European Polymer Journal ,1970, Vol. 6, pp. 41–50. Pergamon Press. Printed in England

POLYDISPERSITY OF CELLULOSES AND ENZYMIC DEGRADED CELLULOSES BY GEL PERMEATION CHROMATOGRAPHY*

M. RINAUDO and J. P. MERLE

Faculté des Sciences de Grenoble et Centre de Recherches sur les Macromolécules Végétales, 38, Saint Martin d'Heres, Grenoble, France

Abstract—The first part of this paper concerns the influence of nitration; the proposed 64 per cent NO_3H/36 per cent AcOH mixture is considered to be the best, particularly with native cellulose of high degree of polymerization ($\overline{DP}_w = 4700$); nitration to 13·9–14 per cent N is regularly found for a time of contact of 1·5 hr at 2° with the nitration mixture. The weight average mol. wt., the degree of substitution and the polydispersity obtained by GPC in THF have been determined.

In the second part of this work, the enzymic degradation by a cellulasic extract of Basidiomycete is followed during 0–48 hr periods on different types of celluloses. After nitration, the residual cellulose is characterized by its mol. wt. and polydispersity. The rate of enzymic modification is discussed and related to the structure of the substrates.

INTRODUCTION

GEL PERMEATION chromatography has been used recently on cellulose; essentially Segal,[1,2] Muller and Alexander[3] and Meyerhoff[4] were the first using this technique on cellulose nitrates disolved in THF. More recently,[5] the technique was applied to different sorts of cellulose esters.

In the first part of this work, we propose the utilization of this technique to compare, with the help of other indications, the effect of the nature of the nitration solution on the nitrocelluloses prepared; then, in the second part, we apply this treatment to residual cellulose from enzymatic attack by a cell-free cellulase.

In fact, the macromolecular aspect of the degradation has never been exhaustively studied; Whitaker[6] measured $\overline{DP}$ by viscosimetry and osmotic pressure, after contact of culture filtrate of *Myrothecium verrucaria* on swollen cellulose. Levi and Sellen[7,8] measured the mol. wt. by light scattering for cellulose attacked by *Chaetomium globosum*; the distribution curves are theoretically deduced.

We consider that only on paper[9] is related to the fractionation and polydispersity of native and enzymatically degraded cellulose.

In this work, we shall discuss essentially the GPC and mol. wt. results and only mention the other experiments; we shall disclose the first results obtained by GPC in the cellulose field; we propose an analyse of GPC diagrams obtained for different nitrate samples (from Whatman powder, native cotton, swollen cotton, ramie, rayon) before and after enzymic degradation; we obtain the values of $\bar{M}_w$, $\bar{M}_n$ and polydispersity $\bar{M}_w/\bar{M}_n$ from the chromatogram using the universal calibration proposed by Benoit[10] and the empirical Mark–Houwink equation:

$$[\eta] = 1{\cdot}5\ \bar{M}_w^{1{\cdot}01}, \text{ using THF as a solvent.}^{(11)}$$

* Paper presented at the Third Prague Microsymposium on Distribution Analysis and Fractionation of Polymers, 23–26 September 1968.

In conclusion, we will discuss the interest of this method which we consider as particularly well adapted to our subject. By comparison of the reactivity of different substrates and by following the modification of their own structure, we expect to explain the mechanism of enzymatic degradation.

EXPERIMENTAL

(*a*) *Materials*

Substrates. Native cotton from India was extracted by an ethanol–benzene mixture (40–60) for 4 hours in a Soxhlet apparatus.

Swollen cotton. Native cotton was treated by a 3N NaOH solution for 1 hr at 0° with some agitation from time to time, then neutralized with 3N HCl at 0° and washed with water to neutrality.[12, 13] This cotton is used without drying before enzymatic attack.

Whatman powder 'CF II column chromedia'.

Enzyme. A fungal commercial enzymatic extract from S.E.A.B. (France) was used; the solution was prepared just before incubation by dissolving the powder in the buffer-solution.

