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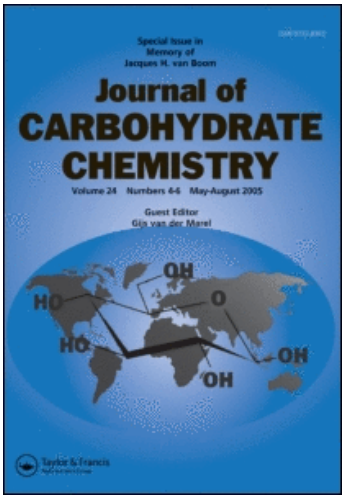

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POLYSACCHARIDES DEPOLYMERIZATION *via* HYDROXYL RADICALS ATTACK IN DILUTE AQUEOUS SOLUTION[1]

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ABSTRACT

The time course of the depolymerization of pullulan, carboxymethylcellulose, welan, scleroglucan and scleroglucan ionic derivatives ("sclerox") in dilute aqueous solution of hydroxyl free radicals (*OH) has been monitored by means of viscosity measurements at 20 °C. Two *OH sources have been employed, namely: Cu(II)/ascorbate and *N*-hydroxy-2-thiopyridone. Singly dispersed polysaccharidic chains undergo depolymerization at a relatively high rate which appears to depend only scarcely on chain structure and charge density, for the experimental conditions employed. Conversely, chains in double-stranded form (welan) or in triple-stranded form (scleroglucan) exibit viscosity decreases which are very small (welan) or undetectable (scleroglucan) whithin the first 1-2 hours of measurements. However, single strands scissions due to *OH attack become manifest when welan and scleroglucan solutions undergo thermal cycles following which their viscosity irreversibly decreases by as much as 70% with respect to that of the initial states.

INTRODUCTION

Exposure to oxygen derived reactive species, especially hydroxyl free radicals (*OH), may bring about severe structural damages in biologically important compounds, e.g., nucleic acids, enzymes, polyunsaturates, etc.[2] The dramatic consequences on the *in vivo* performances of such biomolecules have caused concern and stimulated active research, in particular *in vitro* studies on model systems. The latter quite naturally include aqueous solutions of polysaccharides. In fact, a number of studies on chains degradation of natural carbohydrate polymers brought about by hydroxyl free radicals attack in aqueous media have been carried out using mainly "Fenton" type reagents as *OH sources.[3]

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We have recently undertaken a comparative analysis of the kinetics of depolymerization of a variety of polysaccharides in dilute aqueous solution (33 mM phosphate buffer, pH 6-7) using two different sources of hydroxyl radicals, namely: the Cu(II)-ascorbate couple[4] and *N*-hydroxy-2-thiopyridone.[5]

The time course of chains depolymerization has been monitored by capillary viscometry in a continuous fashion (Cu(II)/ascorbate) and/or in batch experiments (Cu(II)/ascorbate and *N*-hydroxy-2-thiopyridone) at 20 °C. Species considered include non-ionic and carboxylated polysaccharides, namely: pullulan, scleroglucan, "sclerox" (polycarboxylated derivatives of scleroglucan[6]), carboxymethylcellulose (CMC, ds 0.55 and 1.0), and welan.

The main purpose of our work, the results of which are herein described and discussed, is to contribute additional evidence on the correlation between structural/conformational features of polysaccharidic chains and their degradation rates in dilute aqueous solutions of typical hydroxyl free radicals sources.

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RESULTS AND DISCUSSION

The results will be presented in what follows under two main sections: 1) singly dispersed polysaccharide chains (i.e. pullulan and CMC) ; 2) polysaccharides exhibiting double (welan) or triple stranded structures (scleroglucan) in dilute aqueous solution.

1) **Singly Dispersed Polysaccharide Chains.** Representative experimental data on the time dependence of the reduced specific viscosity (20 °C) of pullulan and of CMC (ds 0.55 and 1.0) in dilute aqueous solutions of the two *OH sources considered, respectively, are reported in Figs. 1-3.

A few interesting features stem from our data: these may be briefly summarized as follows.

- For each given polysaccharide, the reduced viscosity decreases faster when the Cu(II)/ascorbate couple is used. This can be simply traced to a higher *OH flux for such couple than in the case of *N*-hydroxy-2-thiopyridone (*OH dosimetry based on the deoxyribose test[7]).

