Effects of Structural Features of Cotton Cellulose on Enzymatic Hydrolysis

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Synopsis

Textile cotton wastes were treated with γ rays and 18% NaOH and 70% $ZnCl_2$ solutions and were subjected to enzymatic hydrolysis. The untreated and treated samples were characterized both before and after hydrolysis by means of parameters concerning molecular structure (degree of polymerization), supermolecular structure (x-ray diffraction), accessibility, and reactivity (moisture regain, enzyme adsorption, and solubility in FeTNa). These parameters were correlated to kinetic parameters of the hydrolysis reaction. The V_{max} and K_m values were evaluated from Lineweaver–Burk plots at different temperatures. The V_{max}/K_m ratio, analogous to the specificity constant, proved to be less sensitive to experimental errors and more suitable for a comparison of the kinetic behavior of the samples. The modifications of both supermolecular structure and morphology of cellulose were of primary importance to attain high yields and rates of hydrolysis. Furthermore, the structural and morphologic parameters chosen to characterize the samples can be correlated to the kinetic parameters of enzymatic hydrolysis, in particular to K_m values.

INTRODUCTION

The biological degradation of cellulose is brought about naturally by some microorganisms that synthesize the enzymatic complex cellulase. In general the accessibility of native cellulose to extracellular enzymes or other reagents is limited in part by its distribution within the cell wall and the nature of structural relationships among the cell wall components.[1,2]

Different pretreatments[3] have been tried to improve the accessibility of the cellulose material to cellulolytic enzymes in order to reduce the cost of the hydrolytic process.

This work examines the relationships among some structural and morphologic parameters of treated and untreated celluloses and some kinetic parameters of the enzymatic hydrolysis.

Cotton fibers from textile wastes were chosen as the substrate since their highly ordered supermolecular structure and morphologic features can limit the accessibility of cellulose to cellulase enzyme.

The three types of pretreatments used in this work included: (1) γ-ray irradiations that modified the molecular structure without significant changes in both the supermolecular structure and the morphology of cellulose; (2) treatments with 18% NaOH that changed the crystalline unit cell (cellulose I to II) and altered the cellulose morphology; (3) treatments with 70% $ZnCl_2$ that affected the morphology of cellulose and determined a structural lateral order variation (decrystallization) without changes in the type of crystalline unit.

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EXPERIMENTAL

Substrates

Textile cotton wastes were washed with water and sieved. The 200–400 mesh fraction was collected.

Portions of the homogenized material underwent one of the following pretreatments:

γ-Ray Irradiation: The cotton, contained in polyethylene bags, was irradiated in a water suspension (10% w/v) by a ^{60}Co radiation source at a dose rate of 270 krad/hr up to a dose value of 50 Mrad. After filtration through a glass filter, the residue was dried at 60°C for 6 hr.

Sodium Hydroxide Treatment: The cotton was treated with a 18% (w/w) NaOH solution, with a bath ratio of 1:50 at 20 ± 1°C for 2 hr. This treatment was conducted in a stoppered flask with slow stirring using fresh NaOH solution. Then the cotton was neutralized with a 5% acetic acid solution and washed with distilled water.

Zinc Chloride Treatment: The cotton was treated with a 70% (w/w) $ZnCl_2$ solution (bath ratio 1:50) at 20 ± 1°C, for 2 hr with slow stirring. The sample, washed with a 50% $ZnCl_2$ solution and subsequently with a 20% one, was regenerated with 0.05*N* HCl and washed with the same acid solution up to negative test for Zn^{2+}. Finally, it was washed with distilled water.

Substrates Characterization

X-Ray Diffraction Analysis: A Siemens D-500 diffractometer, equipped with a scintillation counter and a linear amplifier, was utilized at these operational conditions: $Cu_{K\alpha}$ (Nickel filter), 18-mA fed current at 40 kV.

Degree of Polymerization: It was determined by viscosimetric measurements in cuproethylendiamine (CED) at 25.0 ± 0.1°C.[4]

Moisture Regain: **It** was measured at 20 ± 1°C and 65% relative moisture. The samples, dried at 70°C for 4 hr, were conditioned up to constant weight (ca. 72 hr); then they were dried at 105 ± 2°C for 4 hr and weighed. The equilibrium moisture content of the conditioned samples was calculated as a percentage of dry weight.

