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The kinetics of periodate oxidation of carbohydrates 2. Polymeric substrates[☆]

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday

Abstract

A study of the kinetics of periodate oxidation on a series of dextran oligomers and polymers is carried out by isothermal microcalorimetry. In addition to these substrates, some dimeric carbohydrates and hyaluronan were studied. Rate constants were calculated from the calorimetric decay curves, which, properly corrected for calorimetric response, are proportional to the rate of periodate conversion. The dependence of the kinetic rates on the molecular weight of dextran samples and on the substrate concentration, is described in terms of the much higher rates of terminal reducing units. The presence of two sites with comparable reaction rates makes the analysis of the calorimetric curves difficult, even in the simple overall pseudo-first-order condition. The suitability of a phenomenological treatment of kinetic data is explored. © 2003 Elsevier Science B.V. All rights reserved.

Keywords: Periodate oxidation; Kinetics; Isothermal calorimetry; Dextran; Molecular weight; Hyaluronan

1. Introduction

The kinetics of periodate oxidation reaction on carbohydrates has recently been studied by using a calorimetric approach.[1] The calorimetric method was chosen only for its suitability and sensitivity in comparison with other instrumental methods for concentration determination. In particular, the possibility of following the reaction stages ‘on-line’ through the detection of the ‘thermal power’ at any instant during the reaction, was considered very useful for detailed analysis and statistical data treatment. The rate constants were comparable with other literature findings, and the activation energies were calculated from their variations with temperature. The dependence of the rate constants on stereochemistry was interpreted in terms of the conformational probability of the reactive state. It is also proposed that the accuracy of calorimetric data makes possible the detection of deviations in the apparent rate constant during the course of the reaction.

A short summary on the periodate reaction is given here, referring to the previous paper[1] for a review of other related literature results. The reaction involves a periodate ion and a molecule with vicinal diols, and implies the oxidation of the reaction site with the breakage of the C–C bond and the subsequent formation of two aldehydic groups. The mechanism of the reaction[2,3] can be depicted in the following way: in the first step, one of the I–O bonds of the periodate attacks one of the two hydroxyl groups of the vicinal diol; the second step is the formation of the planar cyclic ester as part of an octahedral intermediate, the rate of which must depend on the acidity of the oxygen of the OH groups and their relative positions.

The oxidation process can be complicated by the presence of two or more sites, the stereochemistry of all substituents having a relevant influence on the kinetics. If sugars differ from each other by characteristics that do not imply the relative position of the –OH groups, only minor differences in the rate constants are de-

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[☆] For Part 1, see Ref. 1.

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tected, possibly related to structural and solvation differences.[4] Depending on the species monitored, the time-evolution of the reaction can be followed in several ways: i.e., by the variation of the substrate concentration, and of the periodate, or of the formic acid that is formed during the reaction. For 2,3,4-triols several rate constants, for the two sites in competition with each other and for the subsequent oxidation, can be defined. When the ratio of reactant concentrations is unity, the amount of singly oxidized substrate first increases, reaches a maximum and then decreases; formic acid is formed as soon as the singly oxidized product is attacked for the second time. Elucidations of all these aspects are derived from a systematic analysis of the change in concentration of the several reactants and reaction products as carried out by Aalmo and Painter.[5,6]

With homopolysaccharidic or heteropolysaccharidic substrates, the reaction leads to an oxidized form that may give hemiacetal forms of two types. Intra-residue hemiacetals are formed by the condensation of an aldehydic group with a hydroxyl group inside the same monomeric unit. Inter-residue hemiacetals are obtained by the condensation between a –CHO group and an –OH group belonging to two different residues. This reaction protects the –OH group[7] belonging to non-oxidized residues adjacent to oxidized residues, thus lowering the oxidation limit compared with theoretical values. The lack of –OH on C-6, as in (1→4)-polyuronates in general or in C-6 derivatives, does not allow the formation of intra-residue hemiacetals, but only inter-residue ones. This situation is described for alginates, polygalacturonates and similar compounds.[8,9]

