 7% to 48%). This means that the residual molecules are essentially untouched while those attacked by enzyme are totally disintegrated into soluble sugar. There is essentially no chain of intermediate length left behind. This type of reaction resembles the action of surface peeling whereby the encountered molecules are completely hydrolyzed regardless of the bi-phasic structure--the amorphous and crystalline regions--of the cellulose. Combining this piece of information with the previously collected pseudo first-order kinetics in weight-loss experiments we can conclude that for certain types of native cellulose, the mode of enzymatic degradation apparently follows the mechanism of a mono-phasic first-order reaction. Thus, the mode of reaction can be written as:

$$C \xrightarrow{k} P \qquad [2]$$

where C is the molecular cellulose at the original chain length, P is the product of soluble sugar including cellobiose and glucose, and k is the rate constant for the disintegration of the whole molecule.

2. Multi-Component Mode of Cellulose Hydrolysis.

While the cotton cellulose may behave as a simple homogeneous substrate for the enzyme, many industrial grade celluloses quite often show a two-component or multi-component type of reaction. The hydrolysis data of Howell and Stuck with the Solka Floc cellulose are typical (14). Figure 2 reproduces the weight-loss plot of these data. The lower three curves do show the two-component behavior typical of the acid hydrolysis.

The upper curve (in solid circles) is a stretching of the initial period of hydrolysis which also appears to be a straight line. Thus, it is evident that both components are first-order but with different rates of reaction. This was postulated by Van Dyke as the multiple components system for the enzymatic hydrolysis of cellulose (15). Brandt and co-workers studied the hydrolysis of milled newsprint. Their result gave very strong support to Van Dyke's model (16).

Summing up the above observations, a general formula can be written for the degradation of cellulose:

$$C_i \xrightarrow{k_i} P_i \qquad [3]$$

where there are i cellulose components, each with its own first-order rate constant, k_i. This can be taken as the typical behavior of an industrially processed cellulose. For the simplest case of pure cotton, i = 1 and the time-course equation is reduced to Eq. 1.

The above analyses still did not cover all the cases of cellulose degradation. For instance, when Huang's experimental data (17) were plotted as a weight-loss curve, they followed neither the simple nor the multiple first-order kinetics. Similarly, some of our hydrolytic data on cotton linters cellulose followed Van Dyke's kinetics (opened circles in Fig. 3). But when the condition of the reaction was changed, the kinetics of the degradation also changed (solid circles in Fig. 3). By merely introducing a mechanical agitation to the reaction, both the reaction rate and the mechanism of degradation were apparently altered. Such phenomenon attracted our attention and prompted a search for a logical explanation.

From the examples given above, it is quite clear that the mechanism of cellulose degradation catalyzed by enzyme can be a complicated phenomenon. To explore this reaction, more information than the conventional sugar

analysis or weight-loss data measurement is needed. To this end, we thought of using gel permeation chromatography to follow the degradation pattern of the long-chain molecule. Combining this information with the weight loss data, we hope that a more in-depth analysis can be achieved.

B. Experimental

1. Material.

The cellulose used in this work was acetate grade cotton linters pulp, SR-169, supplied by Buckeye Cellulose Corporation, Memphis, TN. It was ground in a Wiley Mill to a 20-mesh partical size.

The cellulase used was a crude enzyme preparation of Trichoderma reesei, SP-122, supplied by Novo Biochem. Ind., Inc., [Franklinton, NC]. The enzyme (5 gm) was dissolved in 50 ml NaAc buffer (0.1 M, pH 4.8). The solution was centrifuged and decanted to remove the insoluble material. The clear solution was stored in the freezer ($\sim$-10°C) until it was used.

2. Analytical Methods.

Soluble Sugar. The amount of sugar solubilized by enzymatic hydrolysis was determined by the phenol-sulfuric acid method (18). The amount was converted to % sugar yield by division of the total potential amount of sugar in the cellulose sample. The % sugar yield is also call % weight-loss in the preceeding discussion. According to Welseth (6), the measurement of weight loss based on the soluble sugar measurement is essentially identical to the actual gravimetric measurement.

Molecular Weight Distribution. The molecular weight distribution (MWD) of cellulose was measured by gel permeation chromatography of the nitrated sample dissolved in tetrahydrofuran (19). A set of polystyrene columns containing six Shodex columns (perkin-Elmer, Cincinnati, OH)

of the exclusion limits: 5×10^7, 5×10^6, 5×10^5, 7×10^4, 5000 and 1000 molecular weight (4 A-80M/5, 1 A-802/5 and 1 A-801/5) were used. The corresponding calibration curve is shown in Figure 4.

Enzymatic Hydrolysis. Test tubes each containing 100 mg cellulose sample, 4.9 ml of NaAc buffer and 0.1 ml of crude Trichoderma reesei solution were incubated with constant mechanical stirring in a constant temperature bath (50°C ± 1°C) for 2 to 48 hours. The stirring was done with a 12 mm X 9 mm magnetic bar driven by a magnetic stirrer at approximately 1000 rpm. After the designated reaction time, the test tube was taken out of the bath and centrifuged. The filtrate was analyzed for soluble sugar. The solid residue was washed, freeze-dried and nitrated for MWD measurement.

C. Results and Analysis

1. Change in MWD of Residual Cellulose.

A series of GPC elution curves of the residual cellulose that had been hydrolyzed are shown in Figure 5. The yield of soluble sugar measured for each sample is listed in Table 2.

These data show a major change in the MWD resulting from the mechanically agitated enzymatic degradation of cellulose. Most noticeably, a second peak emerged at approximately 150 DP at the expense of the first and high DP peak. As the hydrolysis progressed, the high DP portion decreased and the low DP portion increased. In the last residual sample, almost all the original molecules were broken to leave a single peak centered at 150 DP. To the first degree of approximation, the latter is very close to the LODP of the cotton linters cellulose observed with acid hydrolysis (20).

From the above change in MWD of the residual cellulose, it appears that a considerable fraction of the original cellulose molecules are first

split to an intermediate chain in the process of degradation, perhaps by the preferential attack at the amorphous regions of the cellulose fiber, which left the straight-chain segments in the crystalline regions untouched. The latter segments were apparently disintegrated at a slower rate or in a later occasion. In this way, there was a gradual accumulation of this intermediate segment in the residue. A bimodal distribution of molecular weights therefore resulted.

2. The cuase of Bimodal Degradation.

The present mode of degradation is apparently different from those of the conventional enzymatic hydrolysis (Table 1) in which a consistent chain length and invariant MWD of the residual cellulose were observed. Efforts have been made to explain the differences. The major difference we could detect was that all the previously reported experiments used either a stationary incubation or a shaker incubator while we used a magnetic stirring bar for the agitation. In our case a mechanical shearing force is exerted directly into the cellulose fiber due to the grinding of the bar against the glass wall of the test tube. Such mechanical force is apparently lacking in the other experiments. To test this explanation we repeated the hydrolysis experiment with identical conditions except for replacing the stirring bar by a oscillating shaker. Some representative GPC elution curves for the residual celluloses are shown in Figure 6.

From this figure, we can see that there is essentially no major change in the MWD from the original cellulose. The profound bimodal distribution recorded for the mechanical agitation is not evident here. There is, however, a small shift of the major peak and a slight extension of the low DP tail, suggesting that a very small fraction of the molecule may be split to low DP segments. This type of degradation agrees very

well with the reported observation listed in Table 1. Since the only change here is the method of agitation, it is logical to attribute the difference to this factor. Thus, in the conventional incubation, the fiber is not subjected to a shearing force; the mode of degradation is mono-phasic. The mode of degradation under the influence of the mechanical shear is bimodal. Considering, however, that any shearing force from a magnetic stirrer can be very small and that the presence of the enzyme is essential for the degradation, we would call this technique "mechano-enzymatic degradation".

In this new type of degradation, an appreciable amount of the chain folds or amorphous regions are exposed to the enzyme that otherwise would not be available. A schematic picture of how that could be achieved is shown in Figure 7. The chain folds or weak bonds of cellulose are normally concealed within the fibrillar structure (20) despite the slight dislocations between the crystallites of cellulose fibril (21). When a shear force acts on the fibril, the dislocation could be enlarged exposing more chain folds. However, because of the small shearing force and the plasticizing action of the water, the enlarged dislocation is not permanent. An in situ mechanical force is needed to maintain such exposure. A similar effect was proposed by Kelsey for the wet-milling of cellulose (22).

3. Kinetic Analysis of Bimodal Degradation.

The GPC elution curves in Figure 5 are apparently composed of two major components: one at the original molecular length, C, and the other at the intermediate chain length, I. These curves were resolved into the corresponding components, C and I, with the aid of the digital computer program (23,24). The fraction of each component in the whole sample was calculated (Table 2). Kinetic models were developed to fit these data.

Simple Consecutive Reaction. As observed in this study, the chain folds or amorphous regions of cellulose are preferentially cut. The

linear chain segments emerge as the reaction intermediate. Subsequently, these segments are further hydrolyzed to produce the soluble sugars. Let C represent the amount of molecules of the original chain length; Let I represent the quantity of intermediate chain segments, and let P represent the amount of soluble sugar. The simplest expression of such bimodal degradation can be written as a consecutive reaction:

$$C \xrightarrow{k_1} I \xrightarrow{k_2} P \qquad [4]$$

where k_1 and k_2 are rate constants for the stepwise reaction. By assuming apparent first-order kinetics for each of these steps, the following equations can be written:

$$C = C_o\, e^{-k_1 t} \qquad [5]$$

$$I = C_o\, (e^{-k_1 t} - e^{-k_2 t})\, \frac{k_1}{k_2 - k_1} \qquad [6]$$

$$P = C_o - C - I \qquad [7]$$

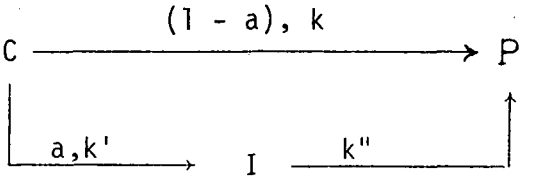
where the amount of each component is expressed as the fraction of total potential sugar. C_o is the initial quantity of the molecular cellulose, which equals 1 in the present case.

By non-linear least squares curve fitting of the experimental data listed in Table 2 (25), the reaction constants, k_1 and k_2, were calculated to be 0.25 h^{-1} and 0.077 h^{-1}, respectively. The sum of squares of the residuals was 76 for this model. The predicted curves and the data points are shown in Figure 8.

The predicted curve C is the only one that follows the general trend of the data points. The predictions based on the other components, I and P, deviated appreciably from the data. Most noticeably, the theoretical yield of soluble sugar was too low in the initial period and

too high after 10 hours of reaction. This S-shaped theoretical curve is characteristic of a simple consecutive reaction. The actual experiment, however, produced soluble sugar much faster in the initial stage and gradually levelled off later in comparison to the theoretical reaction. Thus, this simple model is unable to describe the course of the reaction satisfactorily. A second and more sophisticated model should be considered.

Concurrent Consecutive Reaction. The second model we tried is a combination of Eqs. 2 and 4 and assumes that the substrate can be divided into two fractions and that each fraction may proceed along its own reaction pathway. Thus:



[8]

here a is the fraction of cellulose that undergoes the consecutive reaction to give soluble sugar, (1 - a) is the fraction subjected to surface peeling, k, k' and k" are the rate constants of the surface peeling and the stepwise consecutive reaction, respectively. The kinetic equations based on this model are written as follows:

$$C = C_o \cdot a \cdot e^{-k't} + C_o (1 - a) e^{-kt} \qquad [9]$$

$$I = C_o\, a\, (e^{-k't} - d^{-k"t})\, \frac{k}{k" - k'} \qquad [10]$$

$$P = C_o - C - I$$

$$= 1 - (1 - a) e^{-kt} - a\, \frac{k" e^{-k't} - k' e^{-k"t}}{k" - k'} \qquad [11]$$

The second expression of Equation 8 is reduced for $C_o = 1$ and the concentration of other components are expressed in fractions of total sugar. The kinetic parameters obtained by curve fitting are:

$a = 0.51$
$k = 0.19\ h^{-1}$
$k' = 0.56\ h^{-1}$
$k'' = 0.014\ h^{-1}$

The data points and the predicted time-courses are shown in Figure 9. The sum of squares of residuals for this model is 6.2.

Comparing Figure 8 and Figure 9, we find that, first of all, the concurrent consecutive reaction model is a better fit and is more consistent with the experimental data than is the simple consecutive reaction model. The decrease in the value of the sum of squares of residuals from 76 to 6.2 is a significant improvement in the fitness of the model. Second, this model can satisfactorily predict the sugar yield for up to 24 hours of hydrolysis and 64% conversion. Third, the behavior of the other components, C and I, closely approximate the prediction. For these reasons, we suggest that the concurrent consecutive reaction represented by Eq. 8 can be regarded as the basic mode of molecular degradation of the mechano-enzymatic hydrolysis of cellulose.

D. Discussion

1. Quantum Mode of Enzymatic Degradation.

An outstanding feature can be derived from out present study: the enzyme may normally degrade a particular molecule of substrate entirely, not in parts as has been suggested for the acid hydrolysis. In other words, when cellulose is attacked by the enzyme, these long-chain molecules could possibly be degraded one molecule at a time without

preferential attack on any particular part of the molecule. And until this molecule is totally disintegrated and removed from the reaction site, the enzyme will not leave the first molecule to attack another. A quantum mode of degradation can be assumed. The experimental evidence for this view is the invariant molecular weight measurement previously reported (Table 1) and found in our current study (Fig. 6).

In comparison the mechano-enzymatic reaction is slightly complicated but not different. A fraction of the cellulose is still degraded entirely, i.e., the (1 - a) fraction. The other fraction is rapidly cut to leave the straight-chain segments of LODP. But, from LODP to the soluble sugar (cellobiose and glucose), the transition could also be quantumized. The prevailing and persistent second peak in Figure 5 lends strong support to this hypothesis. Many of the earlier works on the enzymatic hydrolysis of hydrocellulose (such as Avicel) also reported no evidence for reduction of chain length in the residual substrate (7,13).

The cause of the quantum mode of degradation can be explained by surface peeling. Due to the bulky size of the enzyme (whose diameter is at least 20 - 30 Å) (26), these catalysts can only penetrate into the large pores of the cellulose and contact the cellulose molecule on the lateral surfaces on the micro-fibril but not in the spaces between the ends of crystallites (27). Since the cellulose molecules are aligned in parallel to the lateral surface, surface peeling would chop off one layer of these molecules at a time. Molecules in the interior are protected from the enzymatic actions and therefore are intact during the surface reaction.

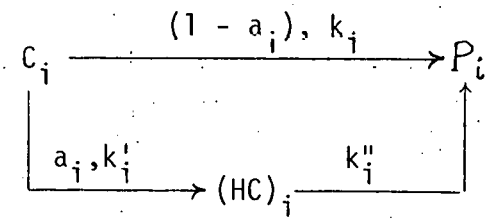
2. A comprehensive Picture of the Enzymatic Hydrolysis of Cellulose.

The hydrolysis of cellulose catalyzed by enzyme is a complex reaction. Many factors relating to the substrate as well as to the reaction conditions

may affect the mode of degradation. According to the folding chain model of cellulose (20), there are three kinds of chains in the elementary fibril, i.e., the highly ordered chain in the crystalline regions, the less ordered chains in the amorphous regions and the chain folds at the end of crystallites. These are shown in Fig. 10-a. Under normal hydrolytic conditions, the enzyme appears to attack only a small fraction of the substrate molecules at a time and leaves the other fraction untouched. There seems to be no preferential cut on the chain folds or amorphous regions. Thus, the molecules left are essentially intact, as represented by Fig. 10-b. In some specific cases, such as in the mechano-enzymatic reaction, many of the chain folds may be preferentially exposed to the enzyme and are cut faster than the linear chains to leave the straight-chain segments at LODP. In this way, the so-called hydrocellulose is formed as the intermediate degradation product. Meanwhile, there are still some of the whole cellulose molecules left intact to co-exist with the newly formed hydrocellulose and, of course, the final degradation product of soluble sugar (Fig. 10-c). This explains the bimodal MWD of the residual cellulose recorded in Fig. 5.

3. A Generalized Kinetic Model of Cellulose Degradation.

Up to the present, we have recognized 3 basic types of molecular degradation of cellulose. These are the apparent first-order reaction of Ghose and Das, the multiple-component first-order reaction of Van Dyke and the currently derived mechano-enzymatic hydrolysis. A generalized model can be constructed to cover all three types of reaction;



[12]

where C_i = cellulose component i, P_i = soluble sugar generated from component i, $(HC)_i$ = hydrocellulose or straight chain segments generated from component i, a_i = fraction of component i that undergoes the consecutive reaction, k_i, k_i', k_i'', = rate constants of component i. Each of the above mentioned mode of degradation can be represented by a suitable assignment of i and a's. The corresponding kinetic equations can be produced by introducing the appropriate subscript i into Eqs. 9 - 10.

For pure cellulose under the normal incubation conditions, i = 1 and $a_i \simeq 0$; Eq. 12 is reduced to Eq. 2 and the kinetic equations are: $C = S_o e^{-kt}$, $I = 0$ and $P = S_o - C$. This last equation is identical to Eq. 1 by Ghose and Das.

For cellulose of multiple components and a specific reaction condition, such as mechanical agitation, both the i's and a's may vary depending on the individual case. In the report immediately following, we will present the results of testing the application of Eq. 12 to the partially and extensively swollen celluloses, which were done in conjunction with the studies of the effect of solvent pretreatment.

4. The Role of Enzyme in the Degradation Process.

The above analysis of the cellulose degradation did not consider the role of enzyme in the reaction. Obviously, the action of the enzyme is of vital importance, otherwise there will certainly be no degradation. And yet the presence of enzyme does not seem to influence the kinetics of the reaction. This situation is possible only if the adsorption of enzyme is much faster than the hydrolysis reaction and if there is not desorption and re-adsorption during the course of hydrolysis. Consulting the literature of the enzymatic reaction, we found that these conditions are possibly met by the cellulose-cellulase system. Mandel and co-workers

reported that once cellulase was adsorbed, it would never be released to the solution phase until the cellulose was disintegrated (28). Ghose and co-workers observed that the adsorption could be complete within the first few minutes of the reaction (29). Castanon and Wilke also suggested that the enzyme once adsorbed remained immobilized on the substrate--even after extensive digestion (30). All these indicate the seemingly chemically active but kinetically neutral role of the cellulase enzyme. We trust, therefore, that our analysis is at least in harmony with these observations.

E. Conclusions

The mode of cellulose degradation catalyzed by enzyme seems to be a complicated system that depends on both the structure of the substrate and the condition of reaction. Under the normal incubation conditions, cellulose is degraded as if it were a homogeneous substrate, and the reaction follows a pseudo first-order kinetics. Under some unusual cirsumstances, however, the reaction shifts to a bimodal type of degradation. A portion of the weak bonds in cellulose may be preferentially cut to give some intermediate chain that reflects the corresponding cyrstalline structure. The other portion can still be degraded directly and completely to soluble sugar. A concurrent consecutive reaction model is proposed for this type of degradation and the predicted kinetic behavior based on this model are found to follow the trend of the experimental data quite satisfactorily. The extension of this model to explain the various modes of cellulose hydrolysis is discussed.

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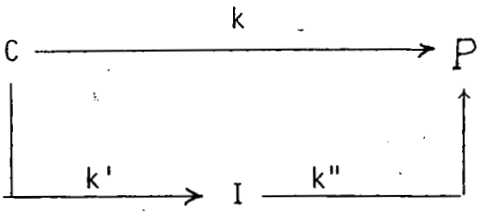
ABSTRACT

The mode of cellulose degradation was studied by analysis of the molecular weight distribution of the residual celluloses during enzymatic hydrolysis. Both the rate and mode of degradation could be altered by a mechanical force acting on the substrate. A bimodal degradation reaction replaced the conventional mono-phasic reaction. The concurrent consecutive model was derived to account for the "mechano-enzymatic" degradation:



where C is the molecular cellulose, I is the intermediate chain or hydrocellulose, P is the product of soluble sugar, and k, k', and k" are the rate constants. This model predicted the kinetic behavior of the reactants, C, I and P, quite satisfactorily for the hydrolysis of cotton linters cellulose. The extension of this model to explain the various modes of cellulose hydrolysis is discussed.

Table 1. Molecular Weight Measurements in Enzymatic Hydrolysis of Cellulose

Year	Substrate	Enzyme	Molecular Weight (DP) Before Hyd.	Molecular Weight (DP) After Hyd.	Reference
1952	Cotton Linters	Aspergillas	1385	1105	6
1957	Cotton	Trichoderma	4970	4200	7
1959	Wood Pulp	Trichoderma	900	800	8
1965	Wood Pulp	Penicillum	1150	400	9
1966	Cotton	Trichoderma	1320	1050	10
1967	Cotton	Basidiomycete	2260	1910	11
1967	Sulfite Pulp	Trichoderma	No change in MWD[a]		12
1978	Cotton	Trichoderma	1800	1490	13

[a]MWD for Molecular Weight Distribution.

Table 2. Sugar Yield and GPC Curve Resulation of Enzymatic Hydrolysis of Cotton Linters Cellulose

Hydrolysis Time (h)	C		I		P
	Position	% Yield	Position	% Yield	% Yield
2	28.0*	52.4	34.0	34.2	13.4
4	27.8	25.9	33.9	42.9	31.2
6	27.3	15.3	34.0	49.7	35.0
8	29.0	15.2	35.0	41.5	43.3
12	29.5	3.1	35.0	48.5	48.4
24	30.0	1.8	35.0	34.1	63.6

*All positions are in elution volume, ml.

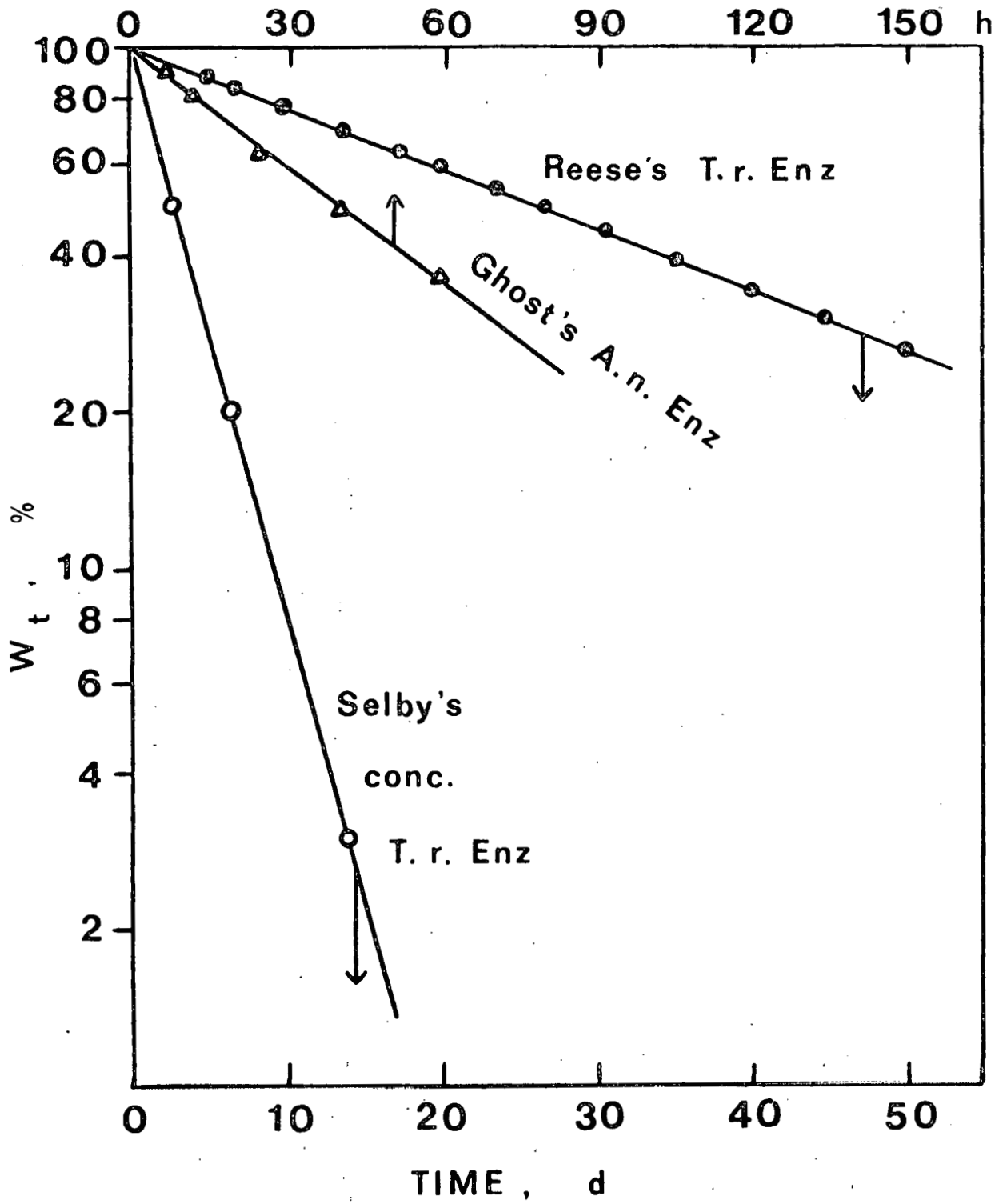


Figure 1. Weight Loss Curves of the Simplest Type of Enzymatic Hydrolysis of Cellulose.