- The rate of reduced viscosity decrease is not influenced by either polymer concentration (in the limited range considered) or added $NaClO_4$ concentration (up to 0.5 M).

- Only in the case of the Cu(II)/ascorbate couple, addition of NaCl markedly reduces the rate of viscosity decrease for all polysaccharides studied. This can be

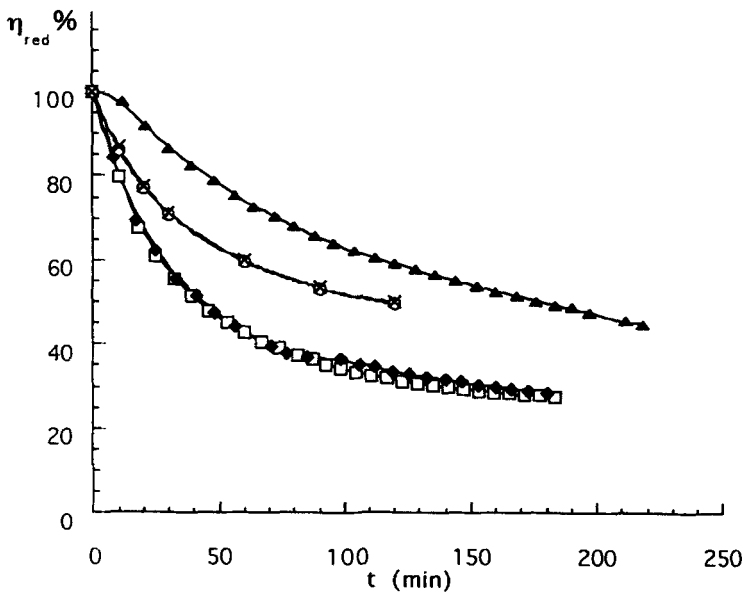

Figure 1. Time dependence of the reduced viscosity (20 °C) of pullulan (1.0 % w/v). Hydroxyl radicals sources (see text) and solvent: Cu(II)/ascorbate in water (❑), in 0.1 M NaCl (▲), and in 0.1 M $NaClO_4$ (◆). *N*-hydroxy-2-thiopyridone in water (○), and in 0.1 M NaCl (×).

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explained on the basis on EPR data (not shown) demonstrating that, for the experimental conditions employed, Cu(II) ions become "silent" upon addition of NaCl by ion-pair formation.

- Comparative inspection of the plots of Figs. 1-3 suggests that the rate of depolymerization should not change much going from pullulan to CMC.

This is better appreciated, always qualitatively though, by elaborating the viscosity data according to the simple statistics of random, single chains scission processes, assuming that the polymer sample, of high molecular weight, exibits a "most probable" chain lengths distribution.[8] If the solutions are so dilute as to allow substitution of intrinsic viscosity with reduced viscosity data, then the following approximate relationship would apply:[3]

$$(1/\eta_{red})^{1/a} = (1/(\eta_{red})_0)^{1/a} + (X_{w0} / ((\eta_{red})_0^{1/a}) \cdot k \cdot t / 2 \qquad (1)$$

where **a** is the Mark-Houwink exponent, X_{w0} is the initial degree of polymerization, $(\eta_{red})_0$ is the reduced viscosity measured at t = 0, and k is the pseudo first order rate constant of depolymerization (t^{-1}).