Some further complications arise for the kinetics involving *vic*-triols, as in the present case.[5] The presence of a second attack (consecutive reaction) may affect the measured rate unless masked by the values of the enthalpies and of the actual rates of the two reactions. Periodate oxidation of dextrans was studied by Ishak and Painter,[10] reporting a value of about 2.5×10^{-2} L mol^{-1} s^{-1} for the initial second-order rate periodate coefficient and the evidence of hemiacetal formation for high-molecular-weight samples (M_w 2×10^6 g mol^{-1}). This work, and the subsequent reports by the same group, did not only clarify some spurious analytical results but also provided the correct understanding of the reaction mechanism.[11,12]

Herein, we present the results of the calorimetric study of the oxidation reaction on several dextran samples, aimed at displaying the differences due to the molecular weight of the substrate and the effects of two concurrent reactions with different kinetic and thermodynamic parameters. Some preliminary data on hyaluronan (HA) are also phenomenologically illustrated to exploit the calorimetric method with this polysaccharide.

2. Experimental

2.1. Samples

All carbohydrate monomers and dimers were purchased from Sigma Chemical Co. (St. Louis, USA), and dextran samples were purchased from Fluka Chemie (Buchs, Switzerland). All were used without further purification. Purity was checked by NMR, GPC and electrospray-ionization mass spectrometry. Molecular weights and molecular weight distributions of the dextran samples were determined by means of GPC–LALLS by using LALLS CMX-100 (TSP-Chromatix) and DRI 410system (Waters) detectors on three TSK columns operating at 40 °C. Only low molecular weights were in fair agreement with the specifications, with large deviations being observed for the high molecular weights (see Table 1). The hyaluronan sam-

Table 1
Characteristics of the carbohydrate monomers and polymers

Compound (abbreviation)	M_r (producer)	$\langle M_w \rangle$	$\langle M_n \rangle$	$\langle DP_n \rangle$	$[\eta]$
Glucose (G)	162	162	162	1	–
Maltose (M)	343	343	343	2	–
Cellobiose (C)	343	343	343	2	–
Trehalose (T)	343	343	343	2	–
Dextran (D1)	1200	1600	1400	8.5	–
Dextran (D2)	6000	–	–	37	–
Dextran (D3)	15,000	21,000	11,000	68	–
Dextran (D4)	70,000	93,000	41,000	253	15.7
Dextran (D5)	110,000	210,000	61,000	376	21.6
Dextran (D6)	200,000	684,000	137,000	844	31.2
Dextran (D7)	500,000	624,000	80,000	493	39.4
HA	160,000	157,000	84,000	210	48

ple (HA Hyalastin) was kindly provided by Fidia Research Laboratories (Padua, Italy). Sodium periodate and sodium perchlorate were pure reagents from Carlo Erba (Milan, Italy). All substrates were used in aqueous solution. Periodate solutions were freshly prepared from stock solutions and always stored in the dark. HA solutions were carefully prepared by stirring the powdered sample overnight in aq $NaClO_4$ salt solutions. Concentrations of polymeric substrates are reported as mole of glucose-ring units (or monomole) per liter of solution.

The polymer samples were also analyzed for their intrinsic viscosity and static laser light scattering. The presence of branching in dextran sample D7 has been confirmed, in agreement with recent findings of literature.[13]

2.2. Calorimeter

A batch-type isothermal microcalorimeter LKB 10700-2, equipped with gold twin cells, was used. Measurements were carried out at 35 °C. One millilitre each of the reactant and the substrate were equilibrated inside the calorimeter for at least 6 h (or overnight). The output of the thermopiles, amplified by a Keithley 150B microammeter, was stored in a PC through a Picolog® A/D data acquisition interface. A frequency of 1 datapoint/s was used for acquisition, averaging 10 raw data collected every 0.1 s. The time scale was set to zero at the mixing of the two solutions. The mathematical analysis of the calorimeter data was made with the ORIGIN™ package. Mixing of viscous solutions containing high-molecular-weight dextrans at high concentration or HA was shown by the irregular raising of the thermal signal, which becomes smooth after 100–150 s. Data points of theses curves were therefore analyzed only for $t > 150$ s.

3. The calorimetric analysis

3.1. Treatment of calorimetric data for simple reactions

The suitability of the calorimetric method to follow the kinetic course of the periodate reaction has been reported previously.[1,14] A brief account of the method is presented.