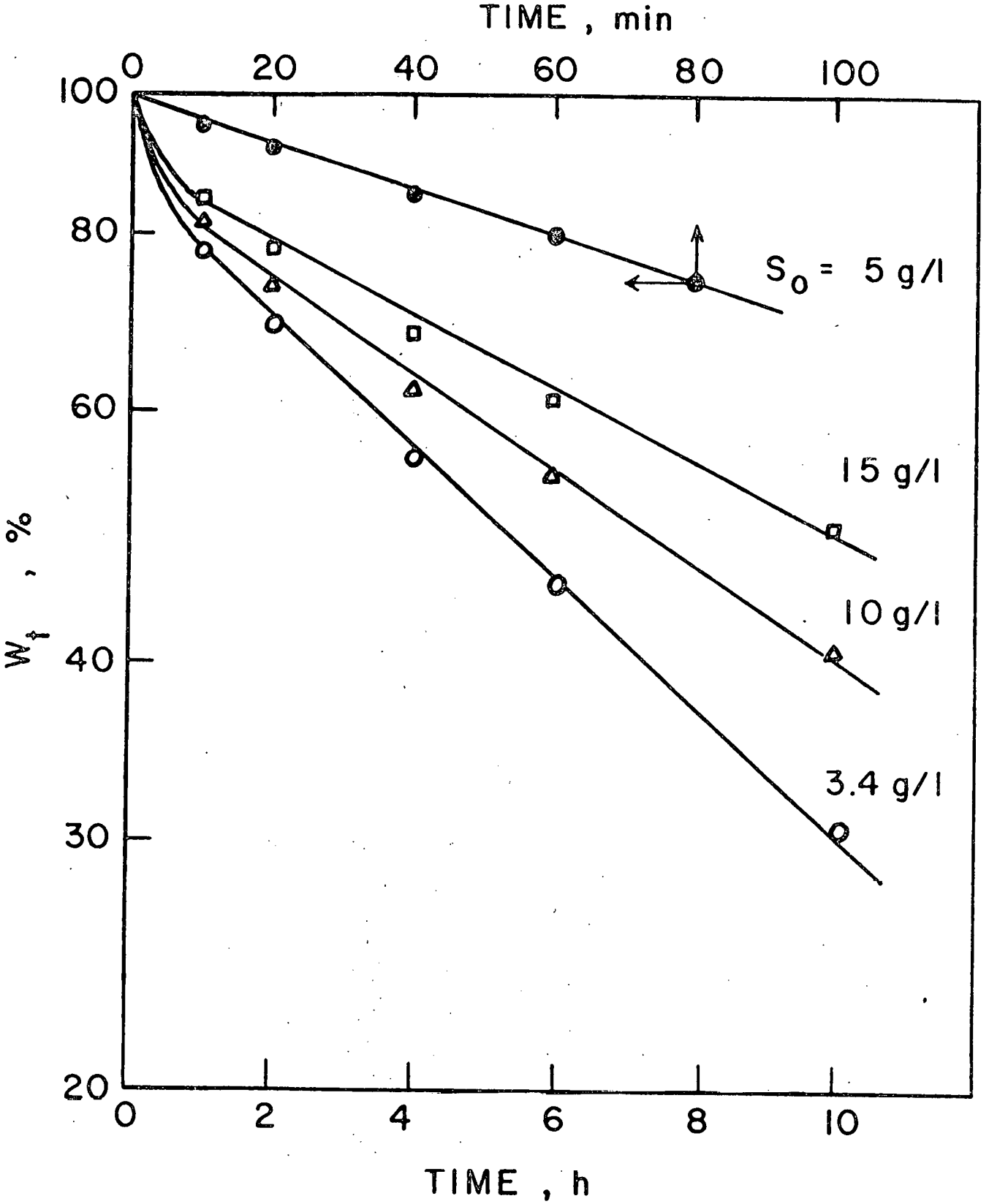


Figure 2. Weight Loss Curves of Enzymatic Hydrolysis of the Solka Floc Cellulose (Howell's Data).

S_o = Initial substrate concentration.

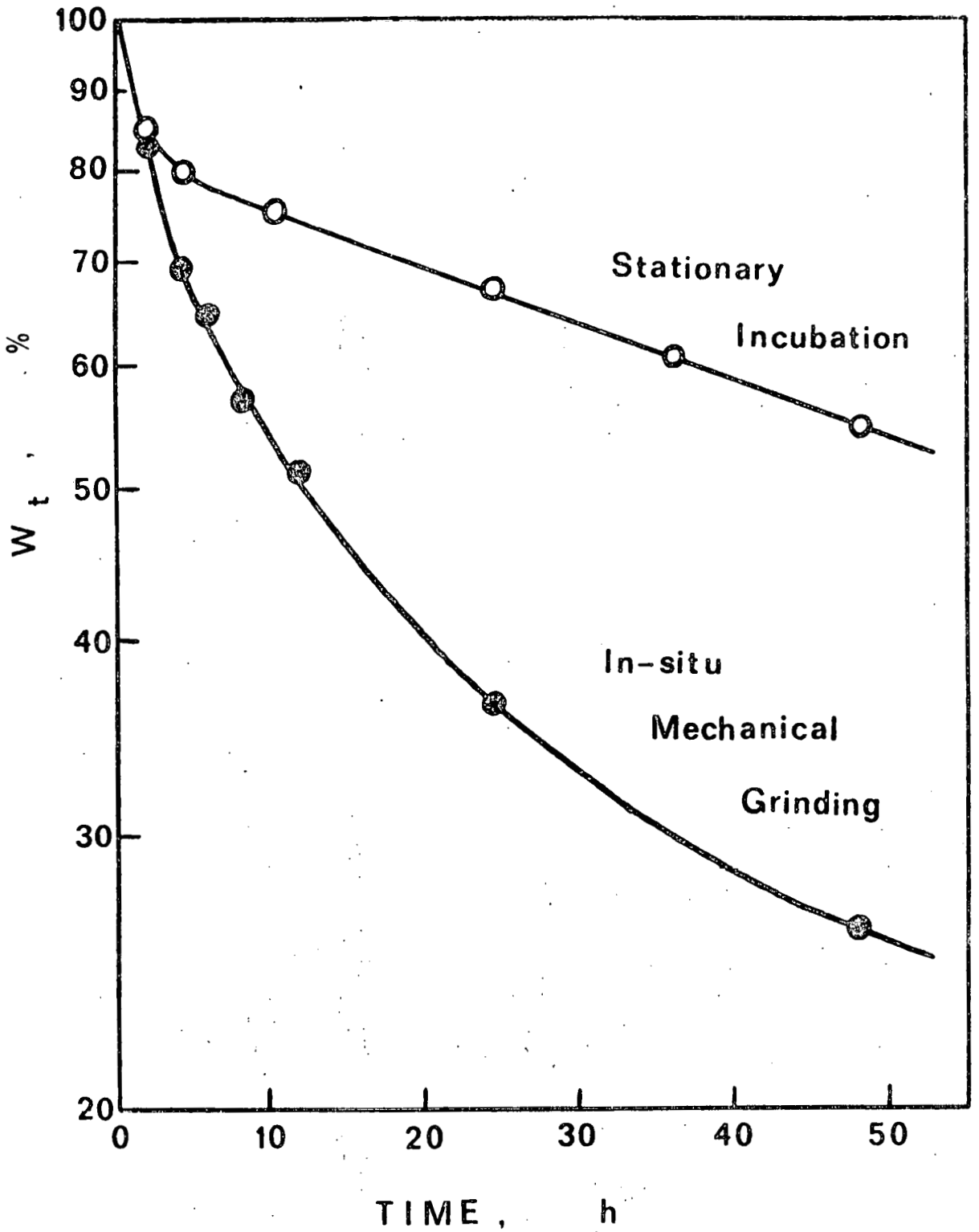


Figure 3. Weight Loss Curves of Enzymatic Hydrolysis of Cotton Linters Cellulose.

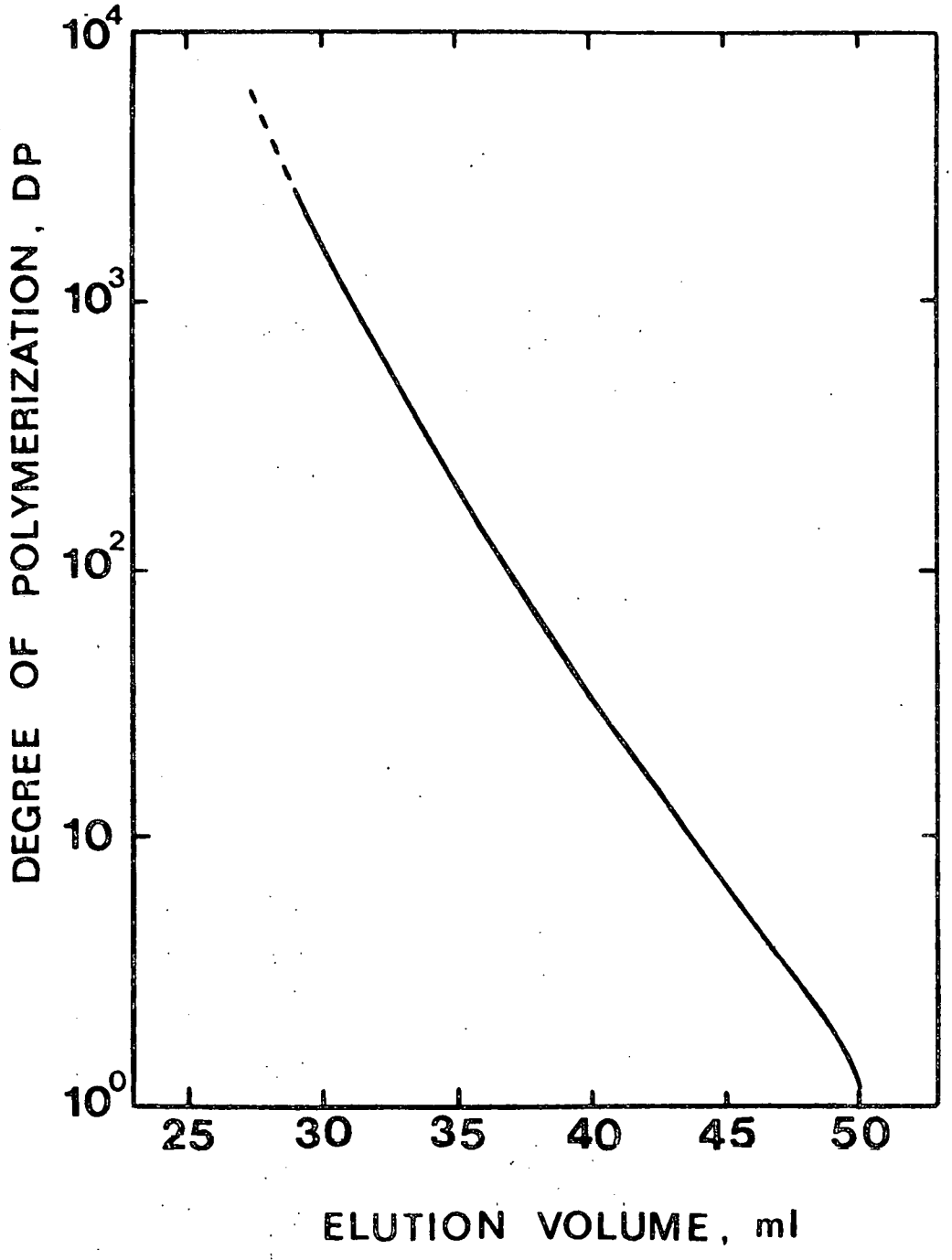


Figure 4. Calibration Curve for GPC.

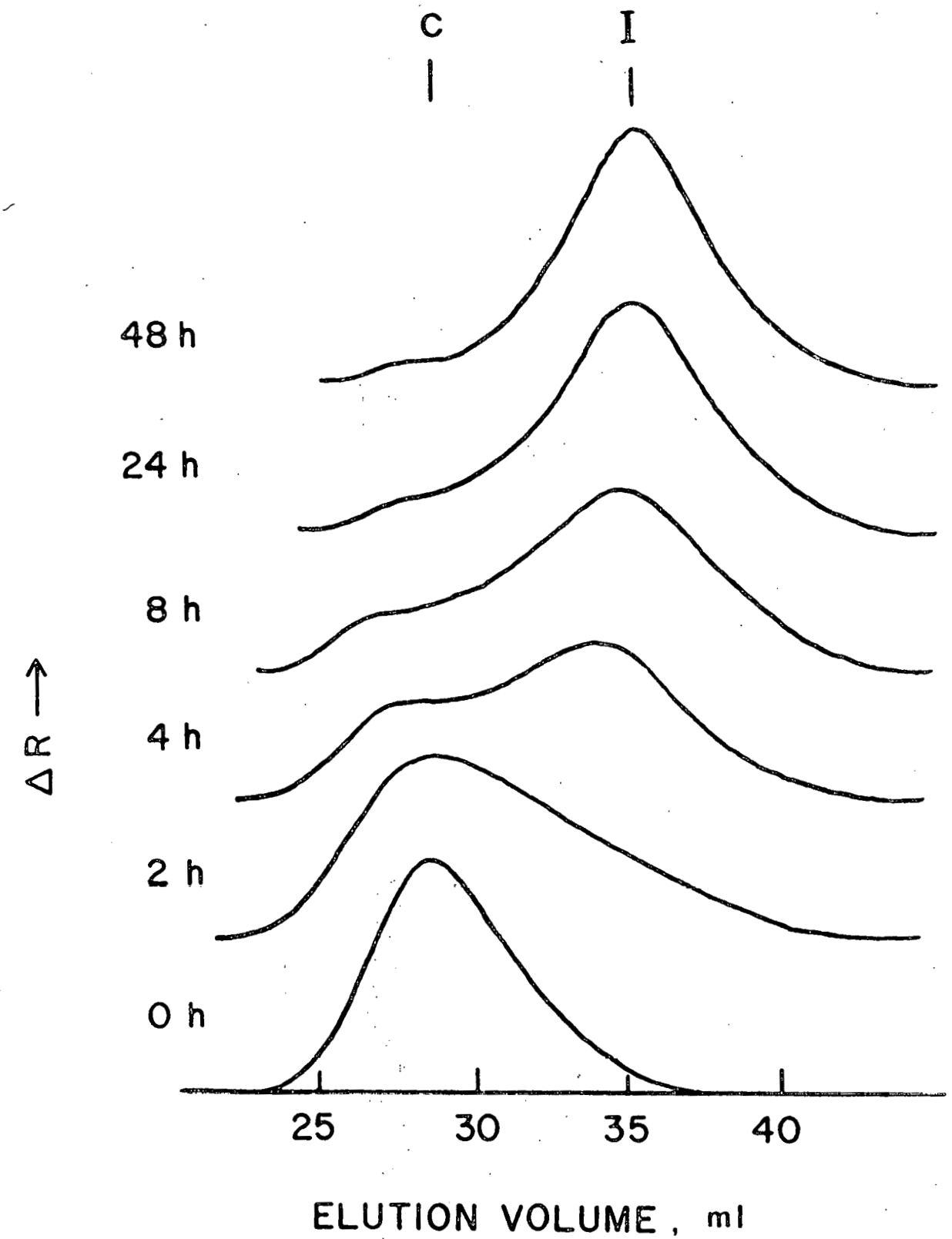


Figure 5. GPC Elution Curves for the Enzymatic Hydrolysis of Cotton Linters Cellulose Under Mechanical Action.

C = Molecular cellulose I = Hydrocellulose.

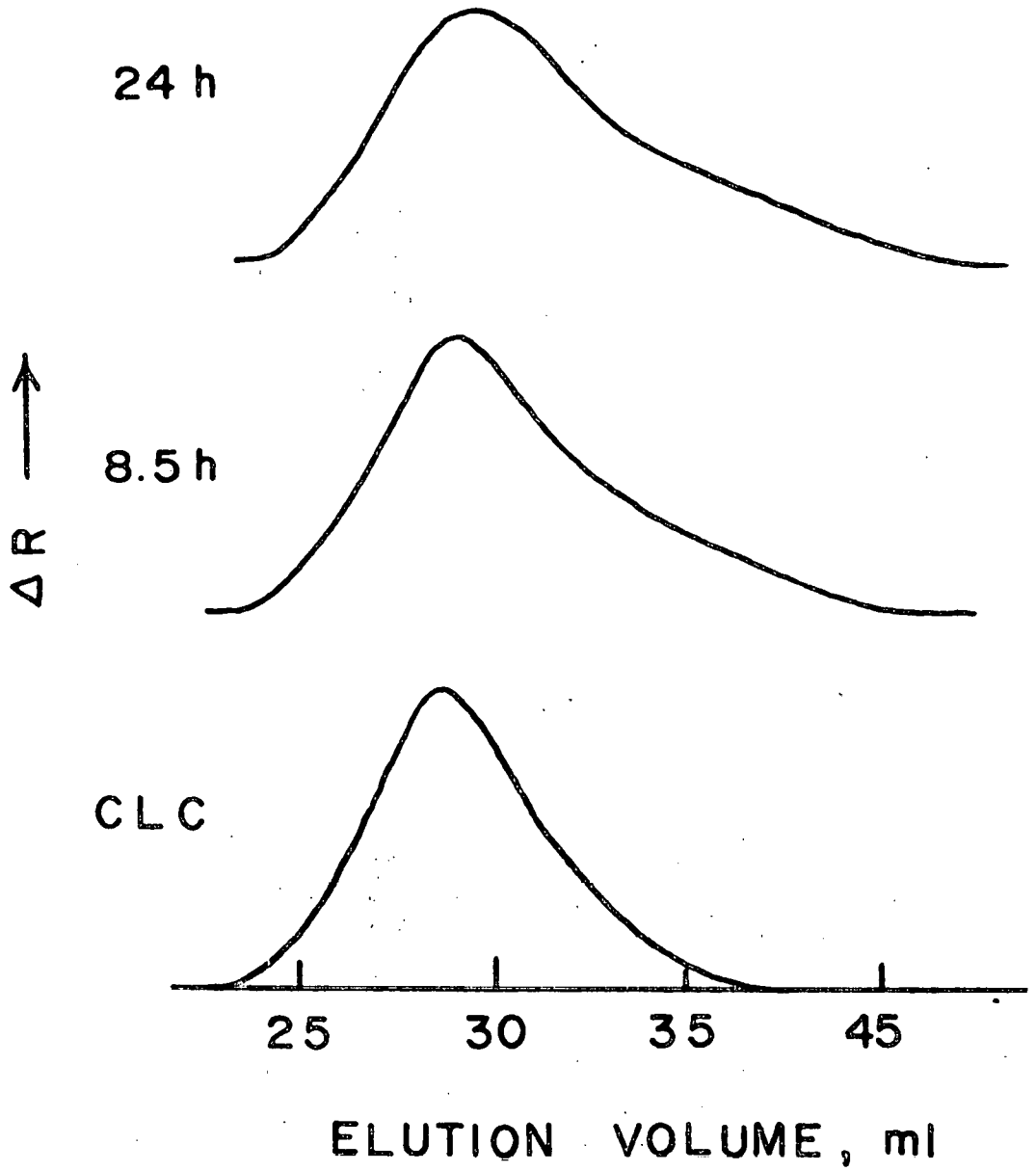


Figure 6. GPC Elution Curves for the Enzymatic Hydrolysis of Cotton Linters Cellulose Under Normal Incubation.

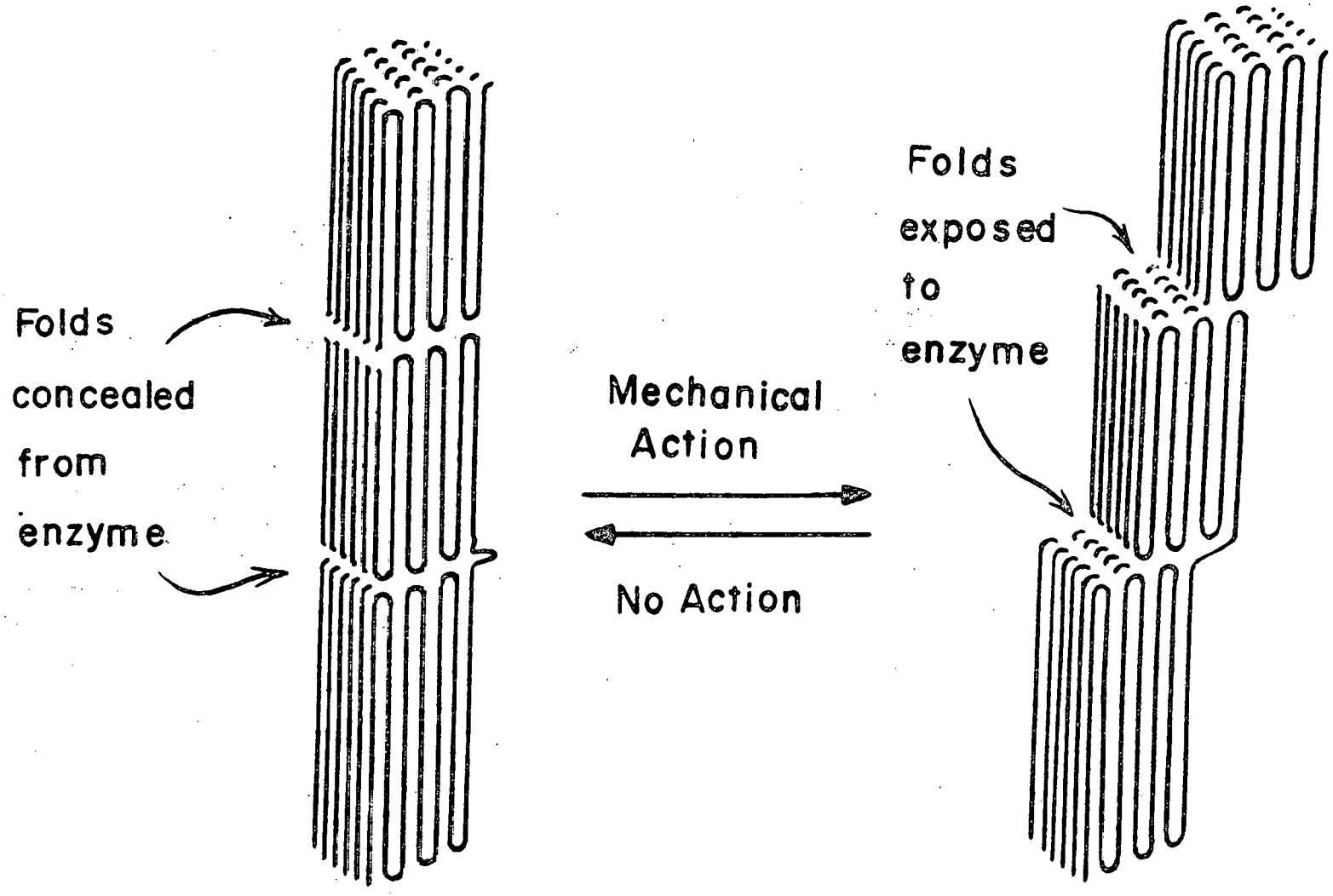


Figure 7. Schematic Deformation of Cellulosic Elementary Fibril by the Action of Mechanical Grinding.