Solubility in Sodium-Iron Tartrate (FeTNa): The dissolution value was determined by dissolving the samples in FeTNa solutions with different percentages of free NaOH, for 15 min with stirring.[5]

Enzymes

Two enzymes (supplied by Novo Enzyme Company) were used: (1) Cellulase from *Trichoderma viride* with a declared activity of 210 IU/g (toward Whatman cellulose CC31) and (2) Cellobiase from *Aspergillus niger* with a declared activity of 250 IU/g (toward cellobiose).

Enzyme Activity: It was defined in terms of International Units (IU: enzyme amount which formed 1 μmole of reducing sugars as glucose/min). C_1, β-1,4 exocellobiohydrolase (EC 3.2.1.91) and C_x, β-1,4 endoglucanase (EC 3.2.1.4) activities were measured toward Whatman powder CC 31 and carboxymethylcellulose (D.S.0.7; Mw 80,000), respectively. The activity of cellobiase, β-glu-

idase (EC 3.2.1.21), was determined in citrate buffer (pH 4.8) toward cellobiose.

Kinetics

The runs were performed at different temperatures (30–60°C) in a thermostated reactor, with mechanical stirring (300 rpm) by using 0.05*M* citrate buffer (pH 4.8). The cellulase and substrate concentrations were 0.3% and 0.75% (w/v), respectively. Samples (2 ml) drawn from the reactor with stirring at different times were filtered through a glass filter (G_4) and frozen to quench the enzymatic reaction.

Sugar Analysis

Reducing sugars and glucose were determined by the dinitrosalycilic acid method[6] and the Glucoquant test (Boehringer), respectively, using glucose for calibration.

RESULTS AND DISCUSSION

Characterization of the Substrates

The characterization of the cotton samples both before and after the enzymatic hydrolysis was made by means of parameters concerning molecular structure (degree of polymerization), supermolecular structure (x-ray diffraction), accessibility, and reactivity of the substrate (moisture regain, enzyme adsorption, and solubility in FeTNa.)

The degree of polymerization ($\overline{DP_v}$) values of samples that were subjected to enzymatic hydrolysis for different times are listed in Table I.

In general, the pretreatments led to depolymerization. This occured particularly in irradiated cotton cellulose. The γ-ray treatment likely gave rise to carbonyl, carboxyl, and peroxide groups, which weaken the glucosidic bond.

The percent reduction in degree of polymerization after hydrolysis was greater in the sample treated with $ZnCl_2$ than in the untreated or NaOH-treated samples. No significant variation was observed in the irradiated sample.

The x-ray diffractograms of the treated or untreated samples before and after 24-hr enzymatic hydrolysis are shown in Figure 1.

The untreated cotton cellulose [Fig. 1(A)] showed the characteristic diffractogram of native cellulose (cellulose I). A similar diffractogram was observed in the irradiated sample [Fig. 1(B)], suggesting that the γ rays did not signifi-

TABLE I
Degree of Polymerization ($\overline{DP_v}$) of Cotton Celluloses

samples	before hydrolysis	after hydrolysis, hrs		
		4	7	24
Untreated cell.	2075	–	–	1640
Irradiated "	60	–	–	60
18% NaOH treated cell.	1000	790	690	–
70% $ZnCl_2$ " "	1430	700	430	–