(*b*) *Methods*

Enzymatic hydrolysis. For each experiment, two samples of 300 mg of substrate, after a 24 hr period of contact with distilled water, are immersed in 30 ml of acetate buffer (0·1 M, pH 5) containing dissolved enzymatic powder (4 mg/ml). The incubation proceeds at 40° from 0 to 48 hr of contact in a thermostated bath with continuous agitation. The enzymatic hydrolysis is conducted in presence of an antiseptic (NaN_3, total concentration M/50).

After a given time of hydrolysis, the cellulose is collected on a glass filter (No. 4), washed with boiling water, vacuum dried and then nitrated with the 66–34 mixture.

Nitration will be described in the first part of the text.

Molecular weight determinations are made by light scattering using a photogoniodiffusometer SOFICA (France), over angles from 30° to 150° and five concentrations; the solvent used for cellulose nitrate is butylacetate; the dn/dc value employed is 0·104, and the concentration varies from 40 mg in 100 ml solution for cotton nitrate to 100 mg/100 ml for rayon and Whatman powder. Monochromatic light ($\lambda = 5461$ Å) is used; the solutions, after dilution, are centrifuged at 25,000 g for 1·5 hr and measurements are made at room temperature.

GPC. Gel chromatography was made using a Waters Instrument equipped with a set of columns packed with polystyrene. The conditions are as follows:

—five columns: porosity 10^6, 10^5, $3 \cdot 10^4$, 10^4, 250 Å.

—the nitrocelluloses are dissolved in tetrahydrofuran; the concentrations are 1 mg/ml for cotton and rayon ester, and 2·5 mg/ml for hydrolyzed cotton and Whatman powder.

—the flow rate is 1 ml/mn.

Acid hydrolysis. Native cotton is hydrolysed by boiling 2·5 N HCl during 15 min, and then washed to neutrality.

PART I

Different mixtures have been proposed for the nitration of cellulose, including a solution of nitric acid, phosphoric acid and phosphorus pentoxide by Alexander and Mitchell,[14] or nitric acid, acetic acid and acetic anhydride solutions; these methods have been reviewed by Cyrot.[15] We used the latter, with various compositions expressed in weight per cent:

	%HNO_3	%Ac_2O	%AcOH
1	50	25	25
2	43	25	32
3	66	—	34

Nitration method adopted

With these three solutions, we nitrate the cellulose after extraction with ethanol–benzene and vacuum drying; 1 g of cellulose needs 100 g of nitration mixture.

The nitration occurs at temperature below 5° and during 1·5 hr, with some weak agitation. The reaction is stopped by filtering on a glass filter; the cellulose nitrate is washed with cold acetic acid and then with cold water to neutrality.

Stabilization is made by contact with methanol (10 ml/1 g cellulose nitrate) with mechanical agitation for 18 hr; then, the samples are air dried and stored in a refrigerator.

Substitution degree is estimated by a method using sodium perborate, sodium hydroxide and Dewarda reagent.[16]

RESULTS AND DISCUSSION

The results are expressed in Table 1; the GPC diagrams are not very reliable because the *DP* of the initial products was too high for the set of columns proposed; in fact the concentration needs to be very low, the signal is small and in addition an effect of

TABLE 1. COMPARISON OF THE DIFFERENT SOLUTIONS USED FOR NITRATION OF CELLULOSE

Results		Solution 50–25–25	Solution 43–32–23	Solution 66–34
N%		13·6	13·8	13·98
D.S.		2·9	2·93	2·96
$\overline{DP_w}$		4400	4750	4700
$\bar{M}_w$		1,300,000	1,410,000	1,400,000
GPC	M_n	644,000	715,000	678,000
	M_w	1,085,000	1,307,000	1,456,000
	$\frac{M_w}{M_n}$	1·68	1·82	2·14

viscosity is very pronounced; for the initial cellulose in THF, the product $[\eta]$ M about $4 \cdot 10^9$ ($[\eta]$ expressed in ml/g).

In conclusion, we consider that the mixture 66–34 is the best: the solutions 43–32–25 and 66–34 give about the same mol. wt. distribution but the degree of substitution is higher for 66–34.

We think that the low temperature and the short time of reaction proposed here are suitable to prevent degradation of cellulose.

PART II

The procedure of nitration has been then adopted as a routine technique for checking enzymatic action. Our purpose in this work is to propose a mechanism for the

enzymic degradation, to show the effect of cellulose structure and perhaps to define the structure of the cellulose after a systematic choice of representative type of structure for the substrate.