Fig. 7

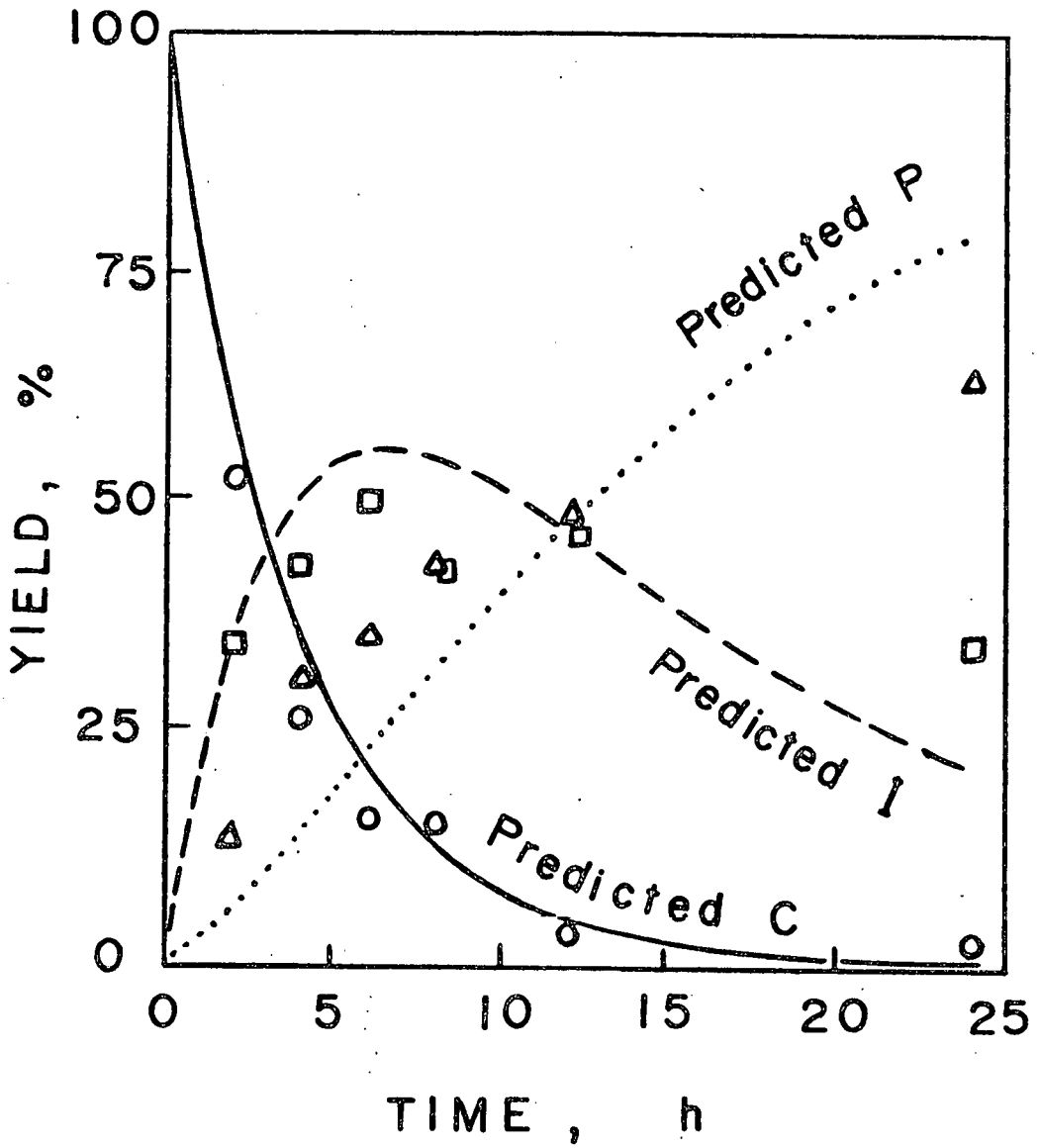


Figure 8. Kinetic Plots of Enzymatic Hydrolysis of Cotton Linters Cellulose According to the Simple Consecutive Reaction.

O, □, Δ : Experimental data points for the molecular cellulose, the intermediate chain and the product of soluble sugar respectively.

C, I, P: Molecular cellulose, intermediate chain and product of soluble sugar respectively.

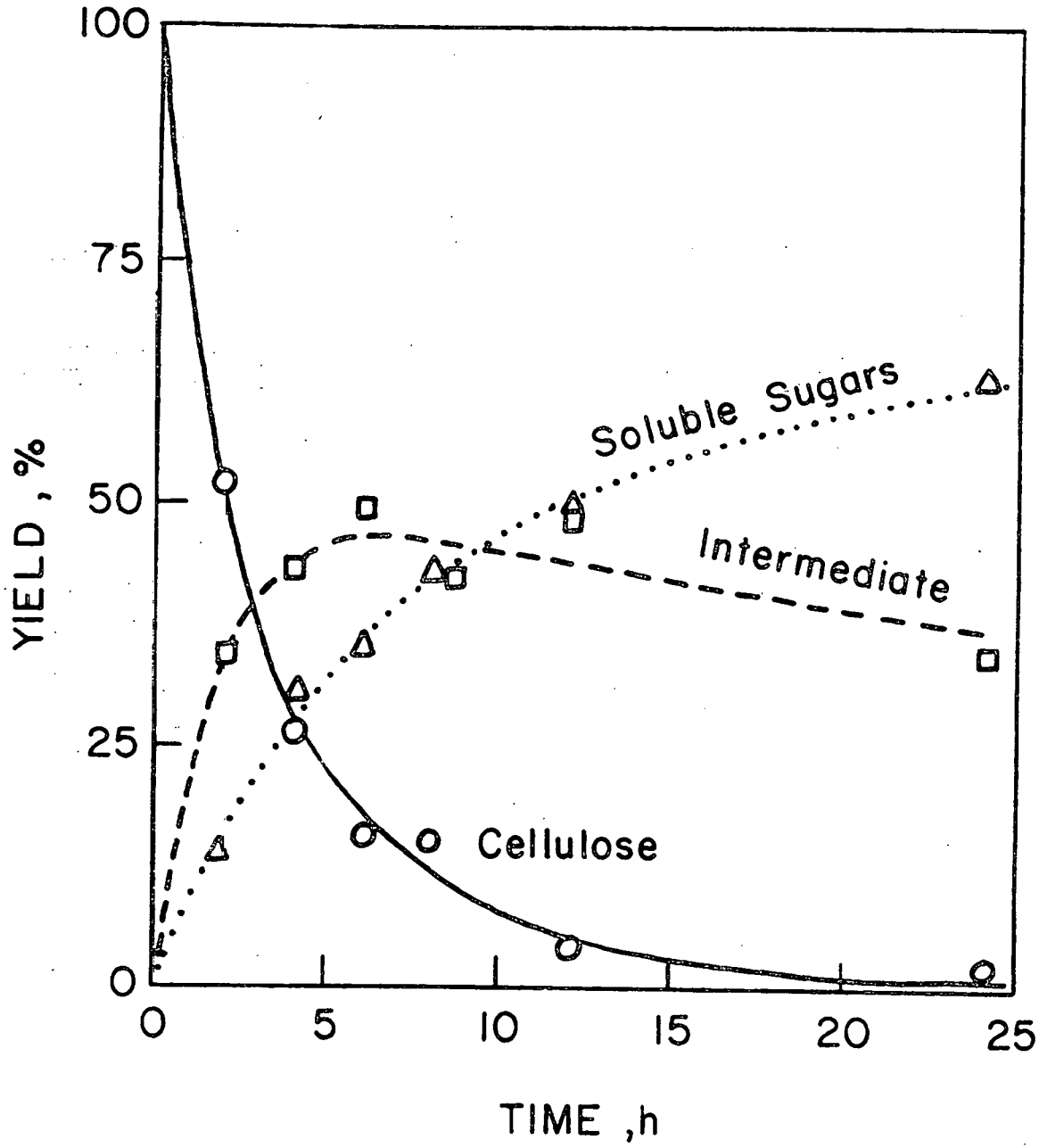


Figure 9. Kinetic Plots of Enzymatic Hydrolysis of Cotton Linters Cellulose According to the Concurrent Surface-peeling and Consecutive Reaction.

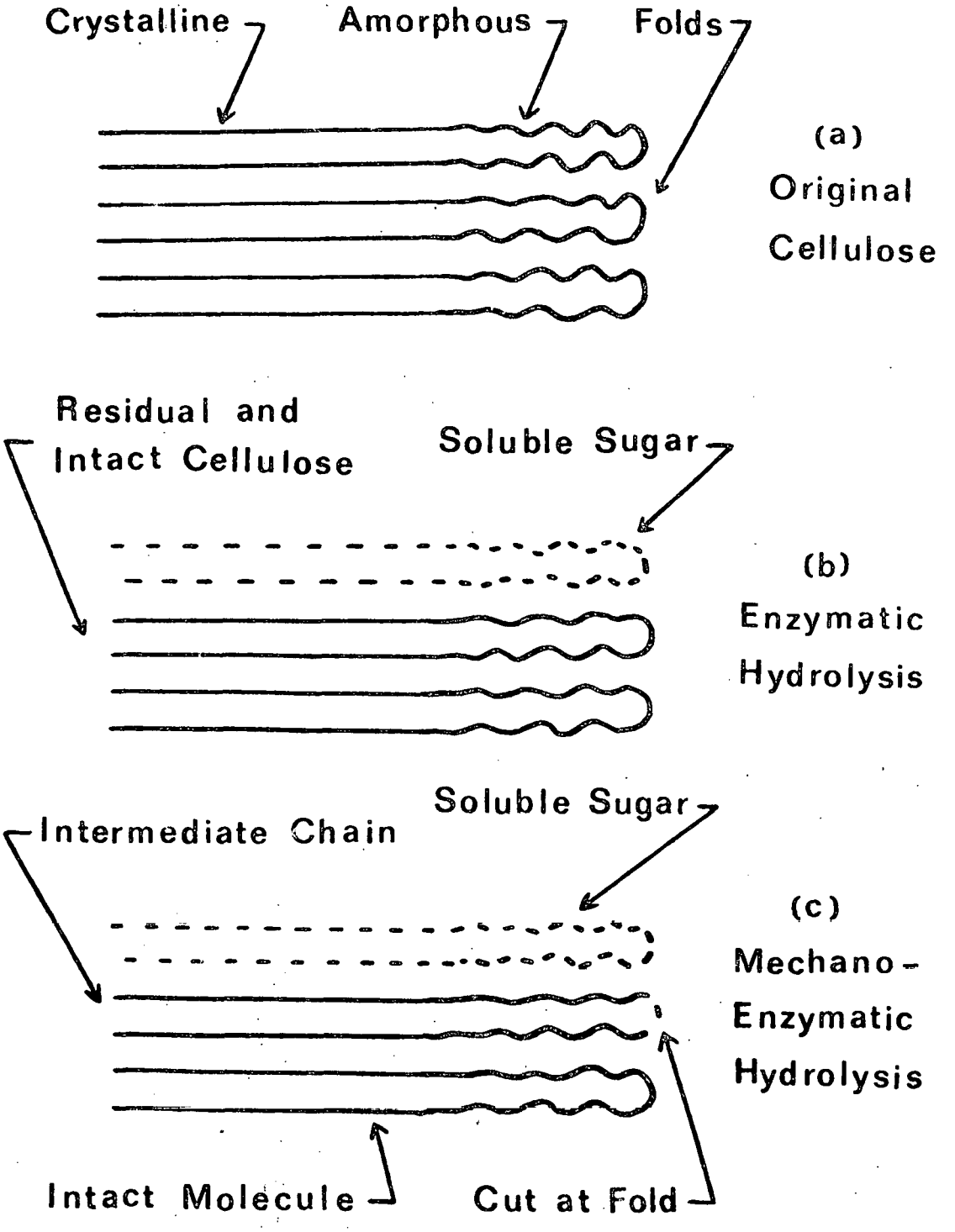


Figure 10. Schematic Mode of Enzymatic Breakdown of Cellulose Molecule.

IV. Helical Structure and Conformation Analysis of Cellulose

A. INTRODUCTION

Cellulose and starch are two major components of the polysaccharide family. They all belong to the poly-condensation products of pure glucose. There is no difference between them in terms of the chemical composition. But the glucosidic bonds connecting the monomeric units are in different conformations. With its β-glucosidic bonds, cellulose forms primarily a linear chain. On the other hand, starch and particularly amylose are conformed to a helix structure for their α-linkages. As a result of this stereo-structural difference, there are ample diversity in bothe the physical and the chemical properties among these two polymers which are well-known in the carbohydrate chemistry.

In our study of the effect of solvent pretreatment of cellulose for enzymatic hydrolysis (1), we observed what we thought was a transition of the linear-chain cellulose molecule into a coil structure strongly resembling a starch molecule (or more accurately an amylose molecule). That is, when cellulose was immersed in a solvent such as concentrated sulfuric acid, the solid substance gradually swelled and eventually dissolved. The cellulose could then be recovered by either neutralization or simple dilution of the acid to give a highly reactive substrate readily accessible to cellulase enzyme. While still in the solution, however, we found that the swollen cellulose could be stained to a deep blue color by an iodine-iodide solution. In addition, we also found a fraction of the regenerated substance could be hydrolyzed to glucose by an amylase enzyme. The latter is supposed to degrade only amylose, not cellulose. These observations are reported in this communication.

B. EXPERIMENTAL

1. Material.

The cellulose samples used in this work were a commercial microcrystalline cellulose, Avicel pH-101, supplied by FMC Corp., Philadelphia, PA, the Whatman cellulose powder CF-11 and the No. 1 filter paper, by Whatman LTD., England.

The enzyme used was a purified glucoamylase, SP105, supplied by Novo Biochemical Industries, Inc., Franklinton, NC. The enzyme (50 mg) was dissolved in 20 ml NaAc buffer (0.1 M, pH 4.8). The solution was centrifuged and decanted to remove the insoluble material. The clear solution was stored in the freezer ($\sim$ 10°C) until it was used.

2. Solvent Pretreatment

Two gm cellulose was immersed in 40 ml solvent (H_2SO_4 75% w/w, H_3PO_4 85% w/w, $ZnCl_2$ 72%, NaOH 30%, respectively) at room temperature with sufficient agitation. Twenty minutes pretreatment time was used for H_2SO_4 and overnight for the other solvents. The regeneration was done by dilution in 1 lt ice-water. The substrate was then centrifuged, decanted and washed free of the solvent with water and freeze-dried. Some substrates had been dried through a different process such as solvent-exchanged to benzene and freeze-dried. These are marked in the Table accordingly. Cadoxen was prepared according to the standard procedure (2).

3. In-situ staining

A drop of the cellulose-solvent mixture was spread over a glass slide. To this was added a drop of 1% I-KI solution and mixed. The sample was then covered by a covering glass and observed under an optical microscope for the color development. In the absence of the solvent, a pure, native cellulose was normally stained to a brown color and a hydrocellulose such as Avicel would not be stained (NS).

4. Amylase Activity

Test tubes each containing 50 mg substrate, 2.0 ml of NaAc buffer (0.1 M, pH 4.8) and 0.1 ml of glucoamylase solution were incubated in a constant temperature bath (50°C ± 1°C). After 24 hours, the test tubes were removed and cooled by vigorous shaking in an ice-water bath. These were then centrifuged. The clear solution was analyzed for glucose content by a Beckman Glucose Analyzer.

C. RESULTS AND ANALYSIS

1. Iodine Staining and Glucoamylase Activity

The in-situ iodine staining of the dissolved cellulose in acid solvents responded instantaneously. Instead of the brown color of cellulose staining, an intensive blue color was developed which was characteristic of the starch solution (3). The cellulosic samples were then precipitated and recovered for the glucoamylase activity test. These results are collected in Table 1.

The table shows that the amylase activity goes hand-in-hand with the blue color devleopment in the in-situ staining experiment. Both the soluble starch (SS) and the cellulosic samples 1-5 reacted positively to the in-situ staining. They were also digested to some extent at least, by the glucoamylase enzyme to give the desirable product--glucose. Secondly, samples that showed a negative response (NS) to the in-situ staining also gave negative amylase activity. Samples 6-10 belong to this catagory. It should be pointed out that sample 8 was also dissolved in a cellulose solvent--Cadoxen. Apparently, the configuration of the dissolved cellulose in this alkali solvent (Cadoxen) was a random coil (4) which does not provide the proper structure required to form the iodine complex for the blue coloration. Thirdly, the possibility of any

cellulase contamination of our glucoamylase enzyme is ruled out. Samples 6-12 contains a variety of amorphous and crystalline celluloses including the control sample of Avicel (#10 of Table 1), the amorphous cellulose (#12) by ball-milling of the Avicel, the dissolved and regenerated Avicel in Cadoxen (#8), and the mercerized cellulose (#6). All these samples show a negative response to our amylase system. Therefore, we are quite certain that there is neither cellulase contamination in our amylase system nor starch contamination in the cellulose sample. The corresponding glucoamylase activities observed for samples 1-5 are the direct results of the solvent pretreatment done on the cellulose.

The blue coloring reaction of the solvated cellulose is a strong indication of the presence of a tight helical coil (2). The iodine molecules are trapped into the cavity of the polysacchride helix to slow an intensive blue color. The positive response in the glucoamylase assay further supports such a spatial structure because enzyme is supposed to recognize the substrate by shape. However, the blue coloring of cellulosic materials had been observed as far back as the turn of this century (5, 6, 7) and the term "amyloid" and even "starch" were named for this type of substances. Presumably it was then thought that a conversion of cellulose to amylose or starch was achieved. We do not think this was the case and our account of the matter is given below.

2. The Conformation Analysis of Cellulose Molecule

As it is well known, the 1, 4-β-glucosidic linkage of cellulose is composed of two single bonds, C1-O and O-C4', connected to the central bridge oxygen atom. In native, solid state cellulose, this linkage is reinforced by a pair of intramolecular hydrogen bonds between the two neighboring glucose units (8). As a result, the molecule of native cellulose is fairly stiff, forming a relatively ordered, straight chain. However,

under conditions whereby hydrogen bonds are released, the adjacent glucose residues may rotate about their bonds to the linkage oxygen. The empirical energy calculated at various rotations about the bonds to the linkage oxygen may be used to define a conformation energy map (8, 9). Figure 1 is a map for cellulose that has not hydrogen bonds. We traced the potential valley in the map to show the possible pathways connecting the theoretically preferred conformations of cellulose (i.e., the energy minima C and L). On top of these, we further incorporated information of the molecular screw sysmmtry according to Ramakrishan (10).

The conformational energy map (or simply ϕ-ψ map) can be regarded as a graphical presentation of the potential energy of bond rotation with the torsion angles ϕ (H1-C1-O-C4') and ψ (C1-O-C4'-H4') as the coordinates. A high potential value means unstable conformation and vice versa. From the figure, we can find two potential minima of interest, C and L, at -4.0 and -1.7 Kcal/mol respectively (U is a relatively unstable form at 1.2 kc/mol). The potential valley (broke line) connecting these two minima has a peak potential of 3 Kcal/mol which is a very low potential barrier for bond rotation. For these two stable conformations, C and L, line n = 2 cross the former and n = -6 passess by the latter. These tell us that the first and the normal β-linkage (C) is a two-fold screw and the second and abnormal β-linkage is a left-handed 6-fold helix (L). The rise per residue or h value calculated for the second conformation has a value of 2 Å. In an earlier analysis (11), this second stable conformation (L) was termed the "looped β-bond" or "β_L". For 3 consecutive glucosidic linkage at this conformation, the molecule would make up half a loop and reverse the direction of the chain. This was the basis for the proposed chain folding in the cellulose crystal. If a series of 6 bonds were to have the β_L conformation, this section of molecule would

assume a complete loop of 360°. Molecules of this shape would be a close structural analogue of the amylose molecule (12). On account of the low potential barrier along the rotation pathway between C and L, we suggest that the transition from the linear chain to the helix structure is possible through the bond rotation of the glucosidic linkages. The blue color development in our iodine staining test is an evidence of such a transformation.

A picture can now be visualized that when cellulose is dissolved (or more accurately swollen) in an acid medium, the molecule may assume a 6-fold helix conformation. If iodine is added at this time, the latter will be trapped into the cavity of the newly formed helix to exhibit the blue color. Chemically speaking, the helix is still the 1,4-β-bond of the cellulosic linkage, not the α-bonds of the starch molecule. But, as far as the stereo-structure is concerned, the cellulosic helix can be in many respects, very closely resembling the amylosic helix. In fact, it is so close that it is indeed perceived, at least in part, by the amylase enzyme we used in the assay procedure.

Because the transition between the linear and the helix form of cellulose is achieved by rotation about linkage bonds, the transition should be reversible.

$$\beta \rightleftharpoons \beta_L$$

The potential barrier for the forward reaction is 7 Kcal/mole and for the reverse reaction, 5 Kcal/mole, according to the corresponding potential minima. The reverse reaction should be proceeded with greater ease than the forward reaction. We indeed found that this is the case--the staining capability of the dissolved cellulosic was gradually reduced through the

washing steps in our sample recovery process. Apparently, the once-helical structure was rotated back to resume the straight chain conformation during the regeneration. Eventually, we got mostly the pure, normal cellulose back in the form of the highly reactive, cellulose-II (13, 14).

D. DISCUSSION AND CONCLUSION

From the above analysis, we are able to attribute the blue color formation of the in-situ iodine staining as being the interaction between the iodine molecule and the helical form of cellulose. In this form, an inner lining of a pure C-H groups is resulted to provide the hydrophobic condition for the entrapped iodine to show the characteristic blue color (15). However, the formation of the helical cellulose could be meta-stable so that upon the removal of the solvent the substrate reassumed the normal form. This accounts for the very low glucoamylase activity we could detected with the recovered samples. If we take the glucose yield as an indication of the amount of helical cellulose presented in the substrate, the best sample in Table 1 has a mere 4% (sample #3). The rest of the substrate is, of course, the normal, regenerated cellulose.

Due to this small amount and unstable nature of the helical form, it is impractical to characterize the substance. Attempts have been made to increase the extent of conversion. One of the promising means we tried was the use of the volatile acid solvent--40% HCl aq. in this case--and the recovery by the direct freeze-drying of the cellulose-solvent system. Substrates recovered in this manner showed glucose yield of about 8% in the standard glucoamylase assay (24 hours incubation) and the yield was continuously increased up to about 20% in 10 days. The improvement in conversion was significant. Further investigation in this area should be continued.

In conclusion, we propose that the pretreatment of cellulose with acid solvent may induce a helical conformation for the macromolecule in solution. This new conformation resembles, in many respects, the structure of the amylose in the amylose-iodine complex. It is responsible for the blue color staining of the iodine solution and can be partially digested by a purified glucoamylase enzyme.

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Table 1.

Amylase Activity and Iodine Staining of Starch and Solvent Pretreated Celluloses

Sample	Pretreatment	Description or Post-treatment of Sample	Amylase Activity Glucose (mg/dl)	Color In-situ Staining
SS	----------	Soluble Starch	500	Blue
1	H_2SO_4	Wash in H_2O, FD[a]	79	Blue
2	H_2SO_4	Acetone wash, AD[b]	73	Blue
3	H_2SO_4	Sol. Exch. FD[c]	97	Blue
4	H_3PO_4	Sol. Exch. FD	35	Blue
5	$ZnCl_2$	Sol. Exch. FD	20	Blue
6	NaOH	Mercerized Avicel	0	NS[f]
7	NaOH	Mer. & Hyd. Cellulose[d]	0	NS
8	Cadoxen	Wash in H_2O, FD	0	NS
9	---------	Watman Cellulose Powder	0	NS
10	---------	Avicel	0	NS
11	---------	Filter Paper	0	Brown
12	Ball Milling[e]	Amorphous Cellulose	0	Dark Brown

a: Freeze-dried

b: Air-dried

c: Solvent-exchange and freeze-dried in benzene

d: Mercerized and hydrolyzed filter paper cellulose

e: Ball-milled 28 hours on Avicel cellulose

f: Not stained

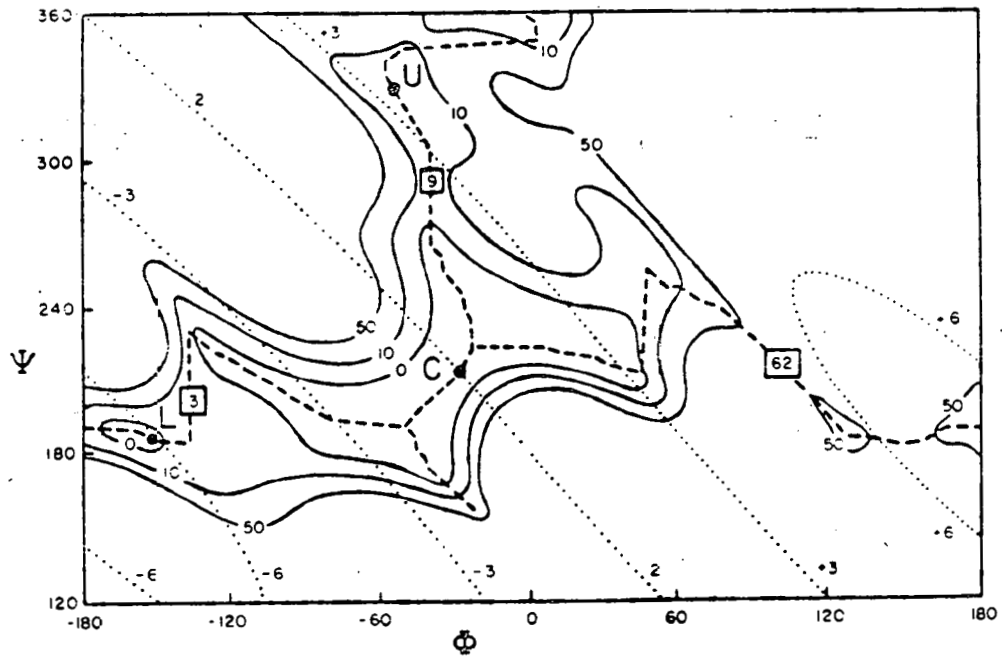


Figure 1. Conformation Energy Map of Cellulose at Zero Hydrogen Bonding. Solid lines: Potential energy contours (numbers 0, 10, 50 in unit cf Kcal/mole).
Broken lines: Potential valley pathways
Dottec lines: line of screw symmetry, [3] : peak potential energy at 3 Kcal/mole, etc.