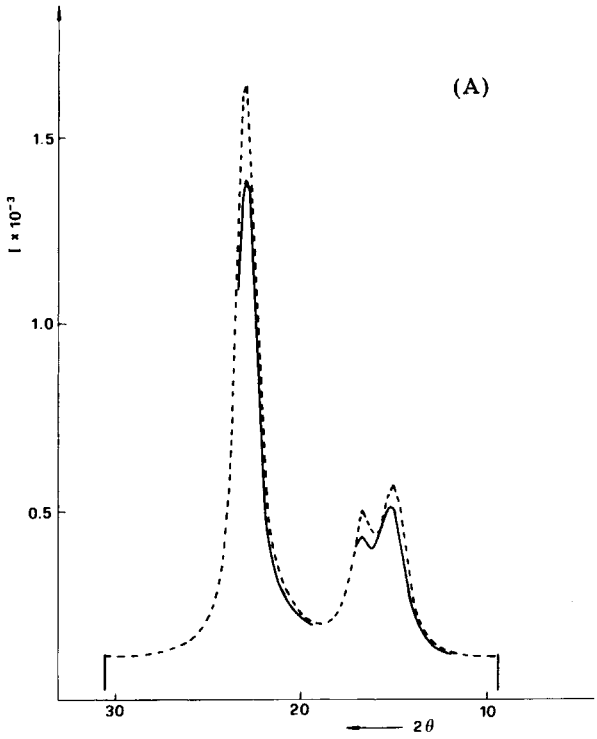


Fig. 1. X-ray diffractograms of untreated and treated cotton celluloses (—) before and after (- - -) hydrolysis. (A) Untreated cellulose; (B) irradiated cellulose; (C) 18% NaOH treated cellulose; (D) 70% $ZnCl_2$ treated cellulose.

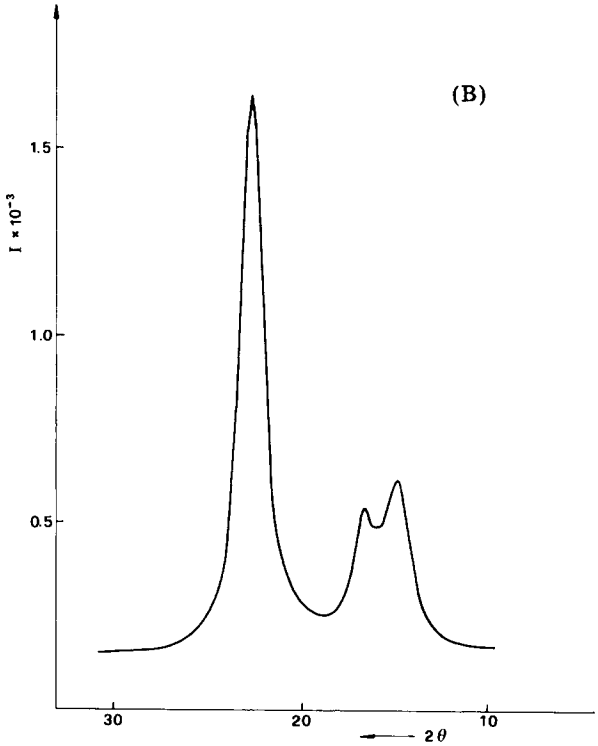
cantly affect the supermolecular structure of cellulose, at least at the dose used in the present work (50 Mrad).

The sample treated with 18%-NaOH solution [Fig. 1(C)] showed a typical x-ray pattern of mercerized or regenerated cellulose (cellulose II). The diffractogram of the sample treated with 70% (w/w) $ZnCl_2$, when compared with the one of cellulose I, showed a marked lateral order modification [Fig. 1(D)]. No cellulose II was observed and the original structure (cellulose I) was not restored by treatments with boiling water. Furthermore, the absence of other, less stable polymorphic structures of cellulose confirms the well-known decrystallization effects of the $ZnCl_2$ solutions at defined concentrations.[7]

After hydrolysis, all the samples with the exception of the irradiated one showed a rise in diffraction intensity with a recovery of crystallinity due to a removal of amorphous material. The anomalous result observed with the irra-

TABLE II
Moisture Regain (%) of Cotton Celluloses

samples	before hydrolysis	after hydrolysis hrs		
		4	7	24
Untreated cell.	6.25	–	–	6.10
Irradiated "	6.35	–	–	6.05
18% NaOH treated cell.	9.20	8.5	7.7	–
70% $ZnCl_2$ " "	9.20	6.35	5.7	–



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diated sample could be due to a partial reticulation or to some chemical change of the cellulose macromolecules. This would account for the reduction in both rate and yield of enzymatic hydrolysis.