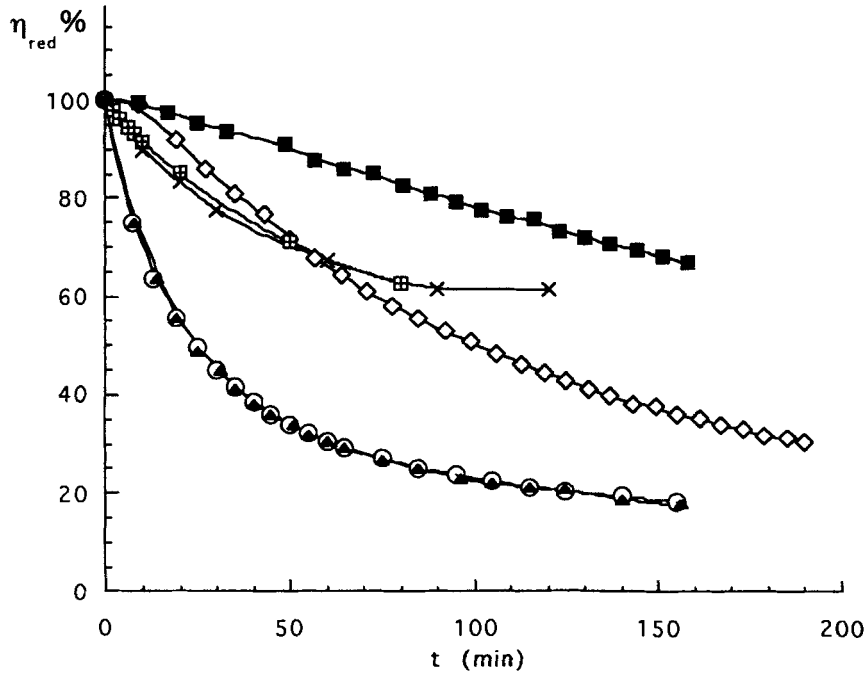

Figure 2. Time dependence of the reduced viscosity (20 °C) of CMC, ds = 0.55, (0.1% w/v). Hydroxyl radicals sources (see text) and solvent: Cu(II)/ascorbate in water (○), in 0.1 M NaCl (◇), in 0.2 M NaCl (■), and in 0.1 M $NaClO_4$ (▲). *N*-hydroxy-2-thiopyridone in water (⊞) and in 0.5 M NaCl (×).

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Using equation (1) and introducing the relevant, approximate **a** values, initial depolymerization data (η_{red} for $0 < t < 40'$) shown in the Figs. 1-3 are linearized in a very satisfactory way, as demonstrated in Fig. 4, allowing evaluation of the associated k values (but see the Experimental part for corrections to the "time axis" in the case of continuous viscosity runs performed using the Cu(II)/ascorbate couple).

The results for pullulan, CMC (ds = 0.55), CMC (ds = 1.0), and hyaluronan (viscosity data not shown) in the order, on the basis of data collected using the Cu(II)/ascorbate source, are:

$$k\,(10^4\,min^{-1}) = 1.1\,;\ 2.7\,;\ 8.0\,;\ 1.6.$$

These figures, approximately an order of magnitude greater than those estimated for the acid hydrolysis of other polysaccharides,[9] do not appear to reflect an important

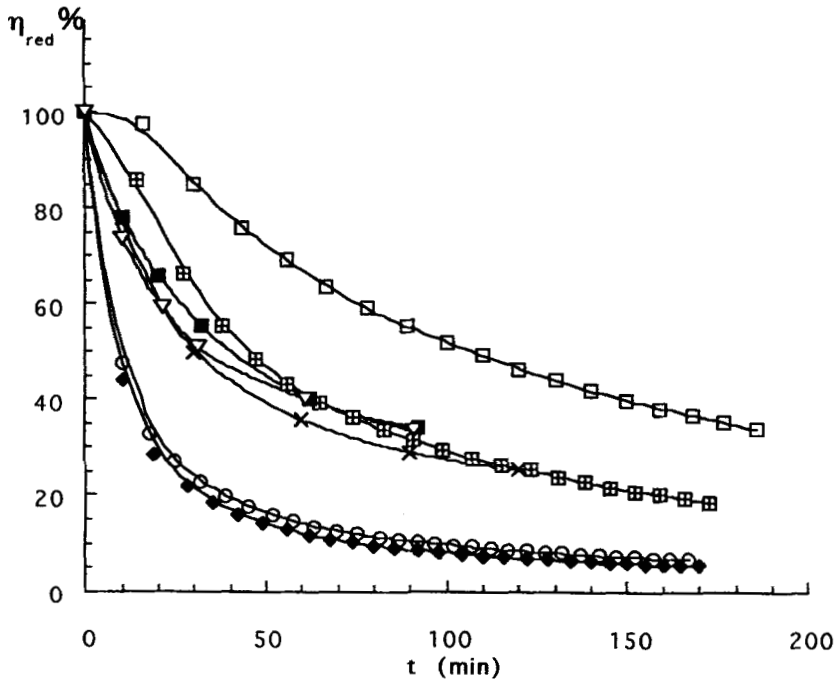

Figure 3. Time dependence of the reduced viscosity (20 °C) of CMC, ds = 1.0, (0.25% w/v). Hydroxyl radicals sources (see text) and solvent: Cu(II)/ascorbate in water (◆), in 0.1 M NaCl (⊞), in 0.2 M NaCl (❑), and in 0.1 M $NaClO_4$ (○) *N*-hydroxy-2-thiopyridone in water (×) in 0.1 M NaCl (■) and in 0.2 M NaCl (∇).