Briefly speaking, the problem is the following: cellulose is a polymer formed of anhydroglucosidic groups joined by β 1—4 glycosidic bonds; cellulases are generally assumed to degrade the macromolecular chain by random cleavage or by splitting cellobiose units from the ends of the chains. We shall compare enzymic hydrolysis with a typically random acid hydrolysis. It is evident that this problem cannot easily be solved, because of the heterogeneity of the substrate. The number of enzymes is also discussed; Selby[17] and Reese[18,19] accept a multienzymatic system with at least a factor (A or C_1) essentially active on native cellulose and able to liberate anhydroglucose chains then hydrolysed by a second one (C_x or B); after that, a cellobiase converts the cellobiose to glucose, final product of the degradation.

RESULTS AND DISCUSSION

(a) *Hydrolysis of different substrates*

In Figs. 1 and 2, GPC diagrams obtained for different substrates are presented; P.W. and rayon are not modified from the macromolecular point of view by enzymatic contact, under the conditions of the experiments.

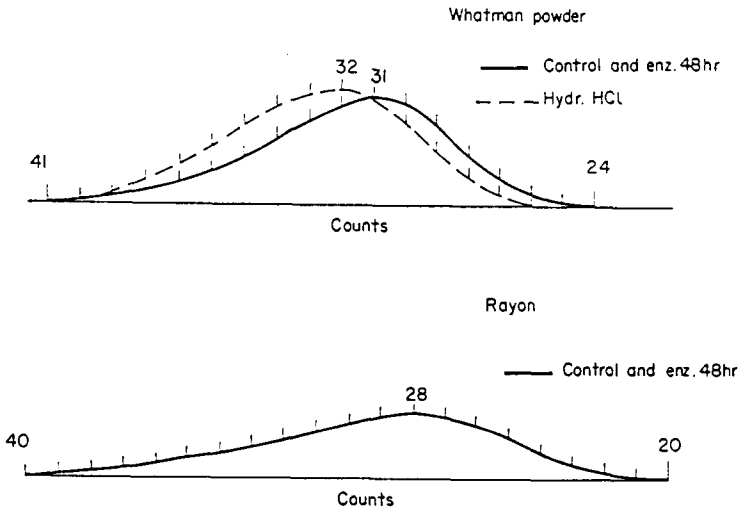

FIG. 1. GPC acid and enzymatic hydrolysis of different types of cellulose.

In contrast, as shown in Table 2, loss of weight is important and respectively 10 and 30 per cent of the initial weight passed in solution ($DP < 8$); ramie and native cotton are high DP substrates with a high degree of order; GPC chromatograms show a little displacement to lower mol. wt.

Swollen cotton, which is a particularly well accessible substrate, presents initially about the same diagram as native cotton, but after 1 hr of hydrolysis is practically completely modified; the distribution of mol. wt. shows two peaks, one corresponding

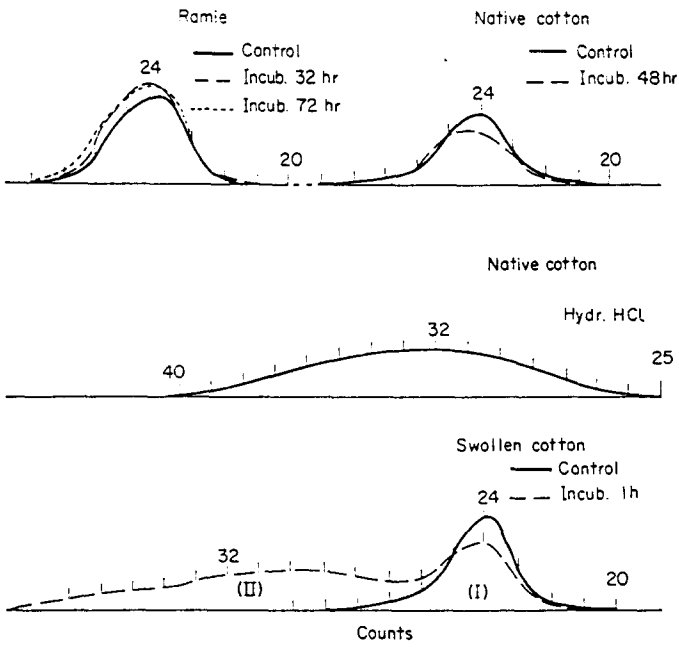

FIG. 2. GPC acid and enzymatic hydrolysis of different types of cellulose.