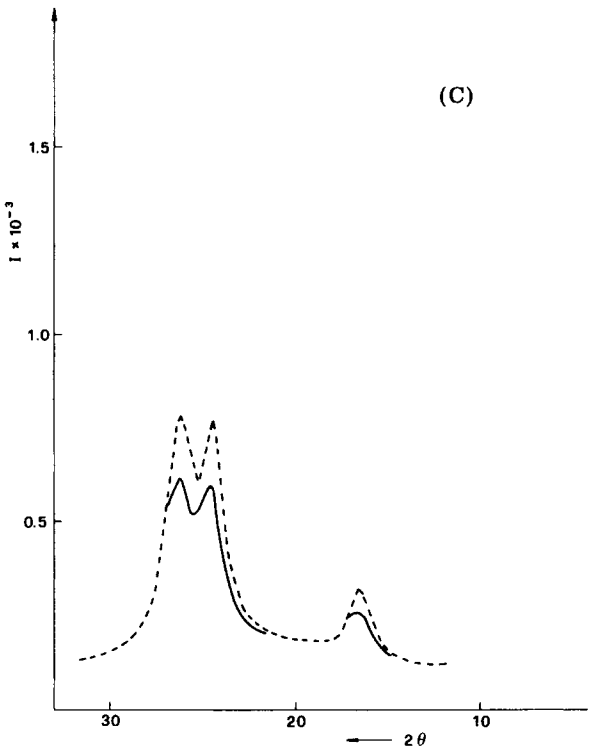
The moisture regain is one of the most effective parameters to evaluate the morphologic and structural changes in polymeric materials subjected to different pretreatments. Hermans et al.[8] obtained a linear relationship between moisture regain and relative amount of the more accessible amorphous portions of different celluloses. Table II shows the values of moisture regain (%) of the treated or untreated samples both before and after hydrolysis for different times. The moisture regain of the samples treated with 18% NaOH or 70% $ZnCl_2$ was considerably higher than that of both the untreated and the irradiated samples.

After hydrolysis the moisture regain remained almost unchanged in both the untreated and irradiated samples, whereas it drastically decreased in the other ones. Particularly, the cotton cellulose treated with $ZnCl_2$ proved to be less accessible than the NaOH-treated one.

The reactivity of cellulose is often defined as the susceptibility of this material to chemical attack.[9]

FeTNa solutions with different contents of free NaOH have been used widely to evaluate the reactivity of different kinds of cellulose materials and the reactivity changes caused by different treatments on the same kind of cellulose material.

The solubility in FeTNa of all the samples before hydrolysis is shown in Figure 2. The significant decrease in degree of polymerization observed in the irradiated sample caused a complete solubilization in FeTNa with lower solvent power.



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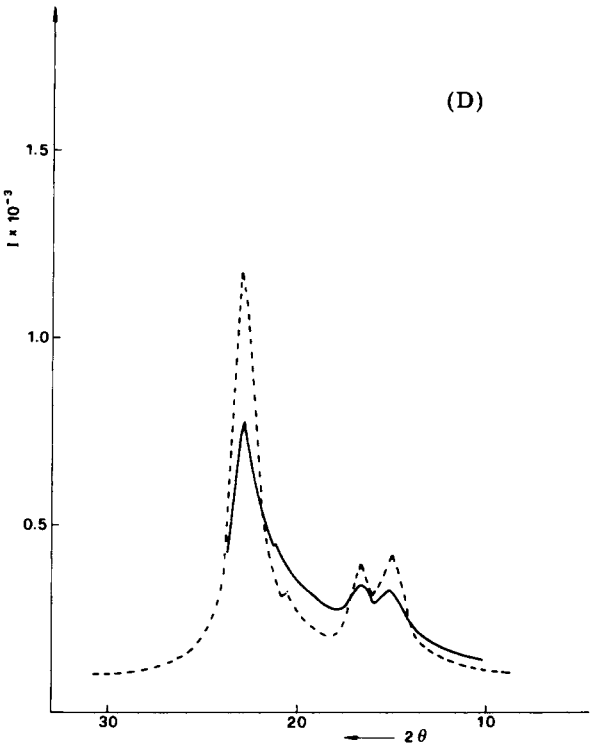
The higher solubility of the sample treated with $ZnCl_2$ compared with that of the untreated one is more likely due to an increased accessibility of the material than to a depolymerization during the treatment. Finally, the anomalous behavior of NaOH-treated cellulose could be accounted for by the phenomena of "passivation" due to molecular rearrangements during the treatment.[10]

Enzymatic Hydrolysis

The synergistic action of the cellulase components C_1, C_x and cellobiase is reported in the literature.[11,12] This interaction is noticeable in fibrous cellulose substrates whose enzymatic hydrolysis requires the presence of a high C_1 component content. In addition, the component with cellobiase activity is important to reduce the inhibition phenomena due to production of cellobiose during hydrolysis.[13,14]

The measured activities of the components of the enzymatic complex were 114 and 57 IU/g for C_1 and C_x, respectively, while the cellobiase activity was negligible. The progress of hydrolysis, measured in terms of reducing sugar concentration and percentage of saccharification, is shown in Figures 3 and 4, respectively. Figures 3 and 4 show that the enzymatic hydrolysis occured faster in the $ZnCl_2$-treated sample than it did in the other three samples. This agrees with the structural parameters obtained by x-ray and moisture regain analysis.