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influence on depolymerization rates of primary structure/charge density of species considered. However, besides experimental errors, the somewhat arbitrary choice of the **a** values (taken from literature data pertaining to different ionic strengths or temperature conditions) adds uncertainty to our k figures.

Similar calculations made using the depolymerization data collected employing *N*-hydroxy-2-thiopyridone lead to k values not much different, if the lower *OH flux of this source with respect to the Cu(II)/ascorbate one is taken into account (see Experimental Part).

A brief comment on the two *OH sources employed appears worthwhile at this stage. *N*-hydroxy-2-thiopyridone has the advantage of being a source of *OH radicals devoid of heavy metal ions and which is switched off simply by stopping the irradiation

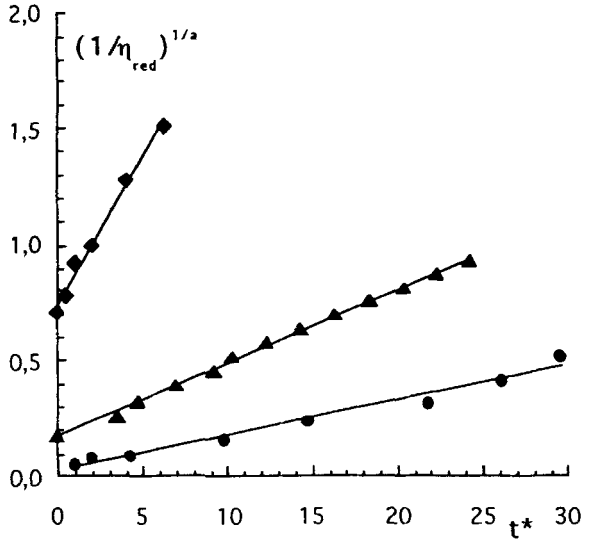

Figure 4. Data for pullulan (◆, a = 0.66) (Fig. 1) and for CMC (a = 0.84, ▲ ds = 0.5; ● ds = 1.0). (Figs. 2, 3) plotted according to equation (1). Actual reaction time, t*, values calculated as explained in Materials and Methods.

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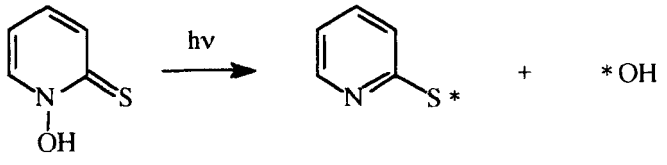
of the solutions. One problem, however, might consist in the simultaneous production of sulfur centered radicals, according to the radiation induced decomposition mechanism:[5]



Thyil radicals, though much less reactive than hydroxyl ones, could interfere with the depolymerization course by, for instance, coupling with carbon radicals created on the polymeric chains by hydrogen abstraction due to *OH radicals. For the polysaccharides indicated above, this seems not to be the case. To the contrary, data obtained in our laboratory suggest that with synthetic polycarboxylates, like sodium polyacrylate and polymethacrylate, such coupling process might take place leading to solution viscosities almost invariant with time under the same experimental conditions as with the polysaccharides. Interestingly, both vinyl polyelectrolytes are rapidly degraded using the Cu(II)/ascorbate source.[10]

With the Cu(II)/ascorbate source, the complex mechanism of *OH production may be schematically approximated as follows.

$$AsA^- + Cu^{2+} \rightarrow AsA^* + Cu^+ \quad ; \quad AsA^- + O_2 \rightarrow AsA^* + O_2^{-*}$$

$$Cu^{2+} + O_2^{-*} \rightarrow Cu^+ + O_2 \quad ; \quad 2O_2^{-*} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^*OH + OH^-$$

where AsA^- is the ascorbate anion and AsA^* is the ascorbyl radical.