TABLE 2. LOSS OF WEIGHT AND MOL. WT. MODIFICATIONS OF THE CELLULOSE NITRATE FOR A 48 HR PERIOD OF ENZYMATIC HYDROLYSIS

Substrates	% loss of weight	$\bar{M}_{w\ init.}$	$\bar{M}_{w\ enz.\ inc.}$	HCl hydrolysis
P.W. $E = 27$ mg/ml	10	100,000	100,000	73,000
Rayon $E = 4$ mg/ml	30	285,000	285,000	
Native cotton $E = 4$ mg/ml	2·5	1,456,000	1,090,000	75,000
Swollen cotton $E = 4$ mg/ml	60	1,279,000	134,000	

to the initial distribution (I), the second localized at about the same place as for hydrocellulose. We must note that the conditions of concentration and temperature indicated for cotton swelling preserve the primary wall; the essential difference with native cotton is the degree of swelling and the nature of the crystalline cell; there are both cellulose I and cellulose II units in the swollen cotton.[12,13]

(b) Rate of the degradation

The particularly interesting modification observed for swollen cellulose has been studied as a function of the period of degradation. For every incubation time, residual

cellulose is weighed and nitrated for mol. wt. determinations and GPC chromatography.

The Fig. 3 shows the development of the chromatogram. The areas under the both peaks permit us to evaluate the proportions of both constituents, and their relative changes with time of degradation.

The results obtained for the chromatograms in two parts, corresponding respectively to the high and low mol. wt. distribution, are mentioned in Table 3.

In Tables 4, 5 and 6, the results are expressed in terms of weight and number average mol. wt. and polydispersity; the Figs. 4 and 5 show the changes of $1/\bar{M}_n$ and polydispersity in the previous tables as a function of period of incubation; the letters T,

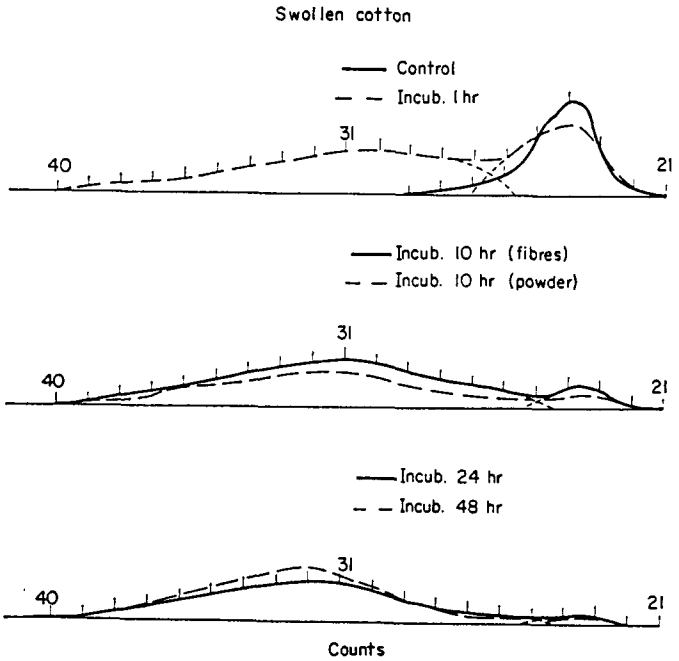

FIG. 3. GPC enzymatic degradation of swollen cotton for different periods of incubation.