An interpretation of enzyme adsorption data at temperatures close to 0°C was



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carried out according to Langmuir analysis. This temperature ensured the absence of hydrolysis. Preliminary results indicated that the numbers of disposable sites in the untreated, NaOH-, and $ZnCl_2$-treated samples are in the ratio 3:4:7. The treatments could modify the preequilibrium state of the enzymatic reaction and, therefore, the rate and the yield of hydrolysis.

As expected, the enzymatic hydrolysis of every sample was rapid in the initial

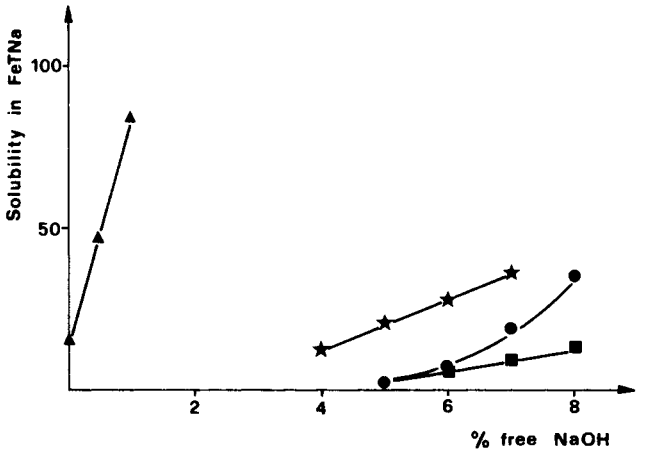

Fig. 2. Solubility in sodium-iron tartrate of (●) untreated, (▲) irradiated, (■) 18% NaOH treated, and (★) 70% $ZnCl_2$ treated cotton celluloses.

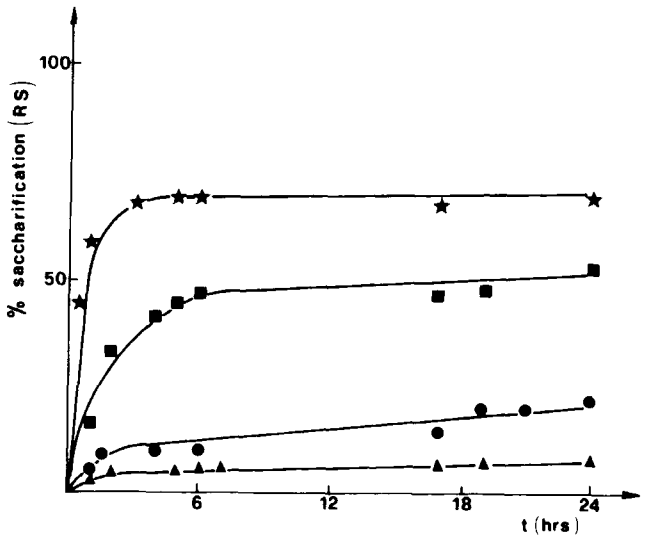

Fig. 3. Enzymatic hydrolysis of untreated and treated cotton celluloses as a function of time at 50°C. (●) untreated cellulose; (▲) irradiated cellulose; (■) 18% NaOH treated cellulose; (★) 70% $ZnCl_2$ treated cellulose. % Saccharification (RS) = [(mg reducing sugars as glucose)/(mg cellulose)] (162/180) × 100

stage of the reaction (Figs. 3 and 4). Nevertheless, the hydrolysis rate reached a nearly constant value in 6 hr but the glucose amount was smaller than the total amount of reducing sugars because of the inhibition by products.