It should be noticed that oxygen is essential in such a mechanism. We have assumed the O_2 concentration of the polysaccharide solutions to be identical and constant in all viscosity runs. With said source, in our experience, the *OH flux can be almost completely suppressed only by adding appropriate amounts of catalase which eliminates H_2O_2, while addition of strong Cu(II) chelating agents just slows down *OH radicals production.

More important, the presence of Cu(II) ions (and Cu(I) ions) could influence the rate of chain degradation at least in the case of polysaccharide polyelectrolytes.

One might in fact assume that Cu(II) binding by polyanions could promote *in situ* *OH radicals production and their immediate attack onto neighboring glycosidic bonds. However, the evidence that addition of $NaClO_4$ (up to 0.5 M) does not influence the observed degradation rates also in the case of CMC with ds = 1.0 indicates that such alleged *in situ* *OH production should not be of primary importance. This is probably due to the very high diffusion coefficient of hydroxyl radicals which would overwhelm any concomitant, local process.

The relatively high k value found for CMC ds =1.0, having the highest "linear charge density" among polysaccharide polyelectrolytes considered, may be at least in part traced to a possible direct reaction of *OH with carboxylate groups[11] contributing to the overall chains degradation process. Very likely, however, the latter is primarily, if not exclusively, promoted by hydrogen abstraction events at the expense of tertiary carbon atoms of the pyranose sugar rings, followed by peroxidation and skeleton bonds breakage.

2) **Polysaccharides in Double or Triple Stranded Conformations.** Results obtained working with welan and with scleroglucan, for identical experimental conditions, are given in Figs. 5 and 6. It is evident by inspection that for these two polysaccharides the change of reduced viscosity with time is much less (welan) than for the polysaccharides considered in the previous Section or almost undetectable (native scleroglucan).

This demonstrates that, despite the unavoidable damage - at least at the single strands level - due to *OH radicals attack, the hydrodynamic volumes of welan and, in particular, of scleroglucan are almost unaffected.

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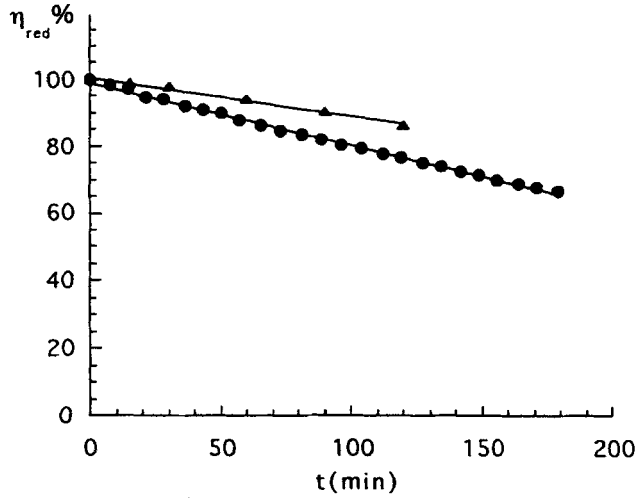

Figure 5. Time dependence of the reduced viscosity (20° C) of welan (C_p = 0.02% w/v) in water. Hydroxyl radicals source: Cu(II)/ascorbate (●); *N*-hydroxy-2-thiopyridone (▲).

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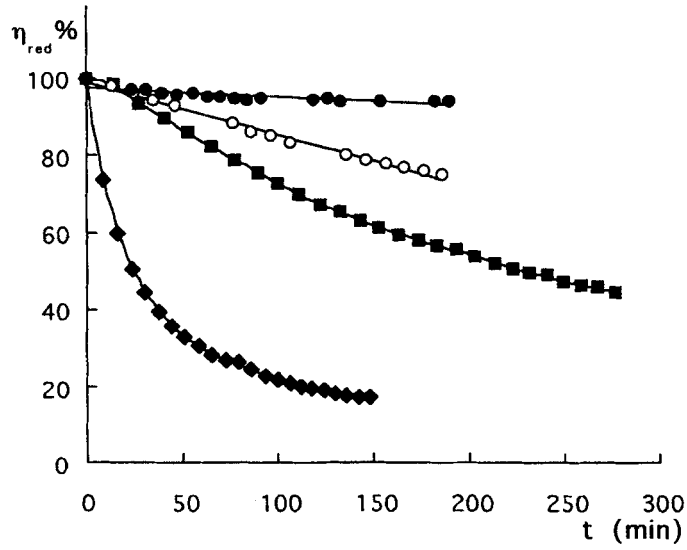

Figure 6. Time dependence of the reduced viscosity (20°C) of scleroglucan and derivatives in water . Hydroxyl radical source: Cu(II)/ascorbate. Native scleroglucan (● C_p = 0.08 % w/v); Sonicated scleroglucan (○ C_p = 0.12% w/v) sclerox" 15% (■ C_p = 0.3% w/v); "sclerox" 100% (◆ C_p = 0.25% w/v).