TABLE 3. RESULTS OF ENZYMIC INCUBATION: LOSS OF WEIGHT AND CHROMATOGRAM'S DECOMPOSITION. (I AND II RELATIVE, RESPECTIVELY, TO HIGH AND LOW MOL. WT. DISTRIBUTION)

Incubation time (hr)	Cellulose solubilized (% of initial weight)	Area of peak I (% of total diagram area)	Existence of species I (% of weight of cellulose)	Ratio I/II areas
0	0			
1	14·7	38	31	0·615
4	34	14	9·3	0·163
10	41·3	10	6	0·115
24	47	8·5	4·5	0·093
48	60	5	2	0·052

TABLE 4. GPC DIAGRAM TOTAL

Incubation time (hr)	Diagram total			
	$\bar{M}_w$	$\bar{M}_w$	$1/\bar{M}_n$	Polydispersity $= \frac{\bar{M}_w}{\bar{M}_n}$
Control	1,279,000	645,000	$1{\cdot}55.10^{-6}$	1·98
1	536,500	82,900	$1{\cdot}20.10^{-5}$	6·47
4 × T	× 204,700	× 50,700	× $1{\cdot}97.10^{-5}$	× 4·03
4 + P	+ 231,400	+ 47,000	+ $2{\cdot}12.10^{-5}$	+ 4·92
4 − F	− 514,000	− 76,900	− $1{\cdot}30.10^{-5}$	− 6·68
10 × F	× 293,900	× 57,700	× $1{\cdot}73.10^{-5}$	× 5·09
10 + P	+ 271,900	+ 49,300	+ $2{\cdot}02.10^{-5}$	+ 5·51
24	182,000	46,100	$2{\cdot}16.10^{-5}$	3·95
48	134,200	43,900	$2{\cdot}27.10^{-5}$	3·05

TABLE 5. GPC DIAGRAM I

Incubation time (hr)	Diagram I			
	$\bar{M}_w$	$\bar{M}_n$	$1/\bar{M}_n$	Polydispersity $= \frac{\bar{M}_w}{\bar{M}_n}$
Control	1,279,000	645,000	$1{\cdot}55.10^{-6}$	1·98
1	1,185,000	622,300	$1{\cdot}6.10^{-6}$	1·90
4 × T	× 1,020,400	× 767,800	× $1{\cdot}30.10^{-6}$	× 1·32
4 + P	+ 1,789,700	+ 1,209,000	+ $0{\cdot}827.10^{-6}$	+ 1·48
4 − F	− 1,361,300	− 720,900	− $1{\cdot}38.10^{-6}$	− 1·88
10 × F	× 1,707,213	× 1,026,900	× $0{\cdot}97.10^{-6}$	× 1·66
10 + P	+ 1,810,256	+ 1,001,000	+ $0{\cdot}99.10^{-6}$	+ 1·81
24	1,495,500	998,300	$1{\cdot}01.10^{-6}$	1·51
48	1,583,000	974,800	$1{\cdot}02.10^{-6}$	1·62

F and P refer respectively to total residual cellulose, fibres and powder fractions considered separately and caused by the heterogeneity of the degradation. Many conclusions may be drawn from these kinetic results:

(*i*) It seems that 2 per cent of the initial cellulose is unattacked by enzyme in the present conditions; but in fact we must consider an inactivation of the enzyme with time.

(*ii*) After only 1 hr of incubation, 70 per cent of the high mol. wt. cellulose has been degraded by random splitting to an intermediate distribution. That means that the accessibility to enzyme is not restricted to surface region but occurs in most of the

TABLE 6. GPC DIAGRAM II

Incubation time (hr)	Diagram II			
	$\bar{M}_w$	$\bar{M}_n$	$1/\bar{M}_n$	Polydispersity $= \frac{\bar{M}_w}{\bar{M}_n}$
Control				
1	114,400	52,000	$1{\cdot}92.10^{-5}$	2·19
4 × T	92,500	44,900	× $2{\cdot}2.10^{-5}$	× 2·05
4 + P	104,400	43,500	+ $2{\cdot}29.10^{-5}$	+ 2·39
4 − F	109,100	53,900	− $1{\cdot}85.10^{-5}$	− 2·07
10 × F	117,300	51,600	× $1{\cdot}93.10^{-5}$	× 2·27
10 + P	93,900	44,400	+ $2{\cdot}24.10^{-5}$	+ 2·11
24	98,300	43,500	$2{\cdot}29.10^{-5}$	2·26
48	79,800	42,400	$2{\cdot}35.10^{-5}$	1·88