Helical Structure and Conformation Analysis of Cellulose

ABSTRACT

A helical molecular structure could be generated by the treatment of cellulose with an acidic solvent. The structure adsorbed iodine to show a characteristic blue color and was partially digestible by glucoamylase. From the conformation energy analysis of the β-glucosidic bonds, it is postulated that the transaction can be resulted from the bond rotations of the β- to the β_L- conformation of the cellulose molecule.

V. Effect of Solvent Pretreatment on the Structure and Reactivity of Cellulose.

A. Introduction.

Cellulose is very resistant to enzymatic hydrolysis to produce glucose. A pretreatment is required to enhance the reactivity. To this end, we have developed a series of chemical pretreatments that use either a basic or an acidic cellulose solvent known as the Purdue solvent process (1, 2, 3). The effect of the basic solvent--cadoxen--on the crystalline structure and reactivity of cellulose has been reported elsewhere (4). This report describes the use of the acid solvents, particularly phosphoric acid and sulfuric acid, and the corresponding enhancement of the enzymatic hydrolysis of cellulose.

Some background information for the study is first presented.

1. Crystalline Structure of Cellulose.

Cellulose is a linear, long-chain molecule. In nature, it is a quasi-crystalline fiber. Figure 1 shows the schematic alignment of the cellulose molecule in the fibrous crystal--the so-called crystallite or elementary fibril (5). Basically, cellulose molecules are visualized as being folded back and forth along the fibrillar direction within the (101) plane of the crystalline lattices. This plane corresponds to the tangential plane of the fiber. The average fold length is associated with the so-called leveling-off degree of polymerization (LODP). This

makes up the basic molecular unit of the cellulose fiber (5). As much as 1000 DP can be accommodated within this basic unit (from "a" to "b" in Fig. 1). The rest of the chain exceeding the 1000 DP will enter into another crystallite unit immediately above or below, along the same elementary fibril. Accordingly, there are straight chains and folded chains in the molecule, and there are crystalline regions and amorphous regions along the fibril. The cross-sectional views of the crystallite also show the relationship of the Mayer and Misch unit cells (solid boxes) in the fibril (6).

Cellulose is also a polymorphous material. Its crystalline structure may exist and co-exist in a number of stable forms. These are known as cellulose-I, -II, -III, -IV and -X (7). In general, the most important structures are cellulose-I and -II (Fig. 1). The former is the crystalline form of the native cellulose, which can very easily be converted to the latter form, i.e., the regenerated cellulose. Treatments of the cellulose, such as those that result in intracrystalline swelling, dissolution, and regeneration, are sufficient to produce conversion.

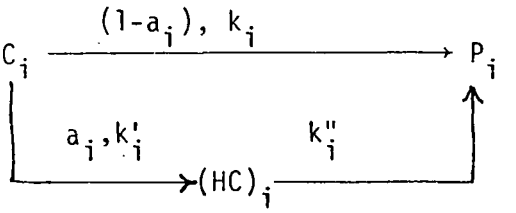
2. Cellulose Solvents.

Due to the strong hydrogen-bonding among the molecules, cellulose is normally insoluble in many common solvents. Almost all cellulose solvents are a strong acid, a strong base or a reagent capable of forming a complex with cellulose. By far, the simplest and cheapest solvents are strong acids (concentrated sulfuric, phosphoric and hydrochloric acid). Sulfuric acid that is about 65% by weight, phosphoric acid that is about 85% and hydrochloric acid that is about 40% are all capable of swelling and eventually dissolving the cellulose (4). Hydrochloric

acid was excluded from our study because of its high volatility and strong corrosive power. For pretreatment, cellulose is first dissolved in the acid for a selected period of time. It can then be reprecipitated by dilution and recovered in a relatively pure and active form.

3. Enzymatic Hydrolysis of Cellulose.

Enzymatic hydrolysis of cellulose is a delicate reaction system. Both the species of enzyme, the state of the substrate and the conditions of the reaction may play a role in the degradation process of the long-chain molecule. In the previous report, we developed a comprehensive model to describe the variable mode of the kinetics for cellulose hydrolysis (8). A bimodal, concurrent, consecutive degradation was proposed:



[1]

whereby C_i = cellulose component i, P_i = soluble sugar generated from component i, $(HC)_i$ = hydrocellulose generated from component i, a_i = fraction of components i that undergoes the consecutive reaction, k_i, k'_i and k''_i rate constants of component i for the various steps of degradation as shown.

Equation 1 was derived for the mechanically induced enzymatic hydrolysis of cellulose. It was extended to cover the variable mode of cellulose degradation. For instance, i = 1 and a_i = 0 for hydrolysis of the pure, native cellulose under the conventional incubating conditions. The pseudo first-order kinetics described by Ghose and Das belong to this

case, C $\longrightarrow$ P (9). When $i \neq 1$ and $a_i = 0$, the mode of degradation shifts to the multi-component reaction described by Van Dyke (10). When $i = 1$ and $a_i \neq 0$, the mode is that of the mechano-enzymatic hydrolysis. We will use this model to study the kinetics, and therefore the reactivity, of the solvent-pretreated cellulose.

4. Dilute Acid Hydrolysis of Cellulose.

Dilute acid hydrolysis of cellulose has been studied extensively. The process can be used either as an analytical tool for studying the crystalline structure of cellulose or as a means for producing fermentable sugar. Our current interest lies in the former function. The cellulose-I crystalline structure is characterized by a high LODP, and that of the regenerated cellulose by a low LODP. These measurements are usually about 100 and 30 DP, respectively, as readily differentiated by gel permeation chromatography (GPC) (4, 11, 12).

B. Experimental.

1. Material and Pretreatment.

Acetate grade cotton linters cellulose (Buckeye Cellulose Corporation, Memphis, TN) was used. Analytical grade 85% H_3PO_4 and 75% H_2SO_4 were used for the solvent pretreatment of cellulose.

The pretreatment was carried out as follows: Two hundred sixty ml of the solvent was placed in a 500-ml beaker, which was in turn placed in a ice-salt bath kept around -10°C to -15°C. When the temperature inside the beaker reached 0°C, 20 gm of cellulose was added. A stirring rod was used to thoroughly mix the cellulose with the acid. After the desired time for swelling, the cellulose-acid mixture was poured into a

large amount of ice-water with strong agitation to minimize degradation. A stirring rod was then used to break the lumps for a good mixing. The solid cellulose was separated by filtering and was washed six times with ice water. The cellulose was resuspended in 300 ml of 1% Na_2CO_3 solution overnight in the refrigerator. Washing was then continued with distilled water at room temperature until the pH of the wash water was identical to that of the distilled water. The wet cellulose was then freeze-dried for two days. The typical pretreatment with 85% H_3PO_4 lasted 2 hours or 18 hours. The corresponding cellulose materials were designated at PSC (partially swollen cellulose) and ESC (extensively swollen cellulose), respectively.

Pretreatment with 75% H_2SO_4 was similar to that with H_3PO_4. The pretreatment periods were 10 minutes or 1 hour. The resulting materials were designated as S1 and S2, respectively.

2. Acid Hydrolysis.

Five hundred mg of cellulose sample was placed in 75 ml of boiling 10% H_2SO_4, in a Erlenmeyer flask and then boiled for 30 minutes with constant stirring. The residue was filtered and washed in a medium-pore scintered glass funnel, which had been oven-dried and weighed. The washed residue was then oven-dried in the glass funnel, at 55°C overnight and the weight was measured. The residue was then nitrated for the GPC measurements as described previously (8).

3. Enzymatic Hydrolysis.

The enzymatic hydrolysis of cellulose was performed according to the procedure described in the previous report (8). Briefly, 100 mg

of cellulose was suspended in 5 ml of 0.1 M NaAc buffer of pH 4.8 by stirring with a magnetic stirring bar. The hydrolysis was carried out at 50°C. Novo Trichoderma reesei crude enzyme (10% by weight of the substrate) was used. The weight loss of cellulose was determined by measuring the total soluble sugar with the phenol-sulfuric acid method (13). The residual cellulose was washed, freeze-dried and nitrated for GPC measurement (8).

4. GPC Analysis.

The molecular weight distribution (MWD) of the nitrated cellulose samples was measured by GPC using tetrahydrofuran as the eluting solvent. A series of 6 shodex columns (Perkin-Elmer Co., Cincinnati, OH) for the molecular weight range 50,000,000 to 1,000 was used for the fractionation. The calibration curve was presented previously (8).

To estimate the chain length, a pead DP was determined from the peak position and the calibration curve. This value represents the most probable chain length of the sample (14,15). Other molecular weight parameters were calculated from the elution curves, which gave a measure of the MWD of polydispersity of the sample.

C. Results and Analysis.

1. Effect of Pretreatment on Chain Length of Cellulose.

A series of GPC elution curves for the solvent pretreated samples and the control are shown in Figure 2. The MWDs of the H_3PO_4-pretreated samples were essentially unchanged except for a small down-shift of the peak positions. The average chain lengths (Table 1) were only slightly reduced. For instance, the chain length was decreased by only 21% with

18 hours of H_3PO_4 pretreatment (ESC); it was decreased by only 14% with 2 hours of pretreatment (PSC). Thus, the degradation assocaited with phosphoric acid pretreatment at low temperature was moderately low (16).

The elution curves of the sulfuric acid-pretreated samples were appreciably moved to the lower molecular weight area. As determined by the peak DP, the chain length was decreased by 80% with the 10 minute pretreatment and by 89% with the 1-hour pretreatment. The polydispersity values were also appreciably increased. Clearly, sulfuric acid acts not only as a cellulose solvent but also as a strong hydrolyzing reagent even at this relatively low temperature (-10° to 0°C).

2. Effect of Solvent Pretreatment on LODP.

When cellulose was hydrolyzed by dilute acid (10% H_2SO_4), the chain folds or amorphous regions of cellulose were preferentially cut. This greatly decreased the chain length to that of the crystalline regions, i.e., LODP. The corresponding GPC elution curves of the cellulose samples are shown in Figure 3. The molecular parameters and the corresponding weight losses are listed in Table 2.

The elution curve for the dilute acid-hydrolyzed cotton linters cellulose (CLC) has a single peak at about 96 DP, which falls in the range of the typical cellulose-I crystal (12). After swelling was produced by phosphoric acid pretreatment for 2 hours, a second peak at about 28 DP emerged creating a dual-peak MWD. With extensive swelling (ESC), the sample produced a single peak at a new and low value of 28 DP, which is typical for the regenerated cellulose (12,17).

The elution curves for the sulfuric acid-pretreated samples also show a drastic reduction of LODP to a chain length of about 20 DP. The LODP is even lower than with the H_3PO_4 pretreatment. The changes in weight loss during the hydrolysis also follow this pattern. These results are comparable to those reported by Warwicker and Clayton (18). Accompanying the decrease in LODP, the crystalline structure changed from Cellulose-I to cellulose-II (19).

From Table 2, the polydispersities are around 1.2 to 1.3 for the extensively swollen cellulose and the sulfuric acid-pretreated celluloses. These values are small enough to assume that they represent the monodispersed species. This means these components may be relatively pure cellulose-II crystals. On the other hand, the polydispersity of the partially swollen cellulose is fairly high at 2.8, and the GPC curve shows two peaks at 96 and at 28 DP. We can reasonably assume that this sample is a mixture of two types of crystals, namely, the cellulose-I and cellulose-II crystals.

3. Enzymatic Hydrolysis of Celluloses.

The results of enzymatic hydrolysis of the solvent-pretreated celluloses are presented in Figures 4 and 5. The time courses of soluble sugar production in the phosphoric acid-pretreated samples are shown in Figure 4. Those of sulfuric acid-pretreated samples are shown in Figure 5. It seems that both solvent pretreatments enhance the reaction appreciably: the longer the pretreatment time, the faster the production of soluble sugar. Quantitative saccharification of cellulose (>90% conversion) in a reasonable time (8-10 hours) can be accomplished with the solvent pretreatment (3).

A figure can be derived from each time course curve to compare the relative reactivity of these substrates. This is the so-called half-life or the time elapsed for production of 50% of the total potential sugars. Thus, the half-life for the cotton linters cellulose is 12 hours, for PSC is 2.8 hours, for ESC is 1.7 hours, for S1 is 2.0 hours, and for S2 is 1.0 hour. A shorter half-life corresponds to a higher reactivity. Comparing these figures with the other molecular parameters listed above we found that these are roughly proportional to the corresponding LODP's. In other words, cellulose samples with a small LODP have a short half-life and, vice versa. This relationship was also found in the acid hydrolysis of the solvent-pretreated celluloses reported by Bose et al. (4). In fact, they suggested that the reduction in LODP of a cellulose sample after pretreatment may be used as an experimental measure of the efficiency of the corresponding pretreatment.

4. Kinetic Analysis of H_3PO_4-Pretreated Cellulose.

In addition to the soluble sugar production, we have measured the the changes in the molecular weight distribution of the phosphoric acid-pretreated cellulose during the course of the enzymatic hydrolysis (Figure 6). The degradation was clearly bimodal. Most noticable is a low DP peak that emerged at the expense of the high DP fraction. This type of degradation closely follows the co-called quantumized "mechano-enzymatic" degradation of the cotton linters cellulose described in the previous report (8). Here we observed that the chain length of the low molecular weight peak measures 36 DP instead of the 150 DP

for the untreated cellulose. Because the LODP of the extensively swollen cellulose has been drastically reduced we can expect that the enzymatic degradation product of this regenerated crystallite should also be reduced to the low DP range. This peak at 36 DP, therefore, corresponds to the hydrocellulose (HC) in Eq. 1.

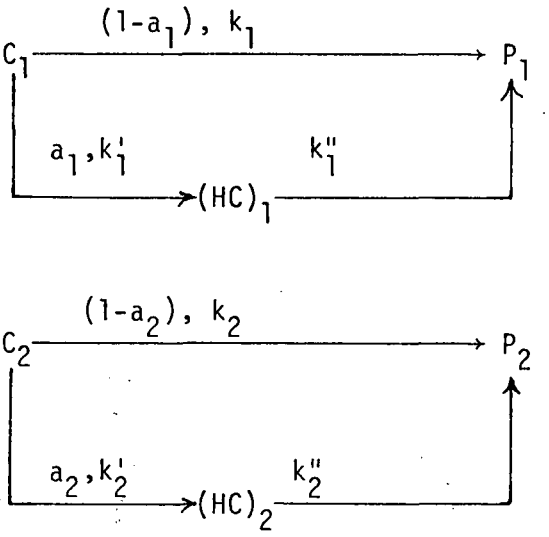
Based on the above analysis, we assumed that the kinetics of the hydrolysis reaction could be fully described by setting i = 1 in Eq. 1. The elution curves recorded for various times of hydrolysis and the soluble sugar data were expected to contain sufficient information for the kinetic analysis. The elution curves were therefore fractionated according to the procedure established previously and the results are presented in Table 3 (8). The plots for the experimental data and for the predicted curves are shown in Fig. 7. The kinetic parameters thus obtained are: $a = 0.44$, $k = 0.72\ h^{-1}$, $k' = 6.6\ h^{-1}$, $k'' = 0.23\ h^{-1}$. The sum of squares of residuals is 3.6 for the curve-fitting process. For a total of 18 regressions points, the value is relatively small, indicating a very good fit.

The change in MWD of the partially swollen cellulose (PSC) was drastically different from that of the extensively swollen sample (Fig. 8). In the very early stage of hydrolysis (30 min), the MWD spread over a wide range of chain lengths. During the next couple of hours, a peak started to emerge in the low DP range at about 36 DP. After prolonged hydrolysis, however, this low DP peak shrank and gradually disappeared. Meanwhile, another peak emerged in the intermediate DP range, at about 150 DP. Eventually, the 150-DP portion became the major component in the residual cellulose. During the entire course of the reaction, the high DP portion remained but the relative quantity

decreased with progress of the reaction. The analytically resolved GPC components confirm a changing pattern such as this (Table 4).

The above degradation scheme obviously follows a quantumized or discrete triple-mode reaction. The molecular cellulose is cut either to an intermediate of 36 DP or to a chain length of 150 DP, and, of course, to the soluble sugar. From the dilute acid hydrolysis, we have found that this PSC sample is composed of a mixture of two crystals (cellulose-I and -II). It is reasonable to expect that they react independently in the hydrolysis. The changing patterns of the GPC reflect faithfully such a mechanism for the reaction.

In the kinetic analysis, i = 2 is therefore assigned. This is complimented with the ratio c, the proportion of the cellulose-II component in the mixed sample. The kinetic model now takes the following forms:



[2]

where $C_1 = (1-c)C_o$ and $C_2 = cC_o$ are the two types of cellulose. In the curve-fitting process, parameters k_1, k'_1, k''_1, k_2, k'_2, k''_3, and a_2 are fixed values obtained from the previous experiments for the cotton linters cellulose and the extensively swollen cellulose. We let c

a_1 float in the least square curve fitting process; the values thus obtained are a_1 = 1.0 and c = 0.75. The predicted curves for the model and the data points are shown in Figure 9, with a residual sum of squares value of 52.

The figure shows that the theoretical curves follow the general trend of the experimental data. The time courses of the two intermediate chain segments are accurately predicted. But the total sugar yield is over-estimated by about 10% at the high extents of conversion. The general fit of the regression curve is also poorer than in the case of the ESC. Considering that the crystalline states of the present PSC are merely a partial transition from cellulose-I to cellulose-II, the reactivity or rate constants of the components might be appreciably different from those for the true mixture of the two distinct crystals. We used the rate constants for the true mixture in our regression process, and this may have contributed to the poorer fit of the model to the experimental data.

D. Discussion

As a polymorphous fibrous crystal, the structure of cellulose is associated with the longitudinal order in the direction of the fiber. On the one hand, native celluloses are in cellulose-I form with a long crystalline order (about 100-200 DP). On the other hand, cellulose-II is the crystalline modification of cellulose-I with a short order (an average of 20-40 DP). In between, a number of combinations are possible depending on the extent of the modification.

In the present study, samples of native cellulose pretreated with 85% H_3PO_4 or 75% H_2SO_4 show a lowering in LODP. This indicates that the solvent pretreatment transformed the crystal from the highly ordered

cellulose-I to the cellulose-II of much lower order. As a result, the amount of cellulose accessible to enzyme was drastically increased. The mode of enzymatic degradation follows that of the concurrent consecutive reaction and reflects the crystallite structure of the substrate. The kinetic models and corresponding parameters have been calculated for the acid-pretreated and the control samples of cellulose (Table 5)(8).

According to the proposed concurrent consecutive reaction (8), there are two simultaneous reaction pathways for the breakdown of the macromolecule--the surface-peeling pathway and the consecutive reaction pathway. The first breaks the substrate molecule directly and instantaneously into soluble sugar. The second cuts the folds first to give an intermediate chain of hydrocellulose which reflects the crystallite structure of the substrate. The reaction then proceeds to break these intermediate chains directly to the soluble sugar. This is the way that cotton linters cellulose reacted in the so-called "mechano-enzymatic hydrolysis" reported previously (8).

The pretreatment of cellulose for 18 hours with H_3PO_4 converted the substrate (ESC) completely to cellulose-II. The mode of degradation was as follows: a portion of the molecules are broken down very quickly by surface-peeling; another portion is cut by the consecutive reaction pathway to yield hydrolcellulose of cellulose-II as the intermediate chain. This acid pretreatment increased the rate constant for the surface-peeling, k, to nearly four times that without pretreatment and increased the rate constants of the consecutive reaction, k' and k", by 12 and 16 times, respectively. With this pretreatment, greater than 90% of the cellulose can be converted to soluble sugar in a relatively short period of time ($\sim$8 hours).

The pretreatment of cellulose for 2 hours with H_3PO_4 led to a partial transition of the crystal. About 75% of the native cellulose may have been

converted to the regenerated form. The mode of degradation now conforms to this new structure. Thus, the native cellulose is degraded according to the pathways for cellulose-I, with the exception that $a_1 = 1$ (which means there is no more surface-peeling because all surface molecules have probably been converted to cellulose-II). The regenerated cellulose is degraded as if it were a pure cellulose-II—disintegrating generally very quickly to produce the intermediate segments of hydrocellulose-II. In this way, the kinetics of the reaction primarily follows a compound bimodal reaction scheme (i.e., Eq. 2). Meanwhile, due to the very low reaction rate of the cellulose-I segments, sugar production stops with the exhaustion of the cellulose-II component. This is quite evident after 8 hours of hydrolysis: the only significant components left undigested are the relatively long chains of the cellulose-I segments (Fig. 9).

The difference in the crystallite lengths (LODP) measured after enzymatic hydrolysis and after acid hydrolysis is noticeable. The former is always larger than the latter (for native cellulose, this is 150 DP as compared to 96 DP; for cellulose-II, this is 36 DP as compared to 28 DP). Three plausible explanations can be given. First, under our experimental conditions, the enzyme may cut only the folds but not the amorphous regions, whereas the acid may cut the folds as well as the amorphous regions so that the acid LODP is low. Second, acid hydrolysis could randomly cut molecules of the crystallite on the surface which would produce an extended shoulder on the low molecular weight side of the elution curve, and therefore, would shift the position of the major peak down. Third, the enzyme may digest the low molecular weight fraction of the crystallite faster so that only the high molecular weight fractions (150 DP) are left. Additional experiments are required to determine the true cause.

Comparing the efficiency of the two solvents used, the sulfuric acid is apparently more powerful than the phosphoric acid. The time course and half-life for the 18-hour H_3PO_4 pretreatment are similar to those for the 1-hour H_2SO_4 pretreatment. Those for the 2-hour H_3PO_4 (PSC) pretreatment are less than those for the 10-minute H_2SO_4 pretreatment. It should be pointed out that the sulfuric acid is not only dissolving cellulose but is also degrading the macromolecule during the pretreatment period. When the degradation is continued enzymatically, the cellulose molecules are much smaller after sulfuric acid pretreatment than after phosphoric acid pretreatment.

E. CONCLUSION

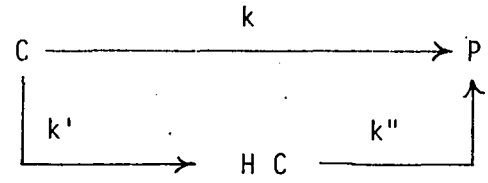
The enhancement of the enzymatic hydrolysis of cellulose by the solvent pretreatment is well documented. The pretreatment itself does not necessarily degrade the cellulose molecule but transforms the crystalline structure and reduces the longitudinal order of the fibrous crystal which are responsible for the increased accessibility.

The kinetics of the enzymatic hydrolysis of the solvent-pretreated celluloses follows primarily the bimodal, concurrent consecutive reaction derived for the mechano-enzymatic hydrolysis. From the results of the kinetic analyses, we have concluded that the most resistive portions of the substrate are the linear chains associated with the large crystallites of the native cellulose.

As a solvent for the pretreatment of cellulose, sulfuric acid is more effective than phosphoric acid.

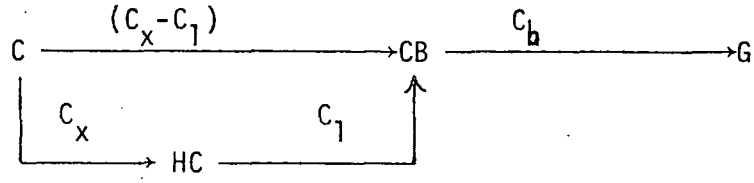
F. SUGGESTIONS FOR FUTURE WORK

In the present work, we have demonstrated that the molecular degradation of cellulose catalyzed by enzyme can be adequately described by the basic model:



The experimental basis for this model consists entirely of the analysis of the degradation pattern of the cellulose molecule; the function of the enzyme has not been considered. How this model can incorporate the action of enzyme is the next question to be answered.

From what we have learned in the literature, cellulose is a composite enzyme basically containing an exo-cellulase (C_1), an endo-cellulase (C_x), and a cellobiase (C_b). The hydrolysis products are primarily cellobiose (CB) and, finally, glucose (G). With this information, we can logically expand the basic model to accomodate the enzymatic actions:



Here (C_x-C_1) represents the synergistic action of the enzyme components. This model combines the structural features of the cellulose with the function of the cellulase components to describe the mechanism of reaction. This model should be a significant step to the in-depth understanding of the enzymatic reaction, provided it can be verified experimentally. We, therefore, suggest that further work be carried out to verify and refine this model.