In order to check the inhibition by cellobiose,[12-15] a commercial product with cellobiase activity was added to the enzymatic complex that was used to hydro-

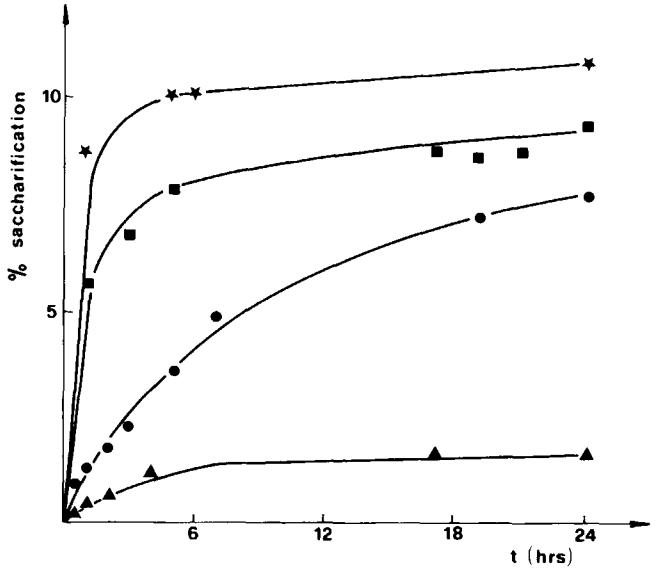

Fig. 4. Glucose production during the enzymatic hydrolysis of untreated and treated cotton celluloses at 50°C. (●) untreated cellulose; (▲) irradiated cellulose; (■) 18% NaOH treated cellulose; (★) 70% $ZnCl_2$ treated cellulose. % Saccharification = [(mg glucose)/(mg cellulose)] (162/180) × 100

lyze both the untreated and the $ZnCl_2$-treated samples (Fig. 5). The percentage of saccharification of both types of substrate had been increasing for more than 24 hr.

The Lineweaver–Burk plots for the three substrates at different temperatures (30–60°C) are shown in Figure 6. The rate of hydrolysis increased significantly from untreated to NaOH- and $ZnCl_2$-treated cellulose.

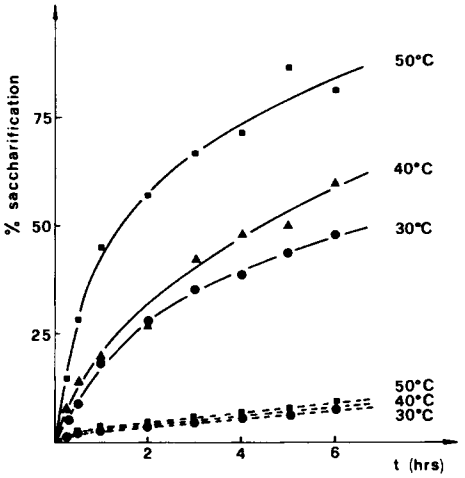

Fig. 5. Influence of the cellobiase activity on enzymatic hydrolysis as a function of temperature of (——) untreated and 70% $ZnCl_2$ (– –) treated celluloses. % Saccharification: see Figure 3.