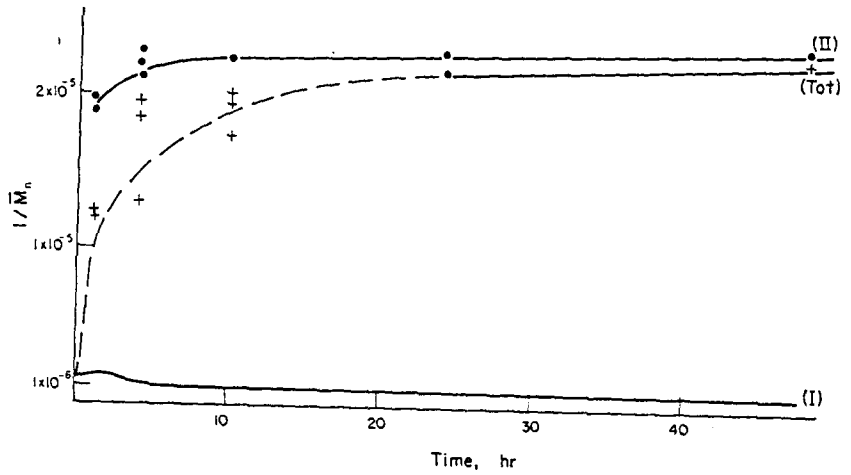

FIG. 4. $1/\bar{M}_n$ obtained by GPC vs. incubation time.

fibre. Levi and Sellen report that, in the particular case of *Chaetomium globosum*, the fungus removes preferentially cellulose from the S2 layer of beechwood-fibre walls,[7,8] the essential part of the cellulose. After 4 hr, more than 90 per cent of cellulose had been degraded, and at the same time important morphological modifications appeared.[20]

(*iii*) The polydispersity value of the both peaks separately is about 2, as in the initial cellulose.

(*iv*) $1/\bar{M}_n$ is practically constant for peak (I) corresponding to high mol. wt. and seems to represent the strictly unattacked cellulose. For the second distribution,[11] $1/\bar{M}_n$ varies slowly and increases to a constant value; that means that there is not a real random splitting; the peak (II) corresponds to a species having $\bar{M}_w \sim 100{,}000$,

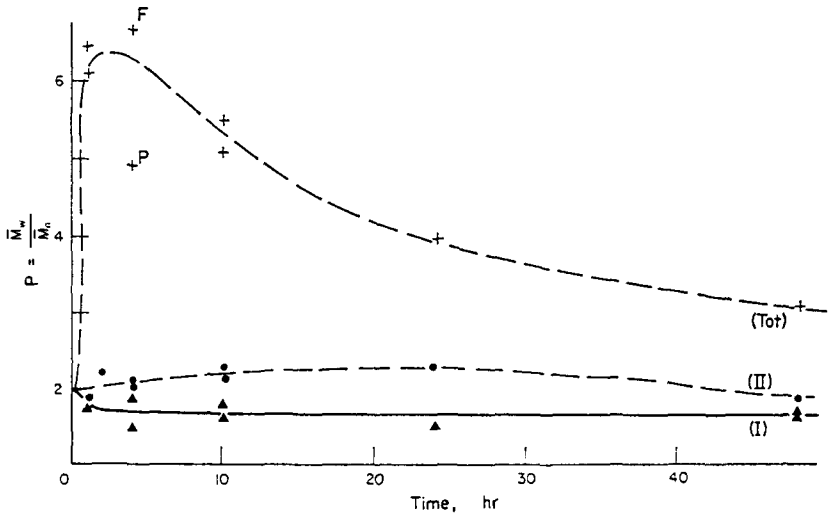

Fig. 5. Polydispersity obtained by GPC vs. incubation time.

or $DP \sim 330$. By comparison of the different substrates, we can see that the diminution of number average mol. wt. is essentially present for substrate of high *DP* of the native cellulose type; for example, Table 2 shows that 30 per cent of the initial weight of regenerated cellulose (rayon) is solubilized but the chromatogram and morphological external aspect are identical before and after incubation; on the other hand, only 2·5 per cent of native cotton is made soluble but the average mol. wt. decreases after enzymatic attack. Swollen cotton is solubilized to 60 per cent of the initial weight, the rest of the residual cellulose having a mol. wt. analogous to the residual product of a random acid hydrolysis; besides, the morphology of the fibre is greatly modified.