Evidently, this must be traced to the high stability of the double and triple stranded structures exhibited by, respectively, welan[11] and scleroglucan[12] in dilute aqueous solution. Given this intrinsic stability, the very limited number of double or triple helices breakages (and hence negligible reduction in average molecular weight) can be explained considering that if the probability of a single strand scission is given approximately by $q = k \cdot t$, which leads, after a number of simplifications, to eqn. (1) reported in the previous Section, then we should write for the probability S of multiple stranded structures breakage:[8]

$$S = (\text{const}) \cdot q^2 \quad \text{(double strands)}$$

$$S = (\text{const}') \cdot q^3 \quad \text{(triple strands)}$$

where (const) and (const') are parameters (greater than unity) dependent on the geometric features of the multiple strands.

If the same velocity constants, (k values), estimated for singly dispersed chains, apply, than the initial rates of reduced viscosity decrease - and hence of molecular weight decrease - must be quite small, as was observed.

Moreover, in the case of scleroglucan the ordered structure features D-glucose side-groups regularly clustered around the core triple helix. These side-groups may act as an efficient shield to *OH attack onto the single strands thus adding extra stability towards degradation.

"Buried" single strands scissions are brought to evidence if the polymers solutions are subjected to thermal cycles (after 1 h treatment with *OH radicals at 20 °C and addition of catalase). In fact, welan and scleroglucan solution viscosity irreversibly decreases after two thermal cycles (20 °C - 70 °C) by as much as 70% with respect to that of the initial "unperturbed" state of native biopolymers, never exposed to *OH free radicals. Conversely, the solution viscosity for both native biopolymers fully recovers even after repeated thermal cycles.

In our opinion, the phenomenon can be primarily ascribed to the inability of oligomeric segments released upon heat "denaturation" from the nicked multiple stranded structures to contribute rebuild on cooling the pristine conformational states featuring long range order. A more detailed description of the phenomenon is however made difficult by the fact that, for experimental conditions employed, in the heating runs some *OH residual production is possible thus contributing to the overall irreversible decrease in viscosity.

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In closing, data reported for sonicated scleroglucan and for a "sclerox" sample (Fig. 6) deserve comment.

The *OH promoted depolymerization of sonicated scleroglucan (average molecular weight : 5 x 10^5) occurs at a detectable rate, different from the native sample case. We suspect that this is due to a few "hidden" single strand interruptions introduced by sonication in scleroglucan triple helices. Abrupt bending of the latter by ultrasound waves may also facilitate neighbouring single strands scissions.

With "sclerox", a polycarboxylate bearing two charged groups every three D-glucose residues in the backbone,[6] the rate of depolymerization is comparable to that observed in the case of CMC ds =1.0. For what said in Section 1), this is in favor of a singly dispersed state of "sclerox" chains in dilute aqueous solution, as previously suggested.[6]

In conclusion, although more systematic data are necessary, the diagnostic power of simple experiments of the type described above in ascertaining the strandedness - which, at least in our case, is synonymous with double or triple helical conformations - of polysaccharides in dilute aqueous solution appears of interest.

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EXPERIMENTAL

The samples of scleroglucan (Actigum CS-11), CMC, welan, hyaluronan and pullulan were kind gifts of, respectively, the following companies: Sanofi BioIndustries (France), F.lli Lamberti (Italy), Kelco-Merck (USA), Fidia Advanced Biopolymers (Italy) and Hayashibara (Japan). CMC, hyaluronan and welan were in the sodium salt form. Catalase was a Sigma product (25.000 units/mg). 2-Mercaptopyridine-1-oxide sodium salt (i.e. *N*-hydroxy-2-thiopyridone) was a Fluka product used without further purification. Inorganic salts employed were pure, analytical grade products. All polysaccharides were dialysed extensively against double-distilled water and then freeze-dried (native biopolymers). For the latter, intrinsic viscosiy, [η], and molecular weight, based on relevant Mark-Houwink constants found in the literature, are listed below. Sonicated samples and derivatives were treated similarly. Iron and copper content of all polymer samples resulted below detection limits (Inductively Coupled Plasma Emission Spectrophotometer, Jobin-Yvon 38).