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EFFECT OF SOLVENT PRETREATMENT ON THE STRUCTURE AND REACTIVITY OF CELLULOSE

ABSTRACT

The effects of solvent pretreatment on the molecular structure and the reactivity of cellulose to enzymatic hydrolysis were studied by acid hydrolysis and by kinetic analysis of the degradation pattern of the cellulose molecule.

When the cellulose was pretreated with a solvent and regenerated, its crystalline structure changed from the native form to the cellulose-II form, which is characterized by a lower LODP. The molecular weight was not appreciably altered by this treatment, but the reactivity to enzymatic hydrolysis was greatly enhanced. The degradation pattern follows a bimodal concurrent consecutive reaction, which is controlled by the crystalline structure of the substrate. Depending on the extent of the pretreatment, the substrate can be a single component of cellulose-II or a mixture of cellulose-I and -II. The mode of degradation and the kinetics of the hydrolysis depend on which type of substrate is present.

For pretreatment, sulfuric acid is a more efficient solvent than phosphoric acid. The resultant LODP can be used to measure the efficiency of the pretreatment.

TABLE 1

MOLECULAR WEIGHT PARAMETERS OF SOLVENT PRETREATED CELLULOSE

SAMPLE	SOLVENT	PRETREATMENT TIME	DP_{peak}	$\overline{DP}_w$	$\overline{DP}_n$	POLYDISPERSITY
Control	---	---	2150	2240	1330	1.7
PSC	H_3PO_4	2 h	1850	2100	1140	1.8
ESC	H_3PO_4	18 h	1700	1780	848	2.1
SI	H_2SO_4	10 m	300	624	213	2.9
S2	H_2SO_4	1 h	230	349	161	2.2

TABLE 2

MOLECULAR WEIGHT PARAMETERS OF ACID HYDROLYZED CELLULOSES

SAMPLE	WEIGHT LOSS (%)	DP_{peak}	$\overline{DP}_w$	$\overline{DP}_n$	POLYDISPERSITY
Control	6	96	85	33	2.5
PSC	13	96/28	69	24	2.8
ESC	15	28	27	21	1.3
SI	15	20	20	16	1.3
S2	17	19	21	17	1.2

TABLE 3

RESOLUTION OF GPC COMPONENTS OF THE EXTENSIVELY SWOLLEN CELLULOSE HYDROLYZED BY ENZYME

HYDROLYSIS TIME (h)	C		HC		P
	POSITION	%YIELD	POSITION	%YIELD	%YIELD
0.5	30.3*	39	37.6	36	24
1	29.8	29	38.2	42	29
2	30.5	13	38.9	29	57
3	31.7	9	39.2	21	70
4	32.0	5	40.0	16	78
6	30.0	2	40.0	12	86

*All positions are in elution volume, ml.

TABLE 4

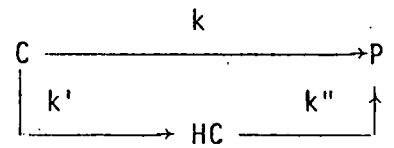
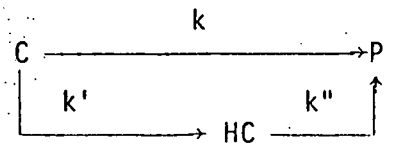
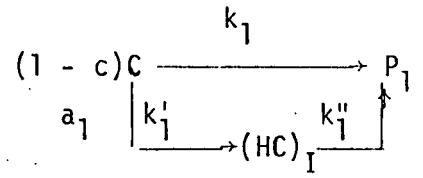
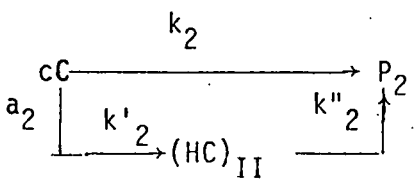
RESOLUTION OF GPC COMPONENTS OF THE PARTIALLY SWOLLEN CELLULOSE HYDROLYZED BY ENZYME

HYDROLYSIS TIME (h)	C		$(HC)_I$		$(HC)_{II}$		P
	POSITION	%YIELD	POSITION	%YIELD	POSITION	%YIELD	%YIELD
0.5	29.2*	30	33.3	15	38.8	31	24
1	29.2	25	34.3	18	39.5	26	32
2	29.0	14	34.7	17	30.4	16	53
4	29.3	11	33.4	21	38.6	14	54
8	29.8	4	35.5	24	40.2	6	66
16	30.2	4	35.5	21	40.0	4	71

*All positions are in elution volume, ml.

TABLE 5

KINETIC PARAMETERS OF ENZYMATIC HYDROLYSIS OF CELLULOSES

SUBSTRATE	KINETIC MODEL	KINETIC PARAMETERS(h^{-1})	
Cotton Linters Cellulose		a = 0.51	k = 0.19 k' = 0.56 k" = 0.014
Extensively Swollen Cellulose		a = 0.44	k = 0.72 k' = 6.60 k" = 0.23
Partially Swollen Cellulose		a_1 = 1	k_1 = 0.19 k'_1 = 0.56 k''_1 = 0.014
		a_2 = 0.44 c = 0.75	k_2 = 0.72 k'_2 = 6.60 k''_2 = 0.23

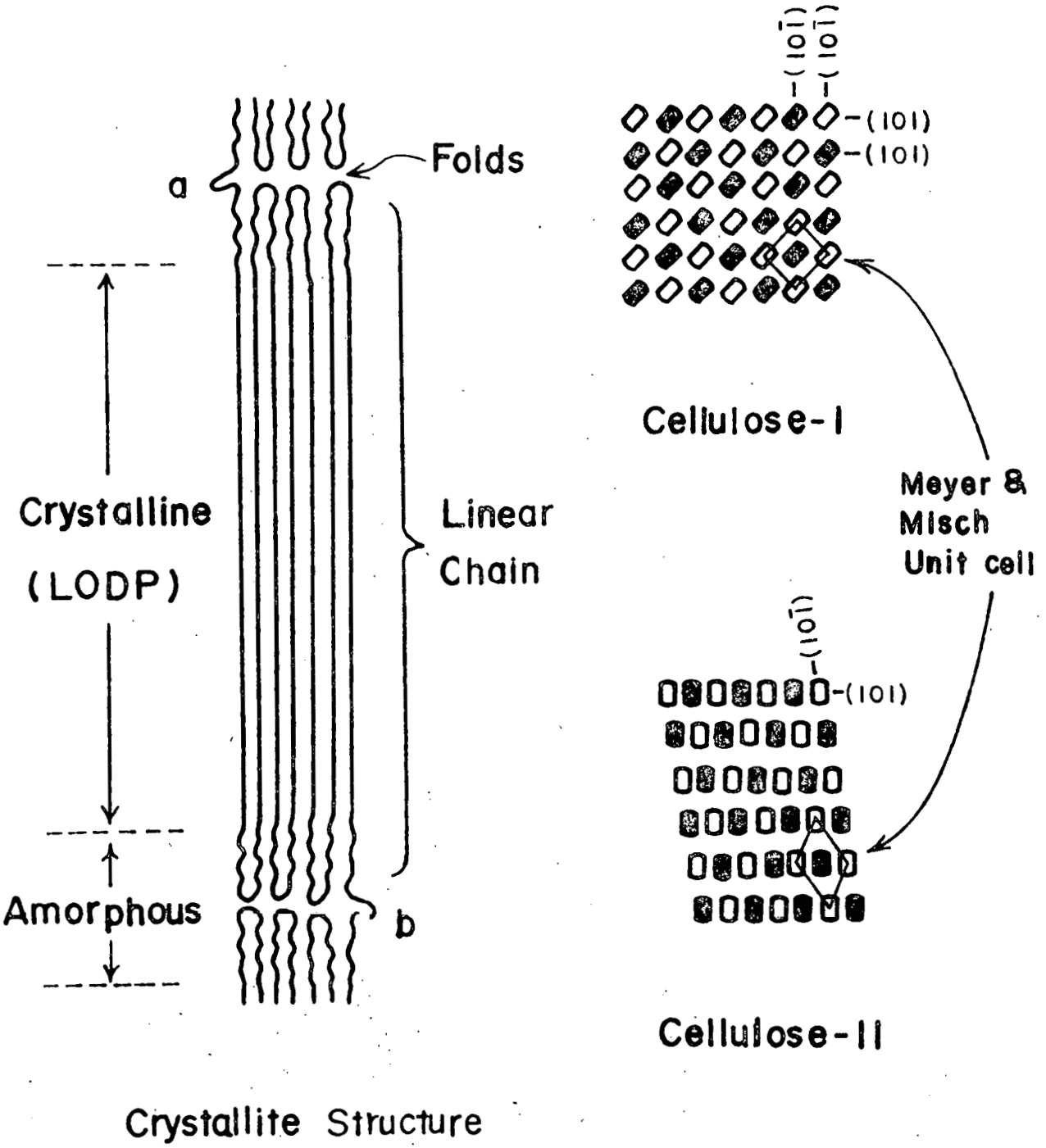


Figure 1. Schematic Crystallite Structure of Cellulose in the Longitudinal (left) and Cross-Sectional Directions (right)

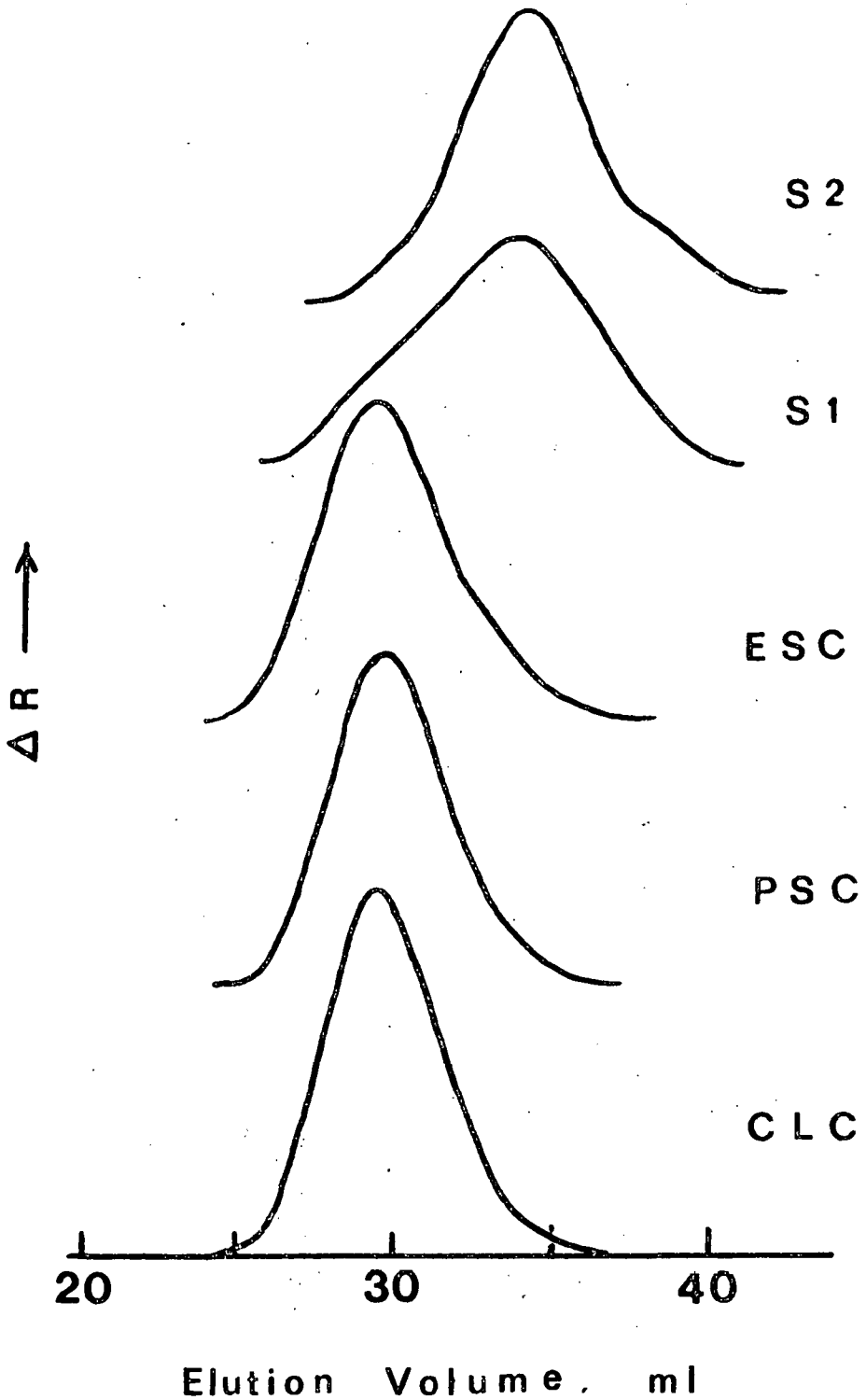


Figure 2. GPC Elution Curves of Cotton Linters Cellulose and Solvent Pretreated Celluloses. CLC = Cotton linters cellulose; PSC = Partially swollen cellulose by H_3PO_4; ESC = Extensively swollen cellulose by H_3PO_4; S1 = Sulfuric acid pretreated cellulose for 10 min.; S2 = Sulfuric acid pretreated cellulose for 1 hour.

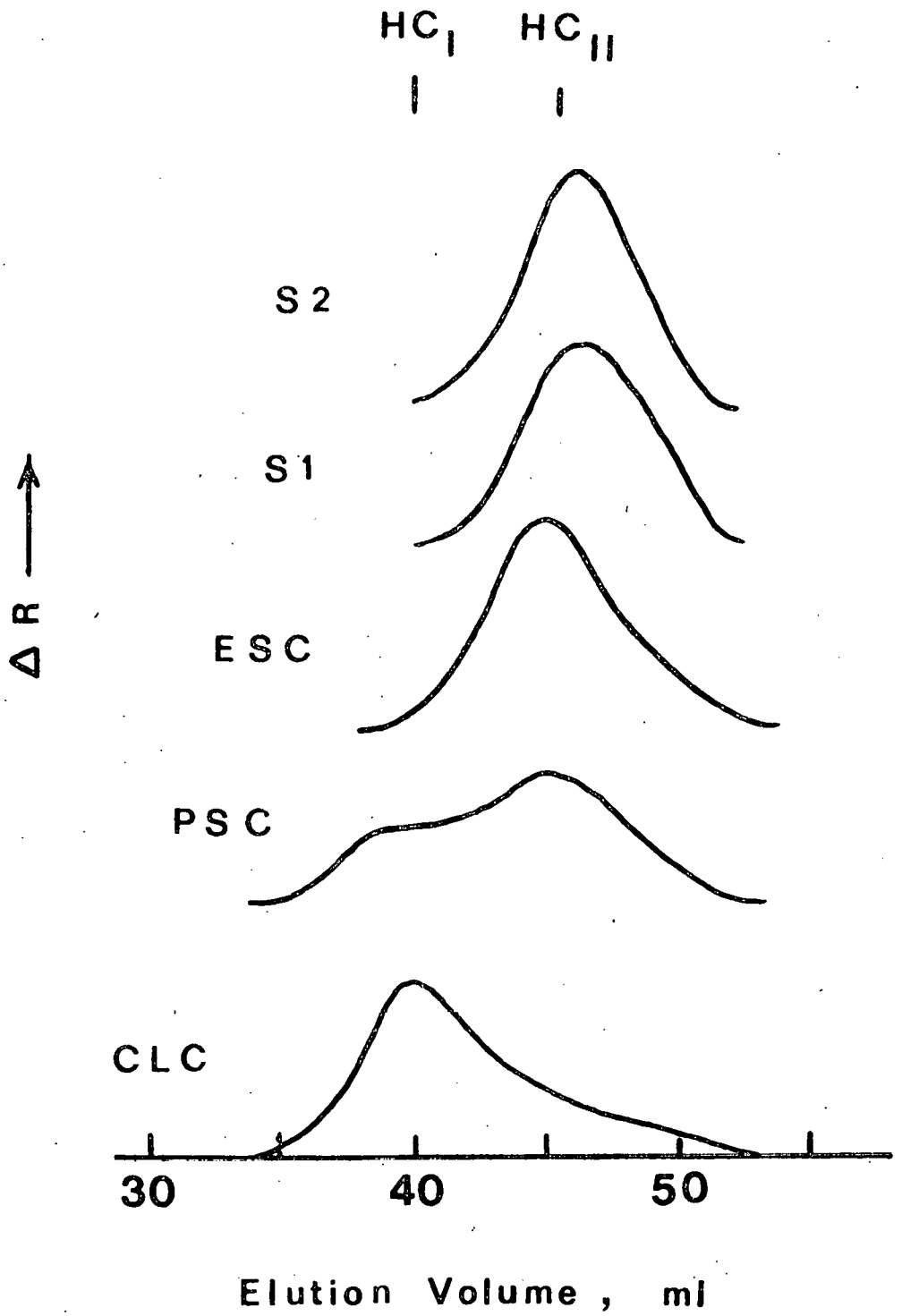


Figure 3. GPC Elution Curves of Hydrolyzed Cotton Linters Cellulose and Solvnet Pretreated Celluloses. Sample abbreviations - see Figure 2.

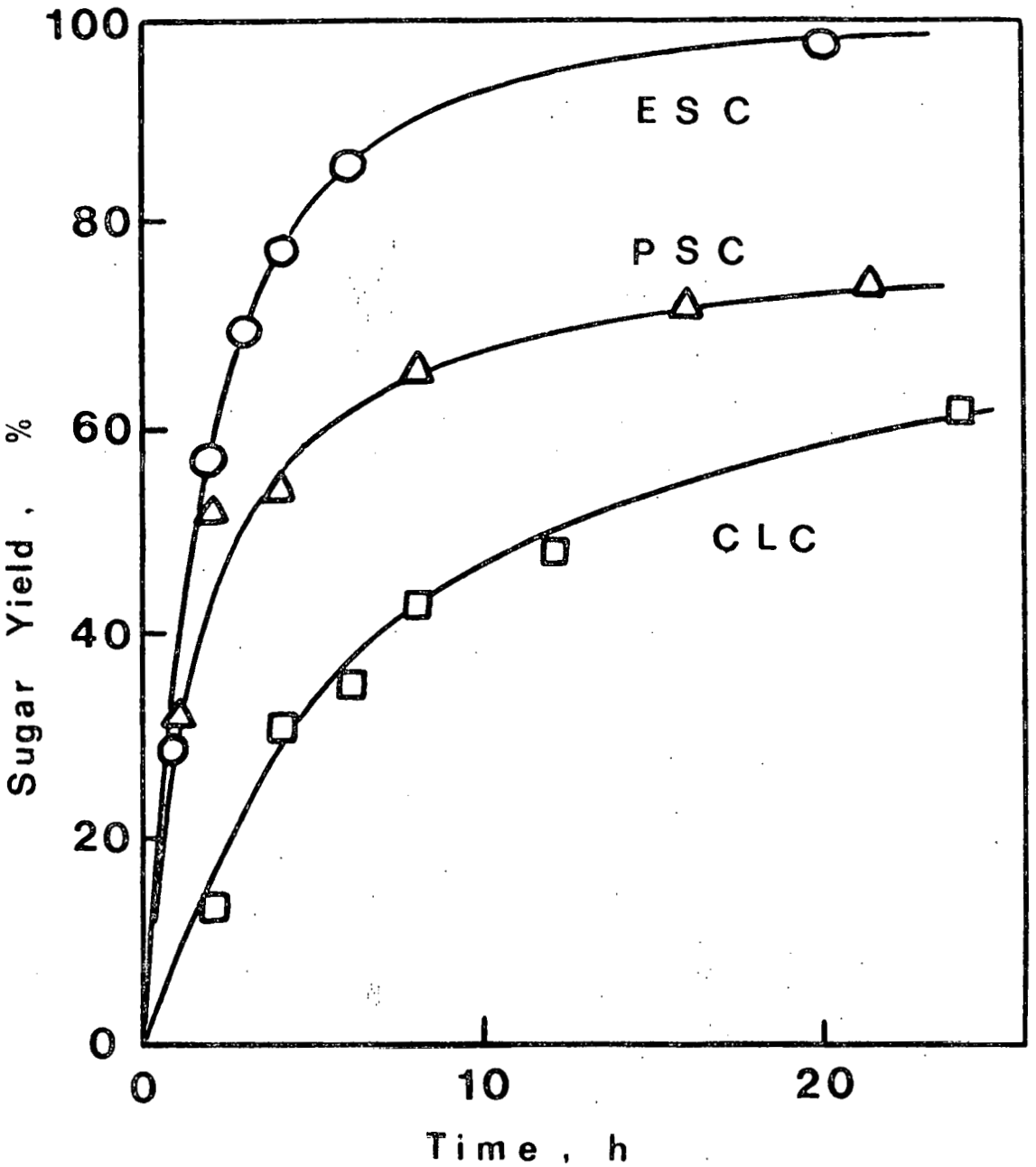


Figure 4. Times-Courses of Enzymatic Hydrolysis of Cotton Linters Celluloses and H_3PO_4 Pretreated Celluloses.

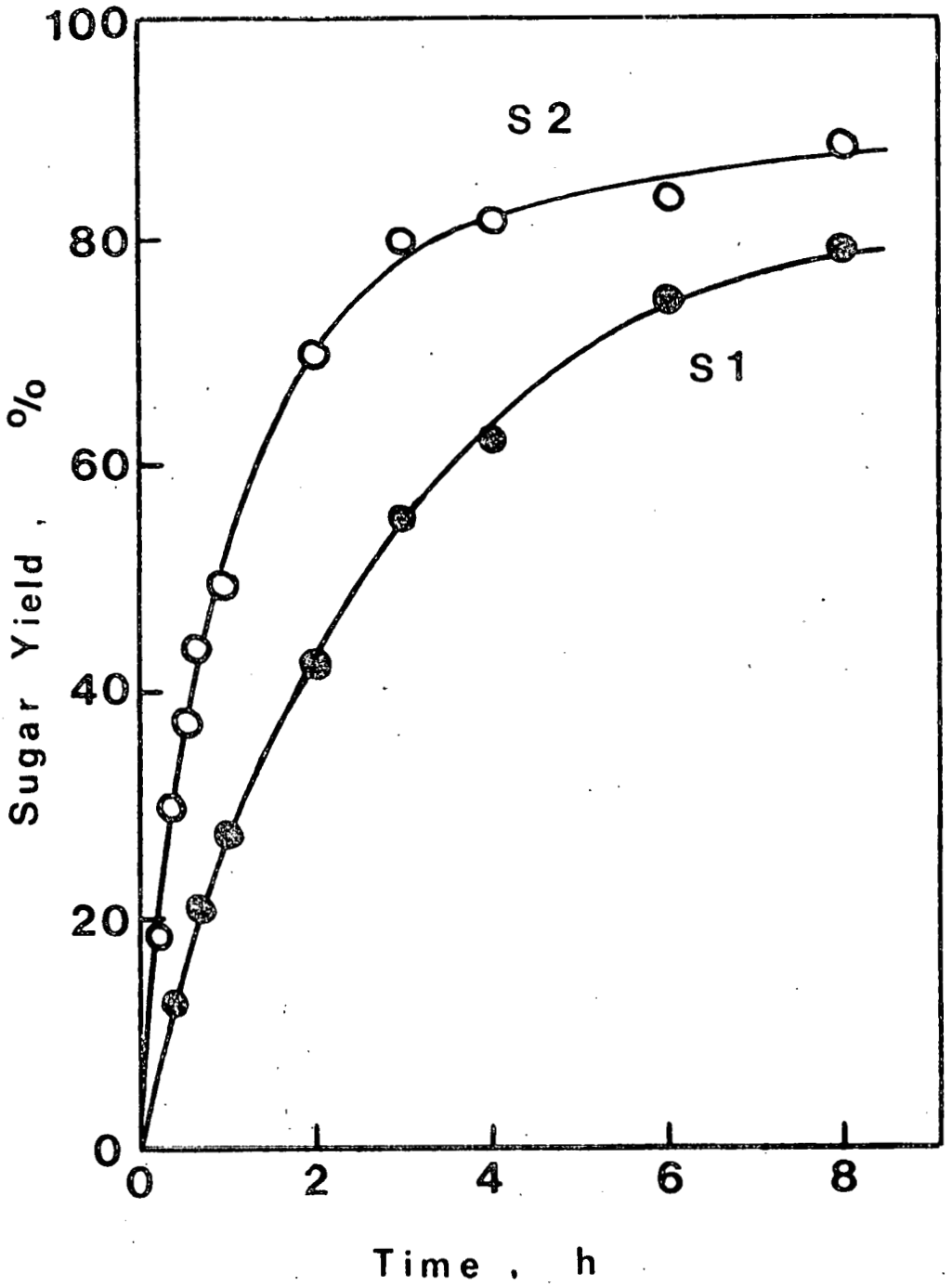


Figure 5. Times-Courses of Enzymatic Hydrolysis of H_2SO_4 Pretreated Celluloses.