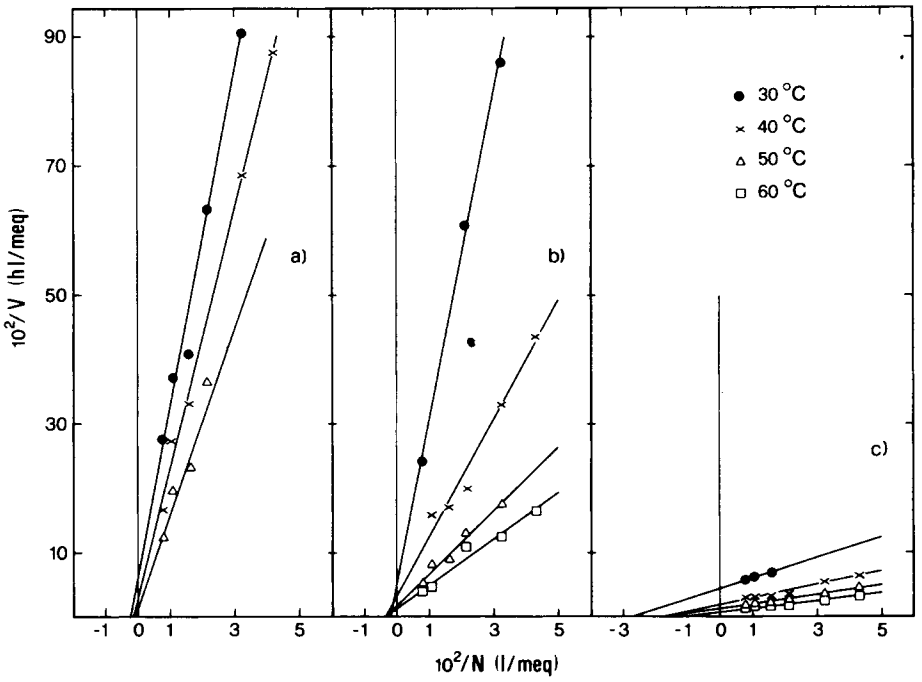

Fig. 6. Lineweaver–Burk plots of untreated and treated cotton celluloses, as a function of temperature. (A) Untreated cellulose; (B) 18% NaOH treated cellulose; (C) 70% $ZnCl_2$ treated cellulose. N: substrate concentration in terms of anhydrous glucose units per volume.

TABLE III
V_{max} (meg $l^{-1}h^{-1}$) Values as Functions of Temperature for the Variously Pretreated Celluloses

samples	30°C	40°C	50°C	60°C
Untreated cell.	17	37	60	–
18% NaOH treated cell.	21	31	56	66
70% $ZnCl_2$ " "	22	48	78	117

According to the Michaelis–Menten equation, the rate of hydrolysis depends on the decomposition rate of the enzyme–substrate complex to give the products (related to V_{max}) as well as on the enzyme affinity for substrate (related to $1/K_m$).

The analysis of the plots (Fig. 6) shows that the changes in the hydrolysis rate resulting from the treatments were essentially due to variations of K_m values and suggests that these pretreatments primarily affected the enzyme–substrate affinity.

The V_{max} values and, to a major extent, the K_m values determined from the plots were scarcely accurate because they were obtained inverting intercepts very close to the origin. V_{max} values are listed in Table III. As observed above, the V_{max} values for the three substrates at the same temperature do not differ significantly. The K_m values showed a slight or uncertain dependence on temperature because of experimental errors. They resulted in the ranges 400–1000, 200–500, and 40–70 meq of glucose/liter for untreated NaOH-, and $ZnCl_2$-treated cotton cellulose, respectively.

In order to compare the three substrates, the inverse of the slope of the Lineweaver–Burk line (V_{max}/K_m) proves to be a more significant parameter, analogous to the specificity constant.[16] It is less sensitive to experimental errors and directly correlated to the rate of hydrolysis when the substrate concentration is much less than K_m.

The V_{max}/K_m values of the three substrates as functions of temperature are listed in Table IV. On the basis of the Arrhenius equation one obtains values for the activation energy (E_a) ranging from 4 to 13 kcal/mole and values for the logarithm of the preexponential factor (ln A) ranging from 4 to 18 in the sequence: untreated, $ZnCl_2$-, and NaOH-treated cellulose. Analogously, in the case of V_{max}, E_a values ranged from 8 to 12 kcal/mole and ln A values from 16 to 23 in the sequence: NaOH-, $ZnCl_2$-treated, and untreated cellulose.

CONCLUSIONS

The enzymatic hydrolysis of cellulose materials requires an activation pretreatment inducing primarily a structural modification of the substrate. The modifications of both the supermolecular structure and morphology of cellulose

TABLE IV
V_{max}/K_m (h^{-1}) Values as Functions of Temperature for the Variously Pretreated Celluloses

samples	30°C	40°C	50°C	60°C
Untreated cell.	0.038 ± 0.004	0.050 ± 0.002	0.060	–
18% NaOH treated cell.	0.040 ± 0.001	0.110 ± 0.012	0.205 ± 0.020	0.284 ± 0.032
70% $ZnCl_2$ " "	0.605 ± 0.022	1.058 ± 0.088	1.345 ± 0.065	1.798 ± 0.065

were of primary importance to attain yield and rate of hydrolysis suitable for an industrial process of cellulose conversion.

Treatment methods that affected only the molecular structure of cellulose proved to be inadequate in improving the kinetics of the hydrolysis reaction.

The chemical and physical parameters that characterize and differentiate the substrates can be correlated to the kinetic parameters of the hydrolysis reaction. In particular, K_m seems to be the parameter more sensitive to the structural variations examined in the present work.

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