A scleroglucan sample was sonicated (Vibra Cell, Sonics Material 300 W Ultrasonic processor; 60' sonication, power monitor reading 52 W) to reduce its average molecular weight to about 5x10^5 (as deduced from [η] = 5.6 dL/g in water, 25 °C, and a = 1.7 and K = 1.3x10^{-9} dL/g Mark-Houwink constants[13]). Sonicated scleroglucan was

used in the depolymerization experiments (for comparison with the native biopolymer) and to prepare, *via* periodate/chlorite oxidation, two "sclerox" samples of degree of oxidation of 15 and 100 %, respectively, following the procedure already reported in detail elsewhere.[6] The 100% oxidized sample had $[\eta] = 2.15$ dL/g in 0.10 M NaCl and $M_W = 2.6 \times 10^5$ (light scattering).

For the depolymerization experiments using the Cu(II)/ascorbate *OH radicals source, the following experimental conditions have been employed throughout using constantly the same chemicals and double-distilled water, filtered through 0.22 μm sterile Millipore filters).

Each polysaccharide was dissolved (0.1 - 1.0 % w/v) in 33 mM phosphate buffer (pH 7) and aliquots of a standard $CuSO_4$ solution were added (final concentration: 10 μM). These solutions were used to determine the reduced viscosity, η^0_{red}, (20 °C) of the undegraded polymers in the given "solvent". Identical solutions were prepared and sodium ascorbate (final concentration 20 mM) was added: these were immediately introduced in the viscometers and the decrease of η_{red} as a function of time at 20 °C monitored in a continuous fashion.

In order to evaluate from such data the depolymerization rate constants, k (see Results and Discussion), the actual reaction time values, t*, have been calculated as $t^*(i + 1) = t_e(i) + t_e(i + 1)/2 + t_c$, where $t_e(i)$ and $t_e(i + 1)$ are the efflux times of the ith and (i + 1)th viscosity runs, respectively, and where t_c is the time necessary for the apparatus to recharge automatically the solution in the Ubbelohde viscometer after the ith run.

With CMC (ds = 1.0) and pullulan a few batch-type experiments were also performed in order to check the validity of the t* values as mentioned above. In each of these experiments, the polymer solution with added ascorbate was left at 20 °C and aliquots taken at given intervals of time, 1 μM of catalase was added to eliminate H_2O_2 and stop the production of *OH and the viscosity measured with constant efflux times. The η_{red} data obtained by the continuous and batch protocols gave superimposable curves when plotted against t* (or simply t, for the batch experiments) for both CMC and pullulan.

The depolymerization experiments made using *N*-hydroxy-2-thiopyridone as *OH radicals source were carried out as follows to polymer solutions (0.1-0.5 % w/v in glass tubes of 2 cm i.d.) in 33 mM phosphate buffer (pH 7) *N*-hydroxy-2-pyridone (final concentration in all cases: 5 mM) was added and irradiated with a white light source (250 W lamp, at a distance of 10 cm). During irradiation the temperature of the solutions was kept at 37-38 °C by fluxing cold air around the glass tubes.

Irradiation was stopped at regular time intervals (10', 30', 60', etc.) and the solutions viscosity measured at 20 °C (efflux time of phosphate buffer: 200.37 s). A few

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irradiation experiments were also performed in the case of CMC using a UV lamp (1 kW, $\lambda = 300$ nm): the set of viscosity results for CMC are independent of the radiation source.

Using both *OH sources, the experimental results were highly reproducible in all cases examined.

Analysis of the *OH flux in the solutions of the two sources employed by means of the deoxyribose test[7] indicate that the Cu(II)/ascorbate couple produces in 20' an amount of *OH roughly twice (per unit volume) of that produced by *N*-hydroxy-2-thiopyridone after 20' irradiation.

Polymer	SCL nat.	SCL sonic.	Pull.	HA	CMC (ds=0.5)	CMC (ds=1.0)	Welan
M_V x10^5	11	5	2.5	2.1	0.74	1.5	4.4

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