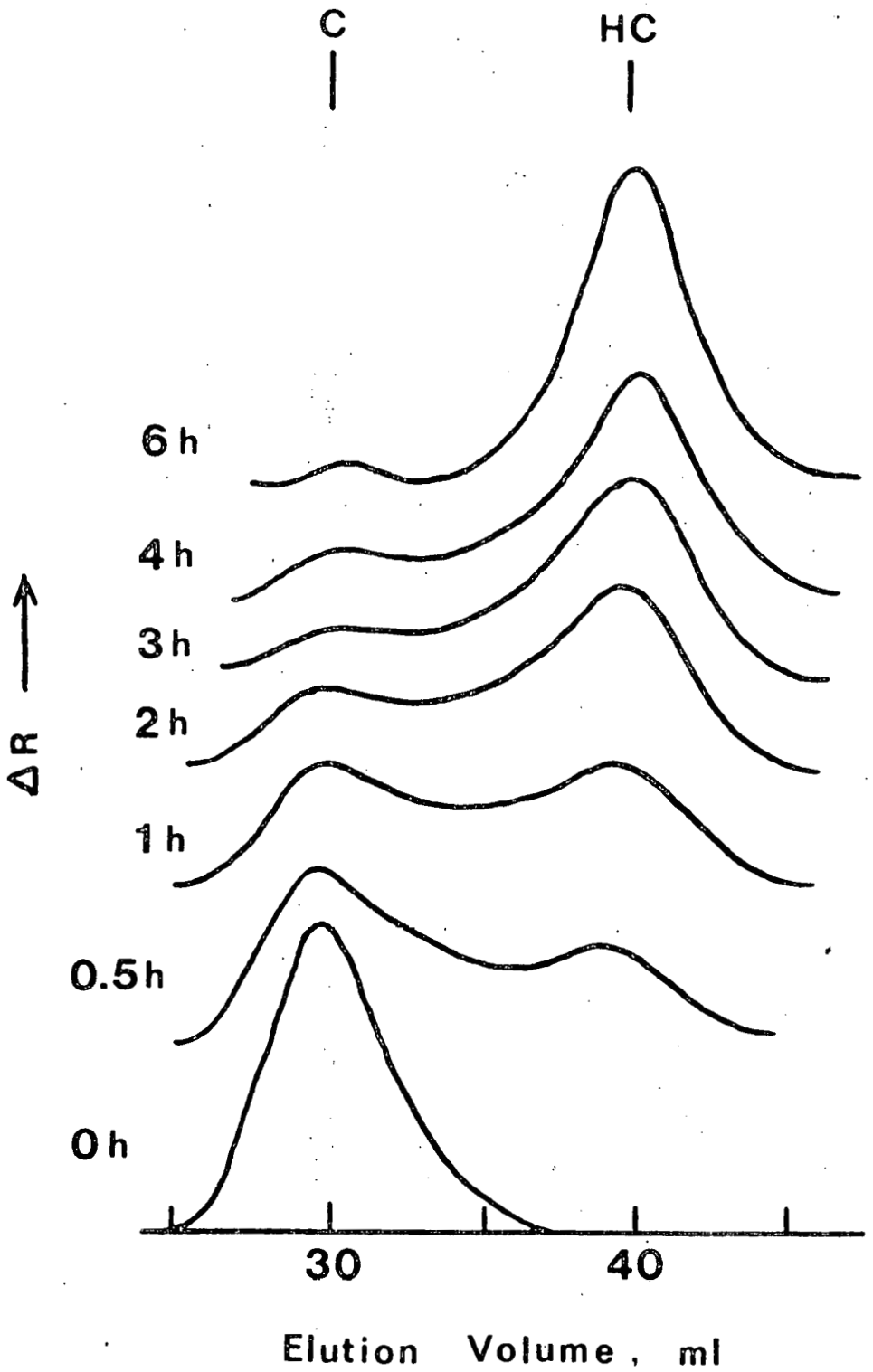


Figure 6. GPC Elution Curves of Enzymatic Hydrolysis of the Extensively Swollen Celluloses by H_3PO_4. C = Molecular cellulose, and HC = Hydrocellulose or intermediate chain.

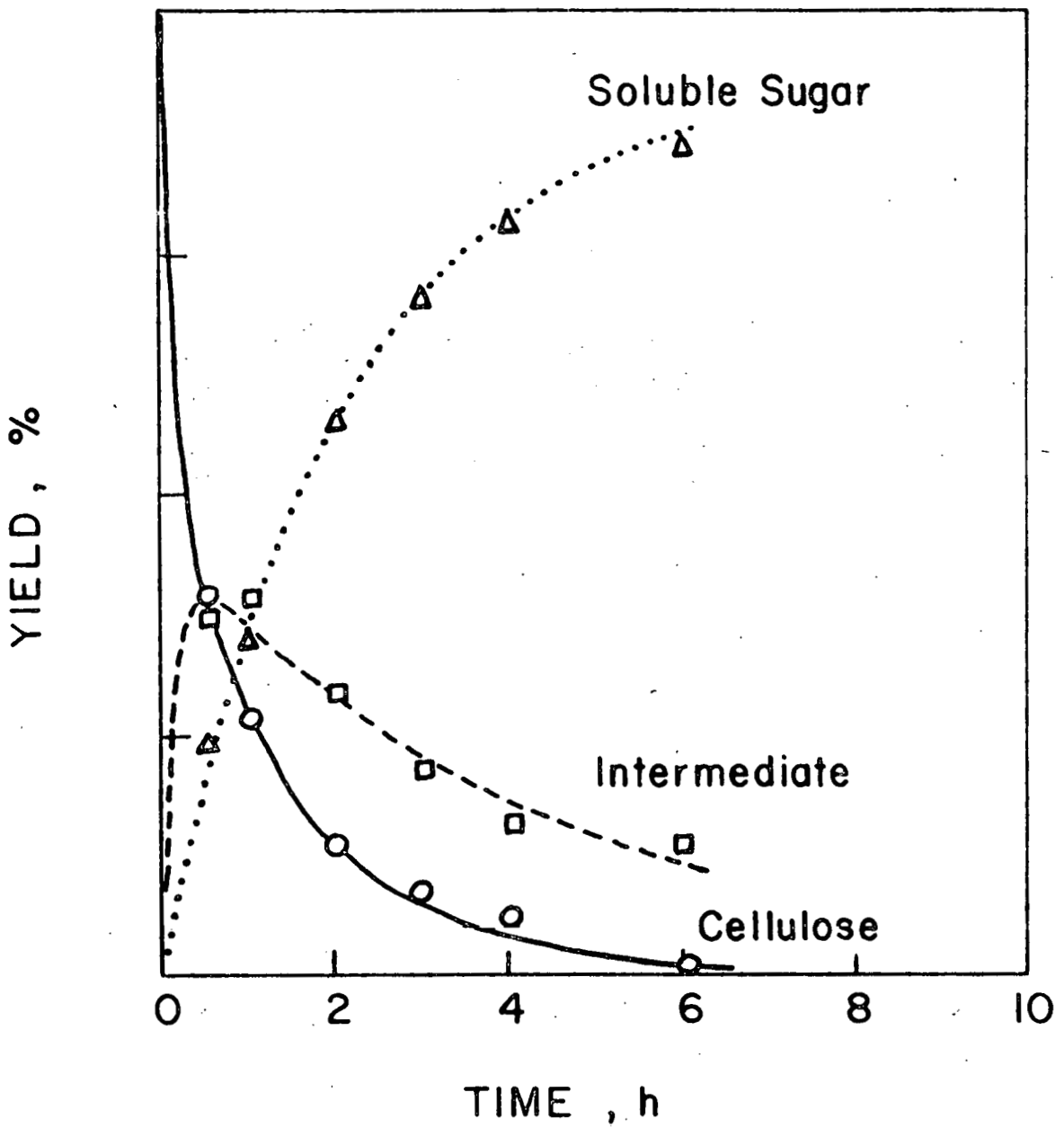


Figure 7. Kinetic Plots of Enzymatic Hydrolysis of the Extensively Swollen Celluloses by H_3PO_4.

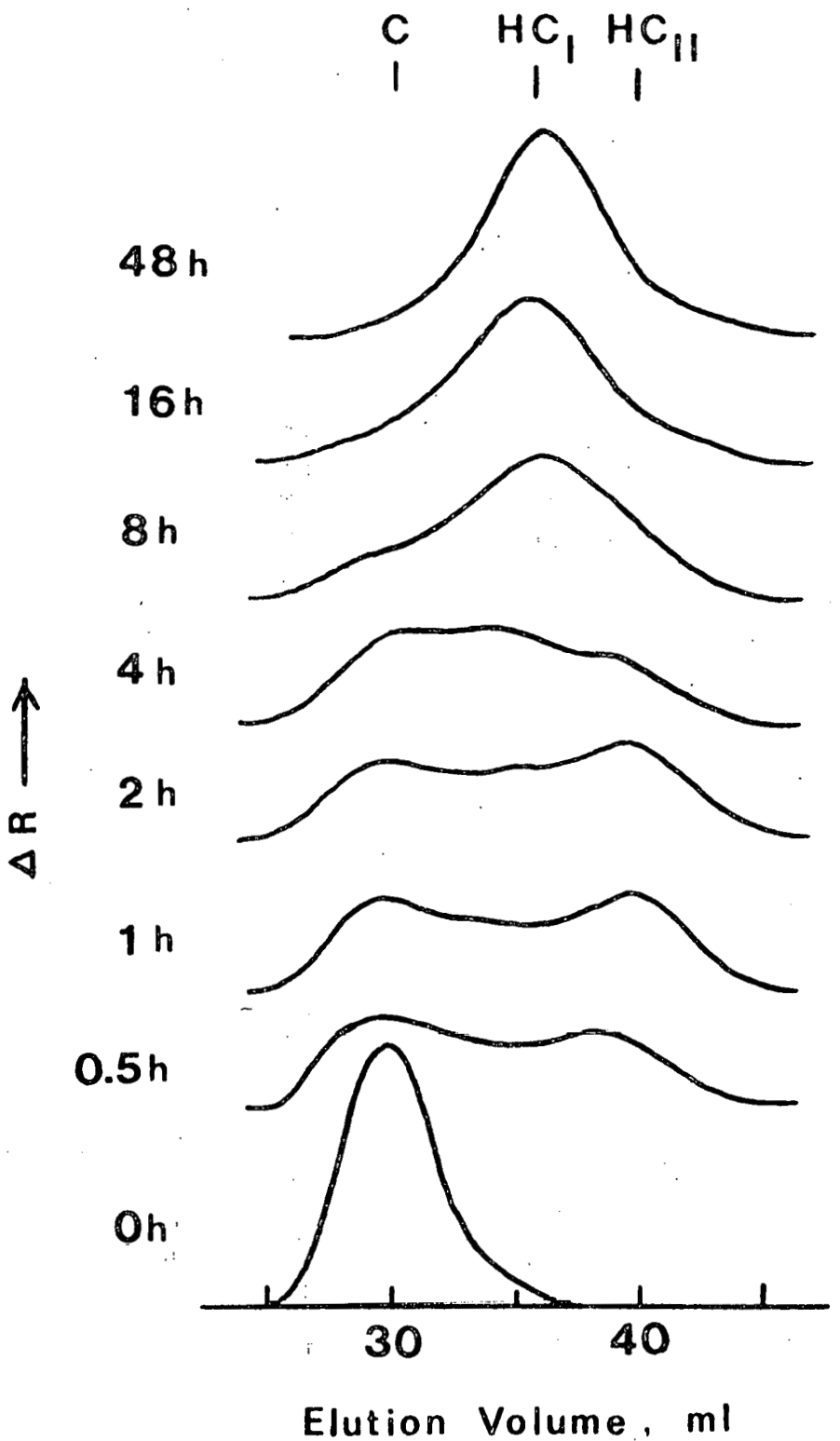


Figure 8. GPC Elution Curves of Enzymatic Hydrolyzed of the Partially Swollen Cellulose by H_3PO_4. C = Molecular cellulose; HC_I = Hydrocellulose-I or intermediate type I; HC_{II} = Hydrocellulose-II or intermediate type II.

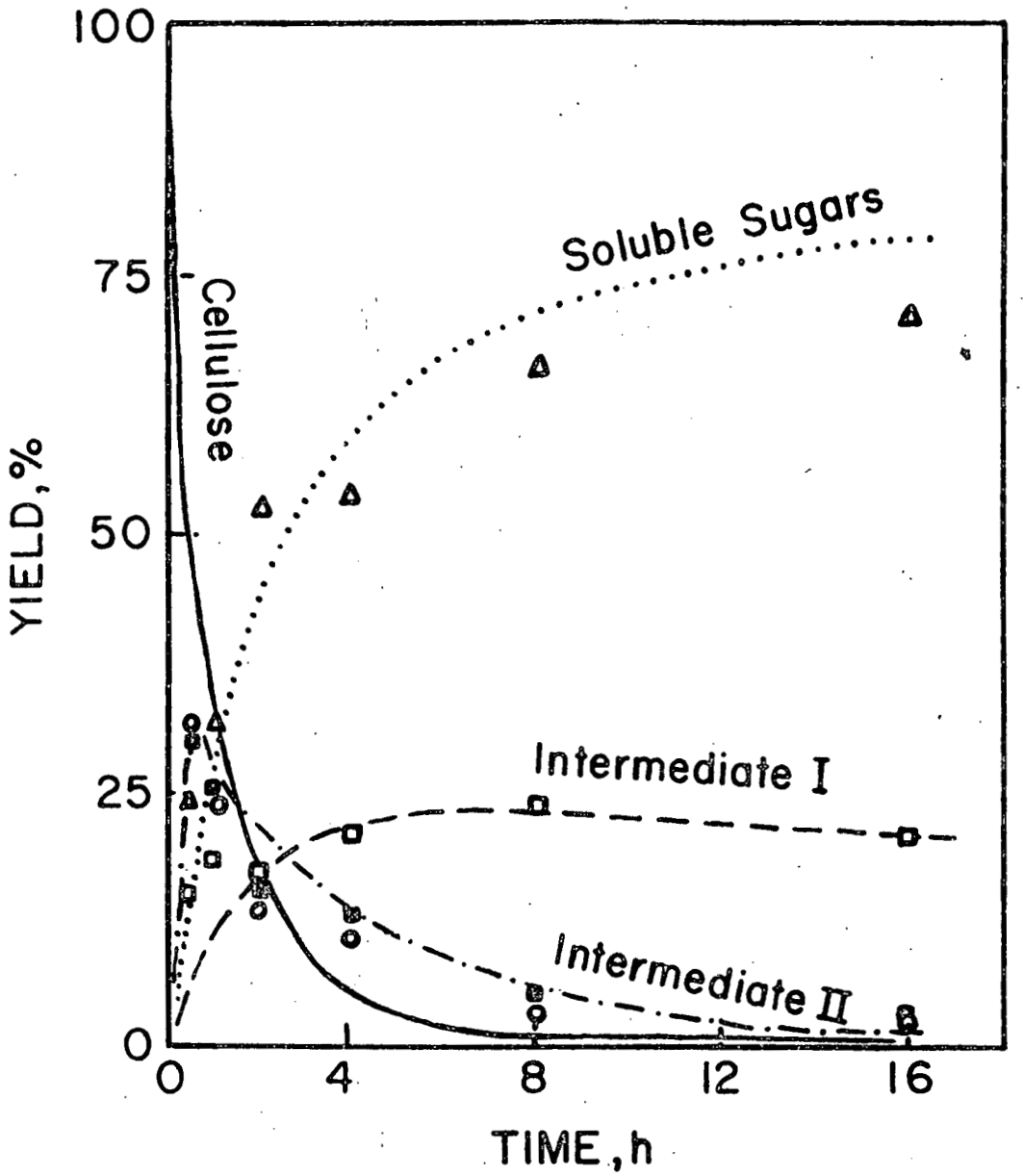


Figure 9. Kinetic Plots of Enzymatic Hydrolysis of the Partially Swollen Cellulose by H_3PO_4.

VI. CELLULASE BIOSYNTHESIS

A. OVERVIEW

Utilization of cellulase by cellulolytic organisms is a very slow biological process. Microorganisms hydrolyze cellulose to metabolizable sugars by way of producing a complicated cellulase enzyme system. Extracellular cellulase synthesis inducers include cellulose, cellulose derivatives, cellobiose, sophorose and lactose.

The response of fungal cells to different inducers varies depending upon the concentration and type of inducers. It is also influenced by other environmental factors such as pH of the medium. The synthesis of cellulase following induction can be inhibited by the presence of glucose or some other sugars in the growth medium. Therefore, the synthesis of cellulase by microorganisms is regulated by the induction-repression mechanism. The basic mechanism of regulation of cellulase synthesis is similar to known inducible enzyme systems. The inducers react with repressor proteins inside the cells causing the derepression of cellulase synthesis.

The known cellulase synthesis inducers have dual functions. They can serve as carbon source for cell growth and they can also serve as inducers of cellulase synthesis. Ideally, the role of an inducer should be limited to the "turn on" of enzyme synthesis. Inducers should not be hydrolyzable nor should they undergo chemical modification once inside the cells. Therefore, the so-called "gratuitous inducers" would be ideal for studying the induction of cellulase synthesis. Unfortunately no such inducers have been found. In some cases sophorose behaves almost like a "gratuitous inducers."

Evidence indicates that cellulases produced by cellulolytic organisms are synthesized either in the cytoplasm or on the endoplasmic reticulum. Thus, the newly synthesized cellulases are cell-bound. However, the majority of fungal cellulases are extracellular. Since the translations occurred inside the cells, the secretion of enzymes must be regulated by either an unspecific releasing mechanism or by a specific active releasing mechanism. Unfortunately, no studies concerning the active releasing of fungal cellulase have been published. The efficiency of the transport and utilization of "potential" inducers by organisms is controlled by the ability of microorganisms to develop a transporting enzyme system and/or the glycosidase enzymes. The transport system and membrane glucosidase regulate the amounts of inducers entering the cells, while the intracellular glucosidase regulates the amounts of "active" inducers. The activity of induction could be determined by the affinity of inducer-repressor that is, protein binding, while the actual activity of transcription and translation are regulated by "catabolite repression" and the half-life of m-RNA. The activity of intracellular glucosidase could be influenced by the amount of intracellular glucose and perhaps by the glucose oxidation product, gluconolactone. Therefore, the regulation of cellulase synthesis appears to be controlled by a complicated mechanism.

Since cellulase produced by organisms is a mixture of enzymes, it is not known whether different cellulases appear in the growth medium sequentially. Hagerdal et. al. (1) reported that all three cellulases produced by Thermoactinomyces appeared in the growth medium simultaneously. Fagerstam and Pettersson (2) analyzed cellulase produced by T. reesei using the immunoelectrophoresis technique and reported that there is no sequential appearance of various cellulases. Lobanok et. al. (3) studying the synthesis

of cellulase in T. reesei, observed that cellobiohydrolase appeared in the growth medium before endocellulase. However, since cellobiohydrolase is the major protein in young culture broth of T. reesei, it is difficult to detect small amounts of endocellulase when using the disc-electrophoretic technique. We observed that both cellobiohydrolase and endocellulase are produced by T. reesei simultaneously and independent of the culture age.

Further investigation of control mechanisms of cellulase biosynthesis by cellulolytic orgamisms would be not only helpful in understanding the regulation of cellulase biosynthesis, but also helpful in pinpointing the obstacle in the development of an efficient way to enhance cellulase production and eventually to overcome the obstacle.

B. BIOSYNTHESIS OF CELLULASE BY T. REESEI

Based on the sequential elution methods we are able not only to cleanly fractionate the three cellulase components, but to do the fractionation with very little loss of enzyme. The total recovery of major enzyme components, summarized in Table VI-1 is considerably higher than those reported previously by other researchers. Table VI-1also gives the molecular weights of the three enzyme components.

Cellobiohydrolase is the major cellulase component produced by T. reesei and comprised over 35% of the high molecular weight soluble proteins produced by fungus (Table VI-1). Total endoglucanase (G-25 fractions) was less than 8% of total high molecular weight soluble protein and cellobiase was present in only trace amounts. The relatively large amount of cellobiohydrolase in crude cellulase of T. reesei is consistent with the

Table VI-1. Summary of Cellulase Recovery

Fractions	Total Proteins (mg)	% of Proteins Recovered	Molecular Weight†
Culture filtrate	1404	100*	
After Sephadex G-25 Desalting	868	61.8*	
Cellobiase	2.6	0.18**	76,000
LMW Endoglucanase	9.75	0.69**	18,000
HMW Endoglucanase	28	1.99**	52,000
Cellobiohydrolase	312	22.2 **	71,000

* Based on total soluble protein

** Based on homogeneous protein

† Estimated from SDS-polyacrylamide gel electrophoresis using proteins of known molecular weight as markers.

results of Berghem et. al., (4). Cellobiohydrolase also makes up the majority of cellulase enzyme in commercial cellulase preparation (obtained from Enzyme Development Corp., NY, NY).

The cellobiase activity in culture filtrates of T. reesei was small relative to that of cellobiohydrolase and endoglucanase. The possibility that cellobiase of T. reesei is either an intracellular or membrane-bound enzyme was indicated by experiments in which cellobiose or other carbon sources were used as the substrate for culture growth. While cellobiose can be taken up rapidly by the fungus, very little cellulase activity could be detected in the filtrate (see Table VI-2-cellobiose as carbon source). Furthermore, the appearance of cellobiase did not parallel the appearance of cellulase activity. Increasing culture incubation time did, however, result in increasing cellobiase activity in the filtrate. This data suggested that at least some of the cellobiase present in the filtrate might have been an intracellular cellobiase which was, perhaps, released when some of the cells underwent autolysis. The existance of intracellular and/or membrane-bound cellobiase has been reported for Sporotrichum pulverulentum (5) and Neurospore crassa (6). Thus, it would not be unreasonable to suggest that other microorganisms might also have intracellular cellobiase.

Preliminary data showing the cellobiase hydrolyzing activity is found inside the cells, as well as outside, for T. reesei is shown in Table VI-3. Cells, when separated from the culture filtrate and broken up by grinding and sonification, showed significant release of cellobiase activity. This phenomenon was observed not only when cellobiose was used as a carbon source during culture growth, but also when other carbon sources such as

Table VI-2. Growth and Cellulase Production of T. reesei

Carbon Source	Growth[a] (Dry weight, gm / 200 ml culture)	% Cellobiase[b] Activity	% Cellulase[b] Activity
Cellulose	0.6	100	100
Lactose	0.69	92	81
Glucose	0.63	2	<1 (trace)
Galactose	0.65	3	<1 (trace)
Cellobiose	0.67	5	<1 (trace)
Glycerol	1.03	4	<1 (trace)
Xylose	0.87	2	<1 (trace)
Lactose and glycerol	1.01	11	3
Lactose and glucose	0.76	26	21

(a) T. viride were grown in 1% carbon source at 28°C for 4 days.

(b) One ml of culture filtrate were incubated with 0.5% substrate at 50°C for 2 hrs. Activity from cellulose as carbon source served as the control (100%).

Table VI-3

Cellobiase Activities[a] of T. reesei Homogenate[b]

Carbon Source	Low Salt Homogenate	High Salt Homogenate
Cellulose	2.12	0.66
Cellobiose	1.13	0.77
Glucose	0.72	0.56
Glycerol	1.12	2.94
Xylose	2.38	0.54

(a) mg glucose produced per ml of reaction mixture after incubation at 50°C for 3 hr.

(b) Mycelia from 200 ml of culture were disrupted by grinding and sonification. Soluble proteins were extracted by 2- ml of 50 mM sodium acetate buffer, pH 5.8. After buffer extraction, the remaining solids were further extracted by high-salt buffer (500 mM NaCl in 50 mM sodium acetate, pH 5.8).

cellulose, glucose, glycerol, or xylose were employed. Although these results clearly show the existence of an intracellular cellobiase, the question of whether or not the intracellular and extracellular cellobiase is the same must still be answered. These results can also be used to explain why cellobiose is not a good cellulase inducer. Since the cellobiose can be hydrolyzed to glucose and utilized intracellularly by the fungus, the fungus is not forced to produce copious quantities of extracellular enzyme in order to have an assured food source.

While cellobiose is a poor inducer, lactose, the milk sugar, is a good inducer of cellulase biosynthesis. Lactose-inducer fungus produces a cellulase complex which is identical to those cellulases induced by cellulose (for SDS gels see Figure I-8 b, e, h, i).