In a calorimeter (heat conduction type) the signal recorded as a function of time is the ‘thermal power’, dQ/dt (in units of $W = J\ s^{-1}$). Thermal power is related to the instantaneous rate $d\xi/dt$ (ξ is the extent of the reaction), while the partial heat evolved, $Q(t)$, is proportional to the extent of the reaction, $\xi(t)$, as a function of time. In this way, chemical units (mole) and thermal units (heat) are related to each other through the rate constant, the enthalpy of the reaction and the actual amount of reacting material.

If the calorimeter has an extremely fast response, then the extent of the reaction $\xi(t)$ at time t is easily measured by summing up the partial area $Q(t)$ and the rate of reaction is then given by:

$$d\xi(t)/dt = W(t)/VC^{\circ}_{A}\ \Delta H_R \quad (1)$$

where $W(t)$ is the power at time t, ΔH_R the enthalpy change and C°_{A} the initial concentration of reactant A at $t = 0$ in a volume V (for a pseudo-first-order process, the concentration of the reactant is in marked defect).

Integration of the kinetic term in Eq. (1) gives:

$$W(t) = VC^{\circ}_{A}\Delta H_R k'_1 \exp(-k'_1 t) \quad (2)$$

which, in logarithmic form, enables a simple evaluation of the rate constant k'_1 from the slope of the linear plot of $\ln W(t)$ versus time t. The advantage of using the power signal mainly resides in the possibility of evaluating k'_1 independently of the slowness of the reaction, and therefore of reaching the baseline signal in a reasonable time. Alternatively, integration of the calorimetric signal gives equivalent equations, which can be used for reactions being completed in the analysis time. In that case, the integral forms of Eqs. (1) and (2) can also be used, writing:

$$\xi(t) = Q(t)/VC^{\circ}_{A}\Delta H_R \quad \text{and}$$
$$Q(t) = VC^{\circ}_{A}\Delta H_R \exp(-k'_1 t) \quad (3)$$

Other recent kinetic analyses have been limited to reactions occurring over many hours or days,[15,16] while the kinetics in the present case take place in a much shorter time. Therefore, the correction of the raw signal for the calorimetric delay time is mandatory for a significant analysis of the time-course of the reaction. As a general rule, corrections are large for fast reactions and short times, the actual limit remaining that of the response time of the calorimeter.

The equations relating the calorimetric signal to the actual ‘power curve’ $W(t)$ have been presented and discussed previously. For calorimeters with a linear response, the correction is given by the inverse of the Laplace (transfer) function, reported in the analysis of the instrumental distortion of impulses, which, in the simplest and approximated form, gives:

$$W(t) = K_c(s^*(t) + \tau\ ds^*(t)/dt) \quad (4)$$

where $s^*(t)$ is the raw calorimetric signal (in V) and K_c takes the meaning of a calorimetric calibration constant (usually determined through electrical calibration). The value of $\tau = 79.11$ s was determined at 35 °C for the heat conduction isothermal microcalorimetry LKB equipped with gold cells. Once the signal is properly corrected for the instrumental delay time, a pseudo-first-order rate constant is easily calculated by using Eq. (2) or Eq. (3).

3.2. Treatment of calorimetric data for complex reactions

In this work, a single kinetic constant could not well fit 'a priori' all the kinetic data because of the presence of several different sites of attack. In these cases, the overall kinetic course is determined by the contribution of each fraction of site times the respective second-order reaction constant. This fact makes the time dependence of the reaction course more complicated, especially when one of the sites, the slower one, is in large excess, and the other, the faster one, is a small fraction. Under such conditions, the overall reaction course may contain appreciable contributions from both a pseudo-first-order reaction and a second-order reaction, with a heat production that cannot easily be separated into the two contributions during the time evolution. From these considerations it becomes obvious that data analysis needs to take into account too many variables and that a phenomenological investigation can be done only if it is independent of the order of the reaction and the effective substrate concentration.[17]