CONCLUSION

This paper gives the preliminary results obtained by gel permeation chromatography to investigate the macromolecular modifications during enzymatic incubation and shows the real interest of this technique to test the hydrolysis effects of cellulase.

This technique was also applied to examine the influence of nitration on cellulose to prepare cellulose nitrate under the best conditions; in fact, in the experimental conditions used, the results are not very interesting but confirm the chemical and other physico–chemical conclusions.

By comparison of the reactivity under the same conditions of substrates of different structural organization, and further, the modifications of this reactivity with modification of incubation conditions, we expect to explain some structural detail of the organization of cellulose and to propose a mechanism for the enzymatic degradation; for example, the relative areas of peaks I and II may be related to the structural heterogeneity of the fibre, i.e. to the accessibility of the substrates to the large enzyme molecule.

With the present results, we can propose the existence of a first enzymatic factor which split the high mol. wt. cellulose to an intermediate distribution (called E_1[11]

and tested by GPC). This factor is essentially active on cellulose of high degree of polymerization (type native cellulose); it would be interesting to compare the degrees of crystallinity with these results; this action is greatly modified by the degree of swelling of the substrate. Then a second enzymatic factor, called E_2, solubilizes rapidly the intermediate cellulose; it is tested by loss of weight of the sample and is active on every sort of cellulose. These results are compatible with the previous data[17,18,21] of enzymologists reported in the literature. It is evident that one cannot assume that the mechanism of degradation of a chain is independant of the morphology of such an heterogeneous substrate; our further research in this field will attempt to extend the present conclusions.

Acknowledgements—We sincerely thank Mr. Laboureur from the Société d'Etudes et d'Applications Biochimiques, of Jouy en Josas, France, who kindly provided the enzyme, and Professor Benoit from the Centre des Macromolécules of Strasbourg, France, who has kindly allowed us to use the GPC Waters Instrument.

REFERENCES

(1) L. Segal, *Polym. Lett.* **4**, 1011 (1966).
(2) L. Segal, *J. Polym. Sci.* **C21**, 267 (1968).
(3) T. E. Muller and W. J. Alexander, *J. Polym. Sci.* **C21**, 283 (1968).
(4) G. Meyerhoff, *Polym. Lett.* **5**, 455 (1967).
(5) R. J. Brewer *et al. J. Polym. Sci.* **A16**, 1697 (1968).
(6) D. R. Whitaker, *Can. J. Biochem. Physiol.* **35**, 733 (1957).
(7) M. P. Levi and D. B. Sellen, *Carbohydrate Res.* **5**, 351 (1967).
(8) M. P. Levi and D. B. Sellen, *Polymer* **8**, 633–642 (1967).
(9) M. Krizanova, E. Oltus and D. Eliasova, *Sb. Vysk. Praci Odboru Celulozy Papiera* **10**, 65 (1965).
(10) H. Benoit, Z. Grubisic and P. Rempp, *Polym. Lett.* **5**, 753 (1967).
(11) J. P. Merle, Thèse Grenoble 1968.
(12) J. O. Warwicker, *J. Polym. Sci.* **A2**, **4**, 571 (1966).
(13) J. O. Warwicker, *J. Polym. Sci.* **5**, 2579 (1967).
(14) W. Alexander and R. Mitchell, *Analyt. Chem.* **21**, 1497 (1949).
(15) J. Cyrot, *Bull. Inst. Text., France* **77**, 27 (1958).
(16) Norme NFT 20 306 pour le dosage de l'azote.
(17) K. Selby, *Biochem. J.* **79**, 562 (1961); *Biochem. J.* **88**, 288 (1963).
(18) E. T. Reese, *Advances in Enzymic Hydrolysis of Cellulose and Related Materials.* Pergamon Press, London (1963).
(19) E. T. Reese and W. Gilligan, *Can. J. Microbiol.* **1**, 90 (1954).
(20) M. Rinaudo, J. P. Merle and F. Barnoud, Sixth cellulose conference, Syracuse (1968) (à paraître).
(21) K. W. King, *J. Ferment. Technol., Osaka* **43**, 79 (1965).