These results, taken together with the data described indicate that T. reesei produces one major endoglucanase and one cellobiohydrolase. Cellobiase-hydrolyzing enzymes are probably located intracellularly as well as extracellularly.

C. THE MULTIPLICITY OF CELLULASES

The multiplicity of cellulases is of fundamental interest because of its implications on the basic understanding of cellulose hydrolysis as well as the regulation of cellulase biosynthesis.

It appears that for the much studied T. reesei multiplicity is a "fact of life." Some of the physical characteristics of multiple component cellulase enzymes for this microorgamism have been elucidated in Brown's laboratory at Virginia Polytechnic Institute. Brown and co-workers purified three cellobiohydrolases from a commercial cellulase preparation. They found these enzymes to be glycoproteins of similar molecular weight with the

largest enzyme (cellobiohydrolase "C") predominating (4, 7) Shoemaker and Brown (8) reported four electrophoretically distinct endoglucanases having molecular weights ranging from 37,200 to 52,000. In addition, Berghem, et. al., (4) have found two major endoglucanases (one having a high molecular weight and the other a low molecular weight) and several minor ones for T. reesei. Okada (9) has reported three endoglucanases of differing molecular weight and randomness of action toward hydrolysis of cellulose.

Based on the work done in our laboratory, we believe that multiple enzymes of the same type are derived from the same enzyme and potentially arise from partial proteolysis of such an enzyme. In previous studies, we have purified three distinct cellobiases from T. reesei which are chromatographically distinct yet kinetically similar.

In a similar context, Nakayama et. al., (10), reported that limit proteolysis of cellulases caused changes in enzyme characteristics and created multiplicity of enzymes. Therefore, it seems to us that if proteolytic enzymes play an important role in causing multiplicity of cellulases, it should be possible to grow the cultures in such a way that proteolytic enzyme production would be limited. Therefore, we analyzed endoglucanase activity of cultures of various ages after fractionation of the endoglucanase components on DEAE-cellulose chromatography. The results are shown in Figure VI-1.

There is one sharp, distinct peak of endoglucanase activity from young fermentation broth. In comparison, the ten-day-old culture exhibited additional endoglucanase peaks, while in the fourteen-day-old culture four different endoglucanase activity peaks were observed. The activity peaks of enzyme obtained from older cultures are similar to those obtained from

older cultures are similar to those obtained from commercial cellulase preparations as shown in Figure VI-1. Similar results have been observed by Nakayama (11).

This data, together with the observation of prolific protease activity in crude commercial cullulase preparations that are probably obtained from older cultures, has led us to speculate that the multiple enzyme peaks in the older cultures could have resulted from protease modification of one parent endoglucanase. This prompted us to discontinue the use of commercial cellulase preparation.

D. PROTEOLYSIS OF CELLULASES

The proteolysis of cellulases has been previously investigated. Nakayama et. al., (10) found that mild proteolysis of endoglucanase from T. reesei by a protease prepared from the same fungus resulted in cellulase enzymes which still possessed cellulolytic activity. Earlier, Eriksson and Petterson (12) investigated the effect of various proteolytic enzymes on the cellulase activities on Pencillium notatum. They found that different proteases affected enzyme activities to different degrees.

It had been reported that T. reesei secreted protease into culture media during cellulase production. We have found that in young culture filtrates of T. reesei, the protease activity is very low, but that this activity increases with increasing culture age. Similarly, for crude commercial cellulase preparations, which are harvested from older cultures, we have detected significant amounts of protease activity by Azocoll assay methods.

It is also instructive to consider some results which have been reported for other enzymes with respect to partial proteolysis. Partial proteolysis

of enzymes has been implicated in post-translational modification of both extra- and intracellular enzymes. For example, RNA polymerase (an intracellular enzyme) from spores of Bacillus subtilis has been found to be different from RNA polymerase of vegetative cells. The difference is due to the partial proteolysis of the subunits of RNA polymerase from the spore stage. Because of this, the template specificity of the spore enzyme is different from the enzyme of the vegetative cells (13). It was later found that this proteolysis could be prevented by careful manipulation of enzyme extracts during enzyme isolation by first removing protease (14). Another example is the proteolytic modification of an extracellular enzyme, staphylokinase (15). It was found that among the three isozymes of staphylokinase, two enzyme components are derived sequentially from one enzyme by mild trypsin digestion. Thus, a total of three enzymes resulted having different isolectric points and different molecular weights. The two enzymes derived from the digestion were identical to those obtained from the culture filtrate.

E. CO-INDUCTION OF CELLULASE

Several lines of evidence suggest that both endocellulase are co-induced by cellulase inducers. We have found that the ratio of both cellulases remained the same with either cellulose or lactose as the inducer. Mutants that were high in cellulase productions produced high amounts of both cellulases (16, 17). The simultaneous induction and repression of both cellulases suggests a coordinated regulatory mechanism analogous to that described for the β-galactosidase enzyme system in E. coli. The cellobiose hydrolyzing enzyme may be regulated by a different regulatory mechanism. Results from the study of cellulase-less mutants of Trichoderma by Nevalainen and Palva (18) suggest that the simultaneous loss of activities

of both endo- and exo-cellulases could be due to a defect in the enzyme secretion mechanism even though the secretion of other enzymes has not been affected. Steward and Leatherwood (19) suggest that the extracellular cellulase is controlled by a different gene site from cell-bound cellulase, since cell-bound enzyme is less sensitive to changes of culture conditions. However, the different sensitivity in response to culture conditions could be attributed to their effect on the specific releasing mechanisms.

Some genetic data are needed before any conclusion can be made, however, at present, it appears that both cellulases are controlled by the same regulatory mechanism while cellobiose hydrolyzing enzyme is regulated independently of the cellulase genes.

F. CATABOLITE REPRESSION

Enzyme synthesis is repressed by the presence of glucose and other readily metabolizable sugars in the growth medium. This phenomenon, known as catabolite repression, is similar to the induction-repression effect of other inducer enzyme systems responsible for the regulation of inducible enzyme synthesis. The structural gene expression is repressed by the accumulation of catabolites. The repression of genes can be derepressed by the inducers which interact with the repressor protein. The catabolite repression by glucose is known as "glucose effect." Glucose appears to repress the synthesis of both endocellulase and exocellulase in the presence of cellulose and other inducers. This indicates that the presence of an inducer is not sufficient for cellulase synthesis. The inducibility of cellobiose, sophorose of lactose depends on the concentration of inducers in the medium as well as the ability of cells to develop transport systems. Inducibility is also influenced by the amount of glucosidases on the cell

membrane or in cytoplasm. When the amount of endogenous glucosidases is high, the hydrolysis of potential inducers occurs, resulting in the accumulation of high levels of glucose, and thus the repression of cellulase synthesis occurs.

Repression of cellulase synthesis in Trichoderma is most profound when glucose is present. Mandels (20) reported that cellulase synthesis could be inhibited when exogenous glucose was added to the growth medium in the presence of an inducer. Cellulase production resumed after glucose had been consumed or after transferring the cells from a glucose medium to a medium with inducer. Nisizawa et. al., (2) studied the induction of cellulase by sophorose and reported that the inhibition of cellulase induction was directly related to the concentration of glucose. The similar effect of glucose inhibition of cellulase synthesis has also been reported in many other microorganisms.

The precise mechanism by which glucose inhibits cellulase synthesis is not known. It could lower the level of cyclic AMP thus lowering the transcription activity of cellulase genes. However, Nisizawa et. al., (21, 22) indicated that glucose inhibited cellulase synthesis by affecting the translation of cellulase m-RNA.

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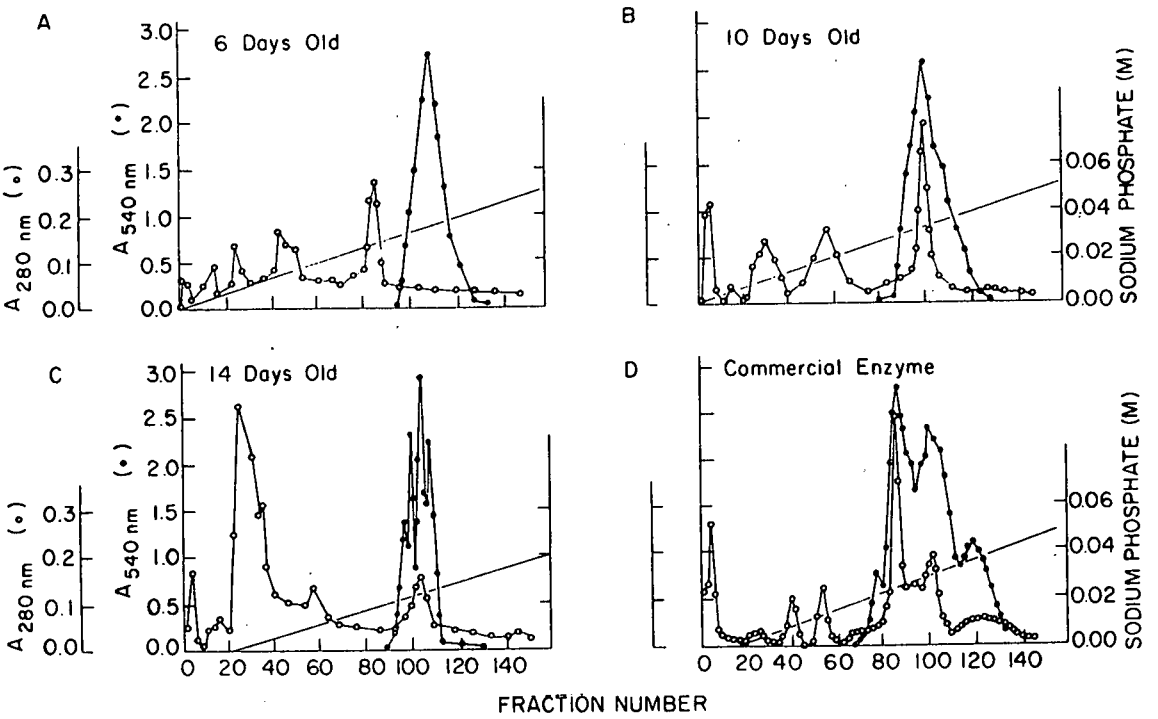

Figure VI-1. DEAE-cellulose chromatography of endoglucanases from T. reesei enzymes from (-O-) Relative protein concentration (-●-) relative activity with respect to CMC. (a) 6-day old culture, (b) 10-day-old culture, (c) 14-day-old culture, and (d) commercial preparation.

VII. PROPOSED MODEL FOR THE REGULATION OF CELLULASE BIOSYNTHESIS

Based on the data we obtained and other petinent information, we propose a mode for the regulation of cellulase biosynthesis.

A unified model for the regulation of cellulase synthesis is difficult to construct due to the wide diversity of responses of organisms to various inducers and growth environments. However, given the known mechanism of induction-repression it is possible to project from the pertinent data, and to postulate a specific model for the regulation of cellulase biosynthesis. The proposed mechanisms of regulation must take into account the importance of glucosidase in the regulation of glucose and also of cellobiose levels within the cells. The glucosidase could be membrane bound or intracellular or both. The glucosidase could be constitutive or adaptive or both. The glucosidase could represent a wide spectrum of enzymes each possessing different substrate specificities, so that one specific glucosidase could have high activity towards hydrolysis of cellobiose but low activity toward sophorose or vice versa.

Several different models for the regulation of cellulase synthesis could be drawn from the known data discussed int this review. However, the main features of all possible models would have to be similar. Hence the model presented in Figure VII-1, would allow for several possible variations as the conditions warrant.

A description of the proposed regulation model is a follows (see Figure VI-1:

(1) Cellobiose and glucose represent the limited cellulolytic products of the "basal cellulase" hydrolyzing cellulase. Cellobiose serves as a carbon source as well as a "potential" inducer.

(2) Active transport of cellobiose across the cell membrane is achieved by the development of an adaptive transport enzyme system (14). Constitutive glucosidase could be present as a membrane bound enzyme, which would hydrolyze part of cellobiose to glucose. Adaptive glucosidase could also be formed in response to the presence of cellobiose or other inducers such as sophorose or lactose in the growth medium.

(3) After cellobiose enters the cells, the intracellular cellobiose, could become the "active" inducer and react with the repressor protein to render the repressor inactive and thus induction occurs. Or the "potential inducer" could be hydrolyzed by intracellular glucosidase resulting in the accumulation of glucose and the repression of cellulase synthesis.

(4) The affinity of the "active inducer" for the repressor protein determines the efficiency of induction.

(5) Induction of cellulase synthesis occurs when the repressor protein has been inactivated by the "active inducer" and transcription and translation follow. The actual amounts of exocellulase and endocellulase are controlled by the activity of translation (in this model, we are assuming that the expression of both cellulase genes is controlled by the same regulator).

(6) The newly synthesized cellulase, in response to induction, could remain membrane bound. The releasing of cellulases across the cell membrane is regulated by a specific releasing mechanism, possibly a specific intracellular acid protease. (Low pH enhances cellulase release and high pH inhibits it (32)).

(7) Newly released extracellular cellulase hydrolyzes cellulose to glucose and cellobiose. The amount of extracellular cellulase released affects of the amounts of glucose and cellobiose produced. Glucose and cellobiose, in turn, support cell growth and are also involved in the induction and repression.

(8) Intracellular glucosidase hydrolyzes part of intracellular cellobiose to glucose.

(9) High levels of intracellular glucose inhibit intracellular glucosidase activity.

(10) Certain organisms such as Sporotrichum possess the capacity to produce adaptive glucose oxidase in response to the accumulation of glucose inside the cell. Glucose oxidase oxidizes glucose to gluconic acid and gluconolactone.

(11) Gluconolactone inhibits glucosidase activity thus lowering the activity of glucosidase which in turn lowers cellulase synthesis repression.

(12) Intracellular glucose concentration influences cellulase synthesis by a repression mechanism.

This proposed regulation model allows for flexibility in explaining the different responses of organisms to different inducers. It also provides an explanation of the various responses which organisms exhibit towards various concentrations of inducers and towards glucose repression. All the known inducers of cellulase synthesis can be utilized by the organisms as carbon sources. The continuous cellulase production is influenced by the continuous supply of active inducers. This indicates that the affinity of inducer-repressor is reversible, and it also indicates that the repressor gene continuously produces repressor proteins. In order

to maintain the continuous production of cellulases it is important to use a non-utilizable inducer (gratuitous inducer) which has a high affinity toward the repressor protein.

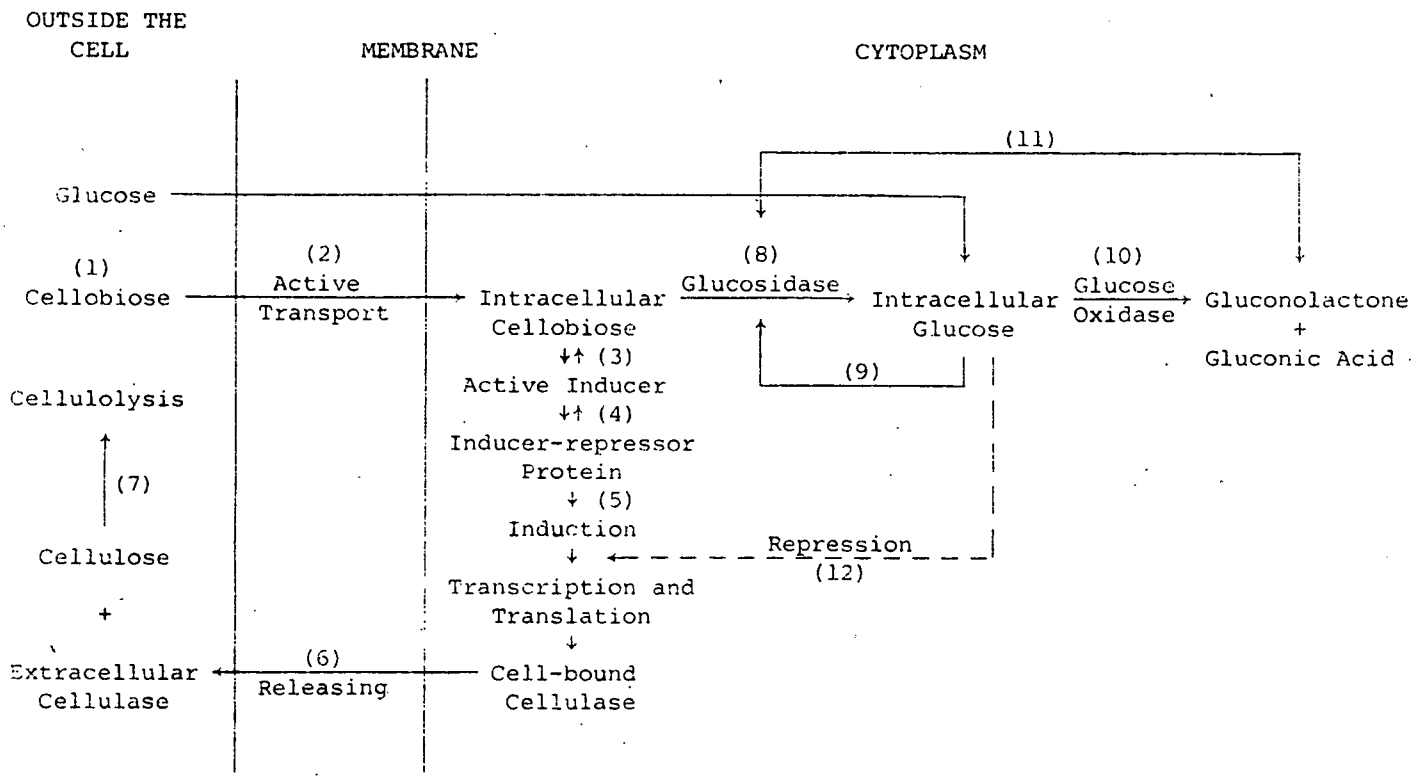

Figure VII-1. Proposed model of regulation of cellulase biosynthesis.

VIII. CELLOBIOSYLTRANSFERASE FROM TRICHODERMA REESEI, A NEW ENZYME

A. Abstract

An independent enzyme entity possessing cellobiosyltransferase activity was found to be present in culture filtrate of Trichoderma reesei. Cellobiosyltransferase mediates the formation of higher oligomer cellodextrins from cellotriose and cellotetraose by transferring cellobiosyl units to the substrate and product acceptors. Therefore, the mode of action of cellobiosyltransferase is different from that of cellobiohydrolase.

B. Results and Discussion

Many glucosidases possess glucosyltransferase activity as part of their innate, reversible enzymatic reactions (5,12). Products of these reversible reactions are known as reversion products. One cellulase enzyme, 1,4-β-D-glucanase, has been shown to not only possess hydrolytic activity toward cellulose, but also transglucosylation activity to catalyze the formation of oligocellodextrins by transfer of glucosyl units to substrate acceptors (9-11,13). Crook and Stone (2) reported that oligoglucosides can be formed from cellobiose by a culture filtrate of Myrothecium. Whitaker (14), concluded that this transfer reaction activity in Myrothecium culture filtrate is indicative of the presence of a cellulase enzyme. Similarly, Okada and Nisizawa (11) and Okada (10) observed a cellulase enzyme, 1,4-β-D-glucanase, from T. viride which was able to catalyze the "condensation" of cellobiose to cellotetraose. This "Condensation reaction" occurred only when high concentrations of cellobiose was used as substrate. Using high pressure liquid chromatographic analysis, Shoemaker and Brown (13) were able to observe the formation of oligocellodextrins from cellotetraose in the reversion reaction catalyzed by 1,4-β-D-glucanase from T. viride. Nevertheless, the existence of cellobiosyltransferase activity was not reported. This communication demonstrates that an independent enzyme entity, cellobiosyltransferase, was present in the culture filtrate of T. reesei.

The techniques used for the preparation of crude extracellular cellulase from T. reesei QM 9414 were identical to those described previously (3). The procedures used for the preparation of partially purified and purified cellobiohydrolase from crude cellulse were similar to those reported earlier (3,6). The concentrated crude cellulase enzyme preparation was applied to a

DEAE-Sepharose column (Pharmacia, 1.5 x 25 cm), and cellobiohydrolase was eluted from the column using a linear salt gradient of sodium chloride in the presence of 0.07M sodium phosphate, pH 6,8 (3). The protein fractions containing cellobiohydrolase activity were pooled, concentrated and the salts removed by gel-filtration with a Sephadex G-25 column (1.5 x 90 cm). The partially purified cellobiohydrolase was further purified by Sephadex G-100 column chromatography.

The cellotriose and cellotetraose were prepared from Whatman CF-11 cellulose. An acid-hydrolysis procedure described by Huebner et al (7) was used with some modifications as described earlier (6). Enzyme hydrolysis was carried out at 50°C in 0.05M sodium acetate buffer (pH 4.8). At various time intervals, samples were taken and transferred to an ice bath to quench the reaction. The enzymatic reaction progression was monitored by analyzing the reactions aqueous solution with a high performance liquid chromatograph (HPLC) as described by Ladisch, et al (8). Cellobiohydrolase activity was determined by methods previously described (3).

Cellobiose, the sole hydrolysis product, is released when cellobiohydrolase acts on the non-reducing end of a cellulose polymer (1,4). In incubation of the purified cellobiohydrolase with cellotriose, both cellobiose and glucose were produced. For up to 14 hours of incubation, no reversion products were detected (Fig. 1). Neither were they detected when cellotetraose was used as the substrate, only cellobiose was produced (Fig. 2). Contamination of the cellotriose was thought to be the cause of small amounts of glucose appearing after 50 min of incubation in an otherwise purifed cellotetraose preparation. These results illustrate the fact that cellobiohydrolase is not able to mediate the production of higher oligomer cellodextrins from either cellotriose or cellotetraose.

When the partially purified cellobiohydrolase was incubated with cellotriose or cellotetraose, a different mode of action was displayed. The

partially purified cellobiohydrolase reacted with cellotriose to produce not only cellobiose and glucose, as did the purified cellobiohydrolase, but cellopentose as well (Fig. 3b). After the reaction was allowed to proceed further, cellotetraose and a small amount of cellohexose could also be detected (Fig. 3c,d). The observations suggest that the partially purifed cellobiohydrolase preparation contains an additional enzyme which mediates the production of higher oligomer cellodextrins by transferring the cellobiosyl units to the substrate and product acceptors. Therefore, the products from the initial reaction of the partially purifed cellobiohydrolase and cellotriose are glucose, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. As the reaction proceeds, all of these oligodextrins are slowly converted to glucose and cellobiose (Fig. 3e). Similar transcellobiosylation results were obtained using cellotetraose as the substrate. After the initial incubation period cellobiose and a small amount of cellohexose were produced (Fig. 4b). Samplings at 90 min of incubation showed that small amounts of cellotriose and glucose had been produced, probably due to the contamination of the cellotriose. The increase in cellotriose concentration after 3 hours of incubation could be due to the transfer of cellobiosyl units to glucose by cellobiosyltransferase (Fig.4c,d). Therefore, the products of the initial reaction of cellobiosyltransferase with cellotetraose are glucose, cellobiose, cellotriose, cellotetraose, cellopentose, and cellohexose. Here again, the oligocellodextrins are slowly converted to mostly cellobiose and some glucose as the reaction proceeds (Fig. 4e). This appearance of oligocellodextrins from cellotriose or cellotetraose clearly indicates that an enzyme fraction belonging to the cellobiosyltransferase is present in the partially purified cellobiohydrolase preparation and this enzyme is different from the generally recognized cellobiohydrolase enzymes.

A distinct difference between cellobiosyltransferase and glucosyltransferase is in the mode of action demonstrated. Glucosyltransferases are commonly associated with 1,4-β-D-glucanases and transfer only glucosyl units to substrate acceptors, while cellobiosyltransferases transfer cellobiosyl units to substrate and product acceptors. Another difference between them in the inability of cellobiosyltransferase to mediate the formation of higher oligomer cellodextrins from either cellobiose (Fig. 5) or a mixture of cellobiose and glucose (Fig. 6). It was reported earlier (10,11) that a cellulase enzyme, 1,4-β-D-glucanase from T. viride, was able to catalyze the "condensation reaction" of cellobiose to cellotetraose in addition to catalyzing the hydrolysis of cellobiose to glucose.

The results indicate that an independent enzyme entity is present in the partially purified cellobiohydrolase preparation. This is the enzyme responsible for the cellobiosyltransferase activity we observed. Although the enzyme preparation used in this study was only partially pure, we believe the results are valid. The existence of a cellobiosyltransferase indicates that T. reesei is able to conserve the cellulolysis product, cellobiose, to produce higher oligomer cellodextrins. The biological significance of this enzyme is not yet known.