Since the calorimetric signal has the dimension of a power, the calorimetric data analysis leads to a direct measurement of the reaction velocity $d\xi/dt$ without any assumption on the initial reactant concentration, enthalpy and order of reaction. To get a kinetic parameter it is necessary to normalize the variation of the heat flux; therefore, the following equation has been used to introduce the Γ parameter that contains the kinetic information:

$$\Gamma = d\Phi/dq = (\Phi_2 - \Phi_1)/(q_2 - q_1) \tag{5}$$

where Φ is the heat flux (dq/dt) and q is the heat output measured by the calorimeter. Since the heat flux has the dimensions of cal s^{-1}, whereas the heat has the dimension of calories, then Γ will have the dimensions of s^{-1}. In the case of a reaction occurring with only one kinetics, the Γ parameter as a function of time is linear, while for a combination of several kinetics it is possible to detect the instantaneous change in the reaction rate. Furthermore, it should be pointed out that $\Gamma \equiv k_1$ for a first-order kinetic process, therefore giving a simple and direct way to compare different kinetics even of unknown order.

As a final comment, let us clarify here that this type of analysis does not itself provide the value of the rate constants, but is highly diagnostic for discriminating the monotonic behavior of the kinetics, since data treatment is independent of any *basic* kinetic model. It will be shown that this approach can be useful for the analysis of polymeric substrates, like HA, where nonlinear behavior is observed.

4. Results and discussion

4.1. Monomers and dimers

Following on and complementing the results of the previous study on methyl glycosides,[1] glucose, and the disaccharides, maltose, cellobiose and trehalose, were used and analyzed under conditions of a pseudo-first-order reaction. The structural differences among these compounds can be summarized as follows:

- Glucose and methyl glucoside differ from each other in their anomeric carbon availability to the oxidation.
- Maltose and cellobiose both have a reducing anomeric carbon, and the only structural difference between them resides in the anomeric configuration at the interglycosidic linkage.
- Trehalose is a nonreducing sugar, both anomeric carbons being involved in formation of the glycosidic bond.

From the curves reported in Fig. 1, it can be seen that the three disaccharides have quite different kinetic behaviors: maltose has faster kinetics than cellobiose and trehalose, the latter being the slowest. The monomer substrates also show a different behavior, since the methylation of the anomeric carbon is a significant modification for the conformational changes necessary to bring the substrate into the planar reactive form. The kinetic results, together with other relevant data, are reported in Table 2, where the kinetic responses for both isomers of methyl glucoside are included.[1] The differences among the values for these sugars are, of course, ascribed to the differences in their structural features. In particular, the lower reactivity of trehalose is caused by the absence of the –OH group on the anomeric carbon, while both maltose and cellobiose are significantly more reactive. At first glance, the different reactivity of these two dimers could be ascribed to differences arising from the different types of glycosidic linkage between maltose and cellobiose (α-(1 → 4) and β-(1 → 4), respectively). However, it is immediately clear that, in both cases, the most reactive site is the terminal reducing group, offering a mixture of α and β anomeric carbons, which have a different stereochemistry. The fact that the fraction of α-anomer is 0.6 in maltose and 0.4 in cellobiose[18] provides the most simple comparison between the kinetic results with the stereochemistry of the reactants, confirming the statement previously made for methyl glycosides. In addition, one cannot exclude a priori that the different linkage may introduce other conformational constraints or hydration changes. A detailed analysis of the oxidation behavior of the anomeric composition of dimeric sugars is not the subject of this work, but these results will be resumed in the interpretation of the following kinetic experiments.