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FIGURE CAPTIONS

Figure 1. Chromatograms of high performance liquid chromatography (HPLC) of cellotriose hydrolysis by purified cellobiohydrolase. The reaction mixture contained 10.9 µg/ml of enzyme and 0.58mM of substrate. Symbols: G_1, glucose; G_2, cellobiose; G_3, cellotriose.

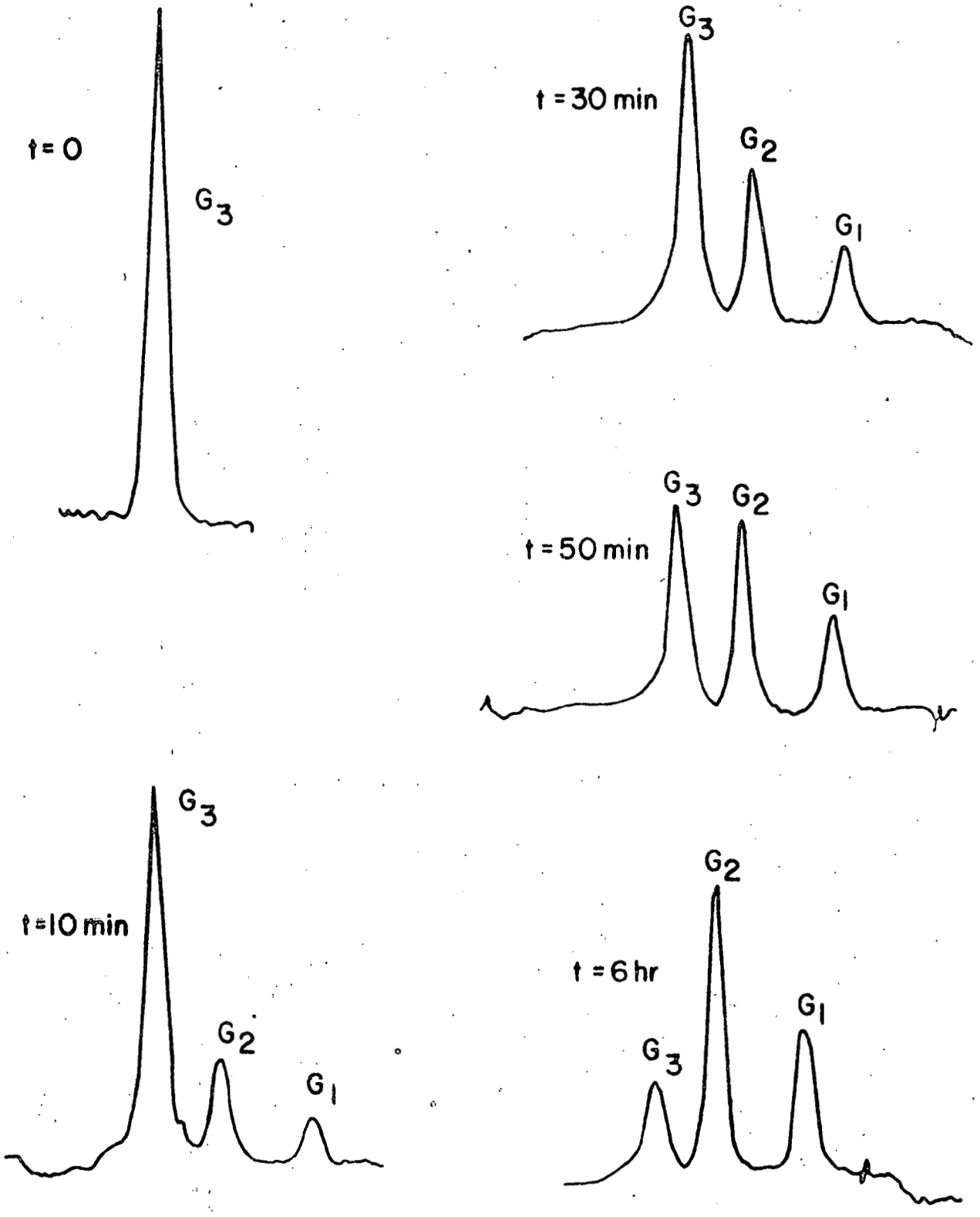
Figure 2. Chromatograms of high performance liquid chromatography of cellotetraose hydrolysis by purified cellobiohydrolase. The reaction mixture contained 10.9 µg/ml of enzyme and 0.63mM of substrate. Symbols: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose.

Figure 3. Chromatograms of high performance liquid chromatography of cellotriose hydrolysis by partial purified cellobiohydrolase. The reaction mixture contained 24 µg/ml of enzyme and 25mM of substrate. Symbols: G_1, glucose; G_2, cellobiose; G_3 cellotriose; G_4, cellotetraose; G_5, cellopentose; G_6, cellohexose.

Figure 4. Chromatograms of high performance liquid chromatography of cellotetraose hydrolysis by partial purified cellobiohydrolase. The reaction mixture contained 24 µg/ml of enzyme and 3.2mM of substrate. Symbols: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentose; G_6, cellohexose

Figure 5. HPLC chromatograms of high concentration of cellobiose in the presence of partial purified cellobiohydrolase. The reaction mixture contained 24 µg/ml of enzyme and 48mM of cellobiose (G_2).

Figure 6. HPLC chromatograms of glucose and cellobiose in the presence of partial purified cellobiohydrolase. The reaction mixture contained 24 µg/ml of enzyme 0.5mM of glucose (G_1) and 2mM of cellobiose (G_2).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G1
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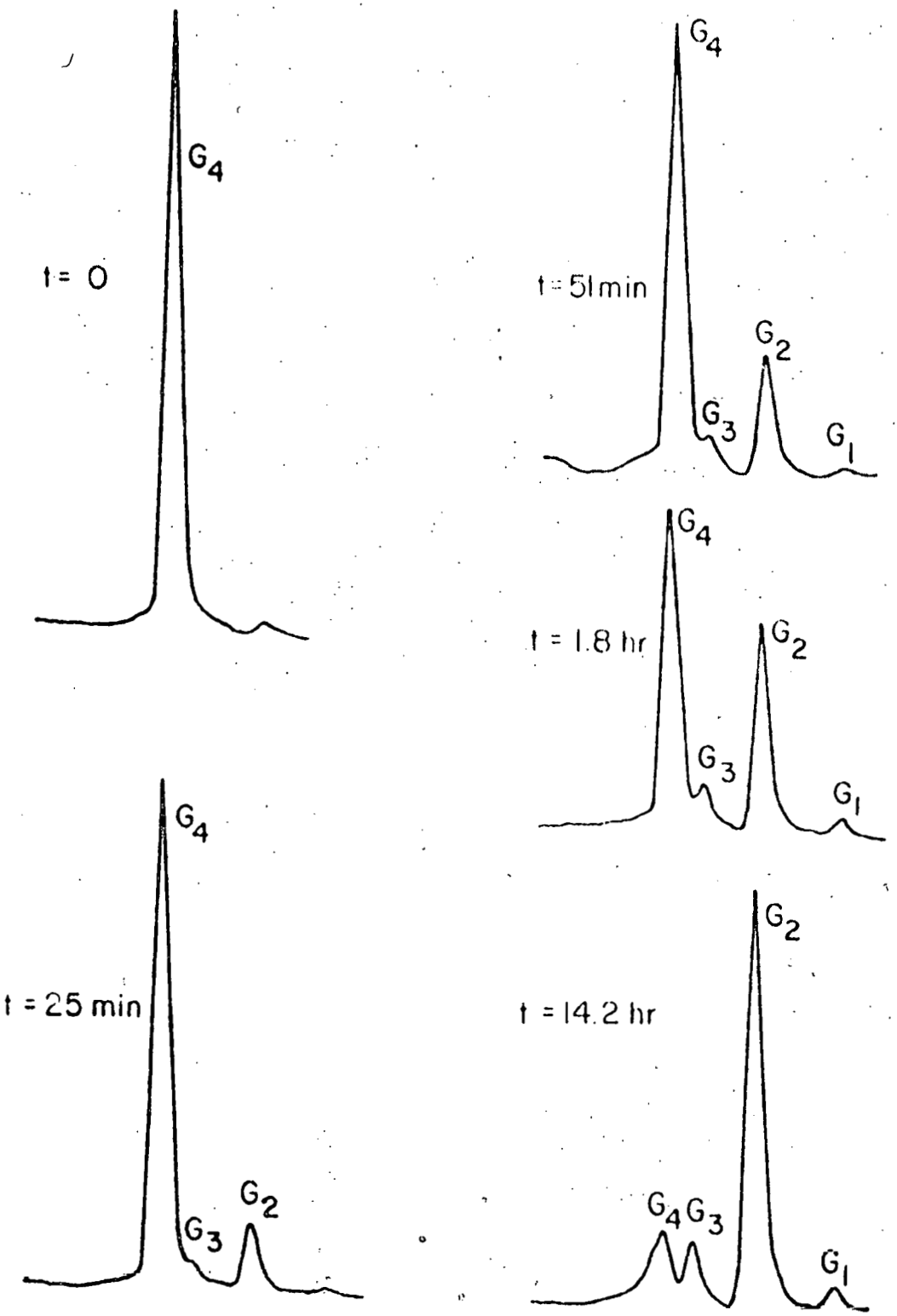
G2
G3
G1

Figure 1.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G1
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G4
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G1
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G3
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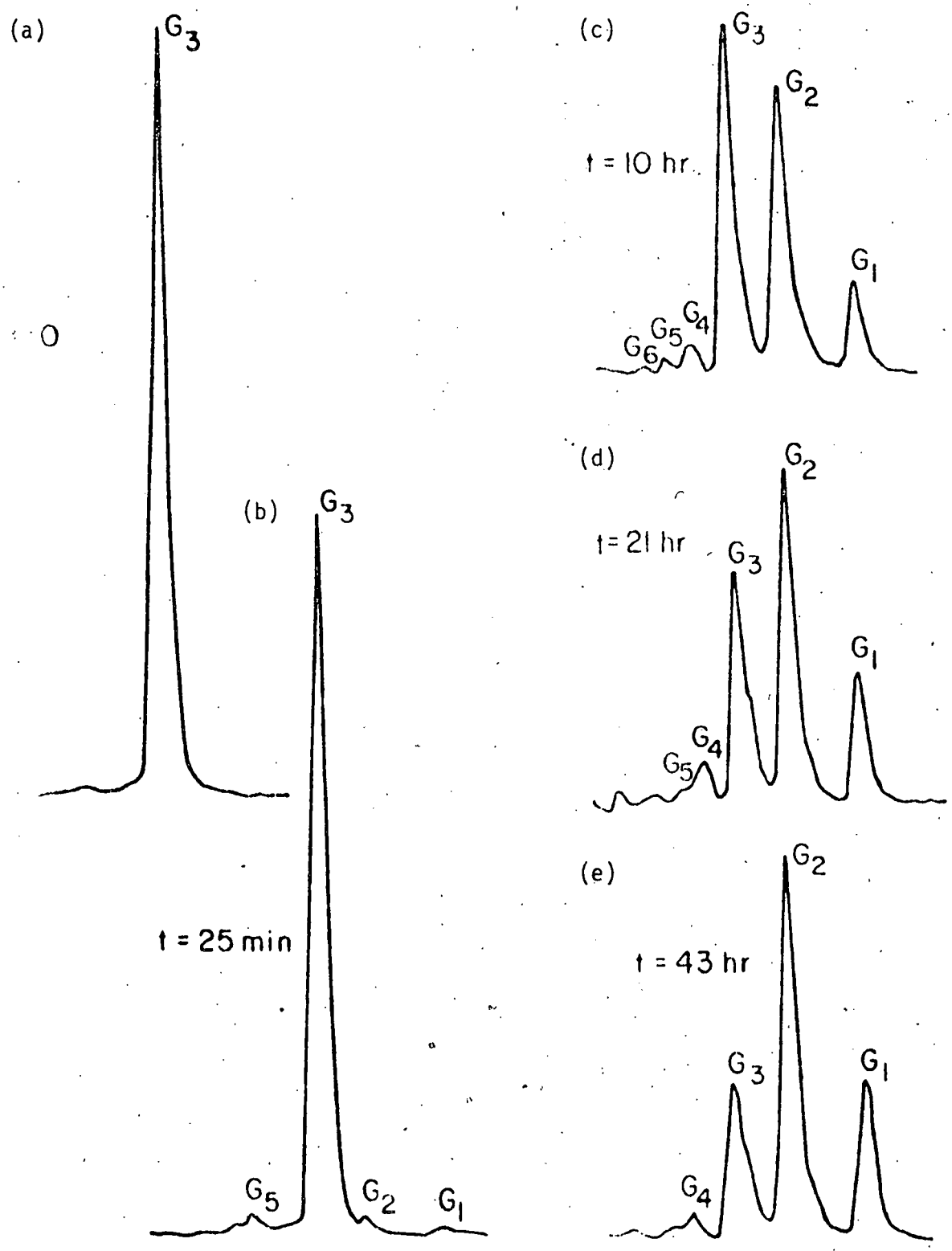
G2
G4 G3
G1

Figure 2.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G6
G5
G4
(d)
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(e)
G2
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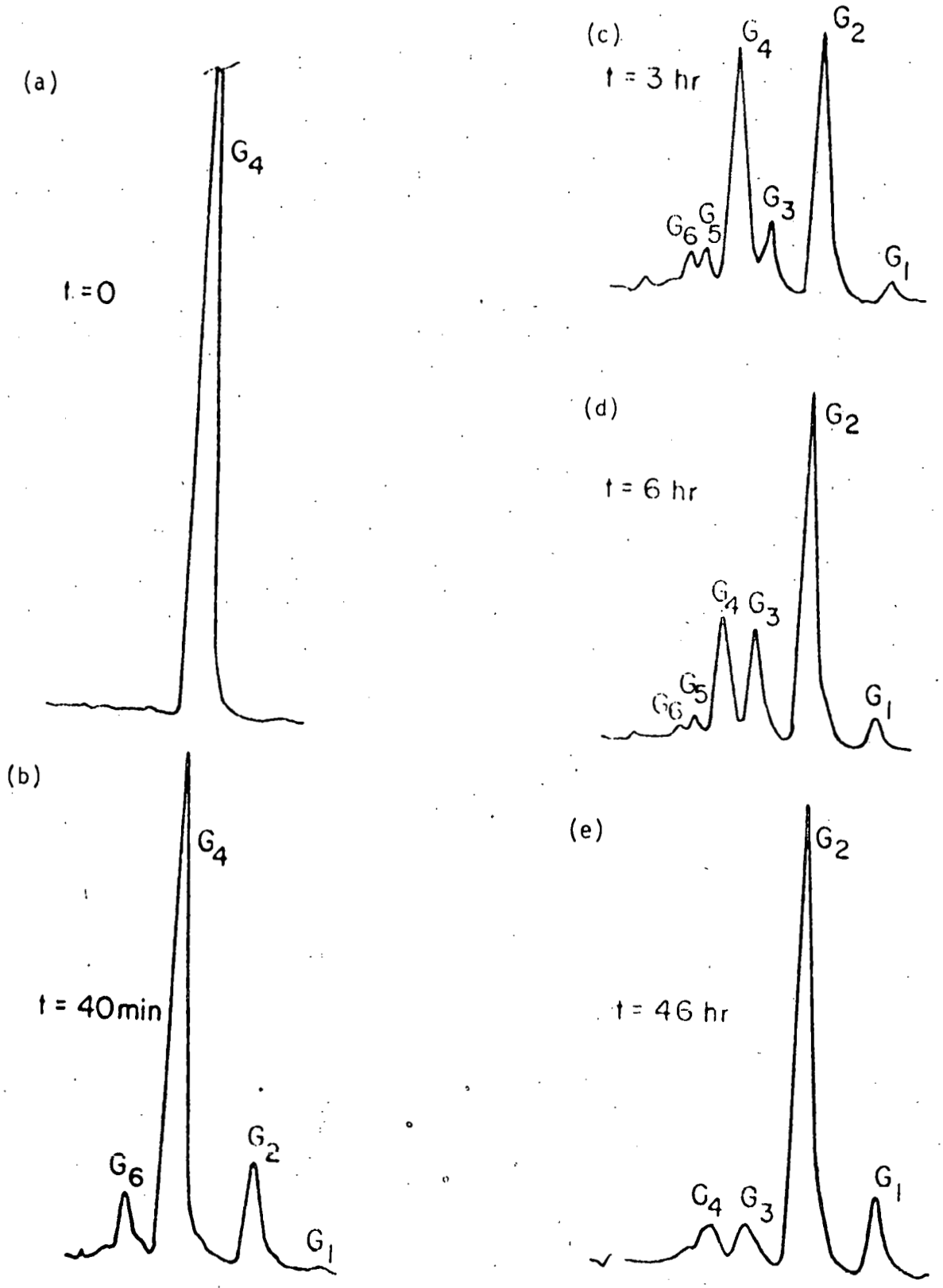
G3
G1
G4

Figure 3.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(e)
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G4
G3
G1

Figure 4.

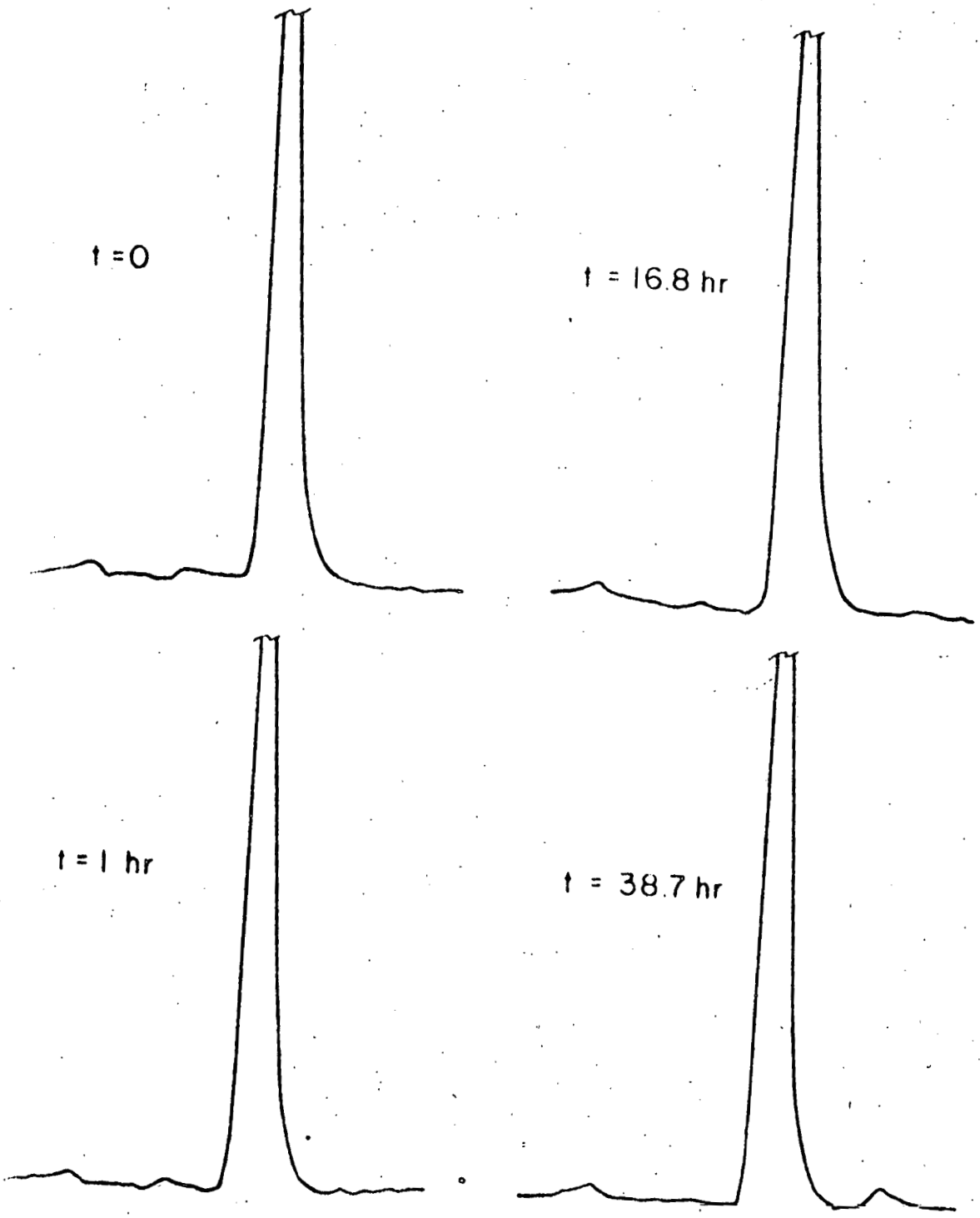

Figure 5.

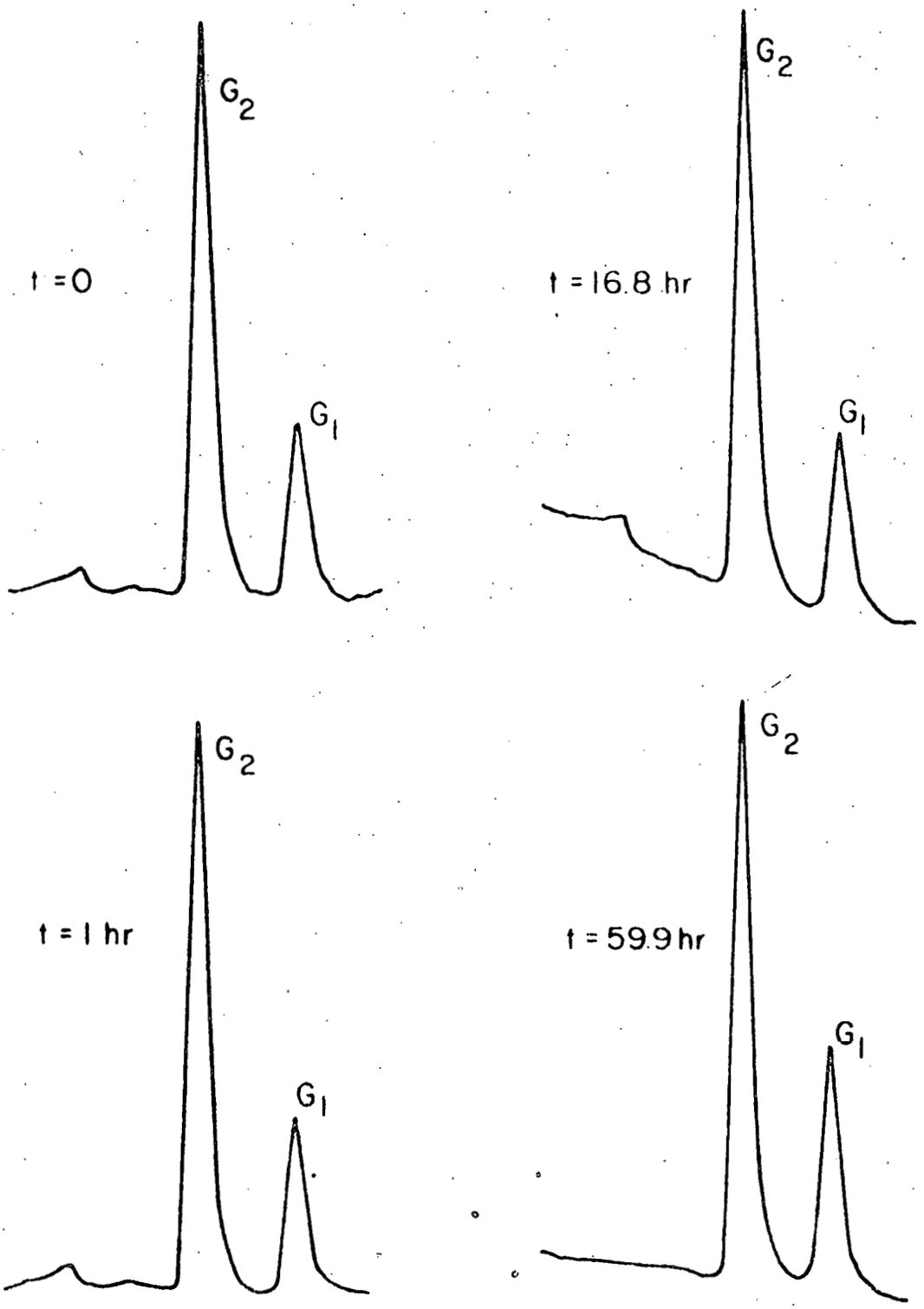

Figure 6.

IX. KEY PERSONNEL SUPPORTED BY DE-AS-02-76ER02755

		Research Interest
Tsao, G. T.	Director	Research Direction and Coordination
Gong, C. S.	Senior Research Group Leader	Cellulase Biosynthesis and Purification
Chang, M.	Senior Research Group Leader	Cellulase Structure and pre-treatment
Ladisch, M.	Assistant Professor	Cellulase Kinetics

Graduate Students

Chou, T.

Hong, J.

Hsu, T.