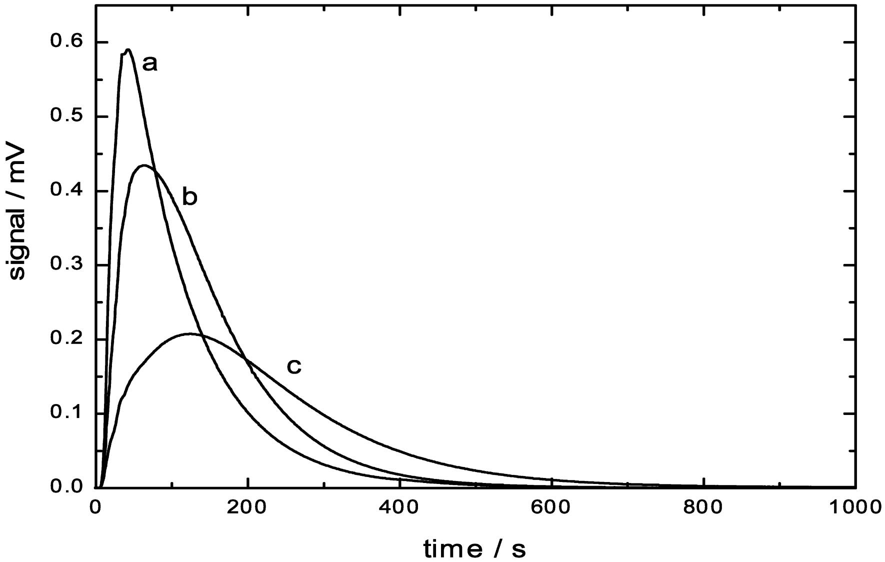

Fig. 1. Calorimetric curves recorded for the reaction of periodate (6.25×10^{-4} M) with disaccharides ($C = 6.25 \times 10^{-2}$ M): (a) maltose; (b) cellobiose; (c) trehalose. Measurements were carried out at 35 °C in 0.1 M $NaClO_4$.

4.2. Oxidation reactions of dextrans

The presentation of the experimental studies on dextrans have been divided into two parts; firstly the ratio (R) between the concentration of the substrate and of the periodate was varied for two samples with different molecular weights; secondly R was kept constant (in the pseudo-first-order conditions), and a wide range of molecular weights was explored.

4.2.1. Kinetics as a function of dextran concentration. To explore the effect of concentration, attention was focussed on two samples of dextrans with very different molecular weights (nominal $M = 1.2 \times 10^3$ and 500×10^3 Da, respectively, see Table 1). Given the large difference in the chain length, the two polymers had to be treated as different mixtures of (1 → 6)-linked glucose moieties and terminal end glucose units. For this reason, the rate constants must be considered as apparent, since they reflect contributions of both types of sites,[19] although to a different extent, as will be discussed below. Fig. 2a and b report some raw calorimetric data and corresponding kinetic curves (a logarithm of the corrected power as a function of time) for the D7 sample at several substrate/periodate concentration ratios R ($R = 25$–160), showing the increasing pseudo-first-order reaction rate upon increasing dextran concentration. Similar trend is observed for sample D1 (data not shown).

For the kinetic analysis, the calorimetric curves were processed according to the method outlined in Part 3, by assuming as still valid the pseudo-first-order reaction for the initial part of the experiment. This approximation, which seems justified from the observed linearity of the plots, was made only at this stage of analysis. The resulting values of the pseudo-first-order rate constants give $k_2 = 0.06$ and 0.20–0.30 L mol^{-1} s^{-1} for high and low molecular weight, respectively (Fig. 3).

An analysis of the calorimetric data has been made to obtain the enthalpy of the reaction (in all cases periodate was markedly in defect). The enthalpic values ΔH_{cal}, calculated by integrating the calorimetric curve and normalizing it for the periodate reactant, show a substantial difference between the two molecular weight samples (Fig. 4). The average value for the enthalpy of reaction results in 66.5 ± 0.5 and 57.3 ± 0.5 kcal/mol for D1 and D7, respectively.

To check the range of validity of the pseudo-first-order rate and to monitor the resulting variations, calorimetric data have been analyzed by using the method of Γ parameter. The results of this type of analysis (some representative curves for very different R-values are

Table 2
Kinetic and thermodynamic data of periodate oxidation on monomers and dimers

Substrates	k'_1 (s^{-1})	k_2^{35} (L mol^{-1} s^{-1})	ΔH (kcal mol^{-1})
Maltose	3.31×10^{-2}	5.35×10^{-1}	70
Cellobiose	2.07×10^{-2}	3.35×10^{-1}	76
Trehalose	8.53×10^{-3}	1.38×10^{-1}	61
Methyl α-D-glucoside	–	7.28×10^{-2}	60.2
Methyl β-D-glucoside	–	5.73×10^{-2}	60.2

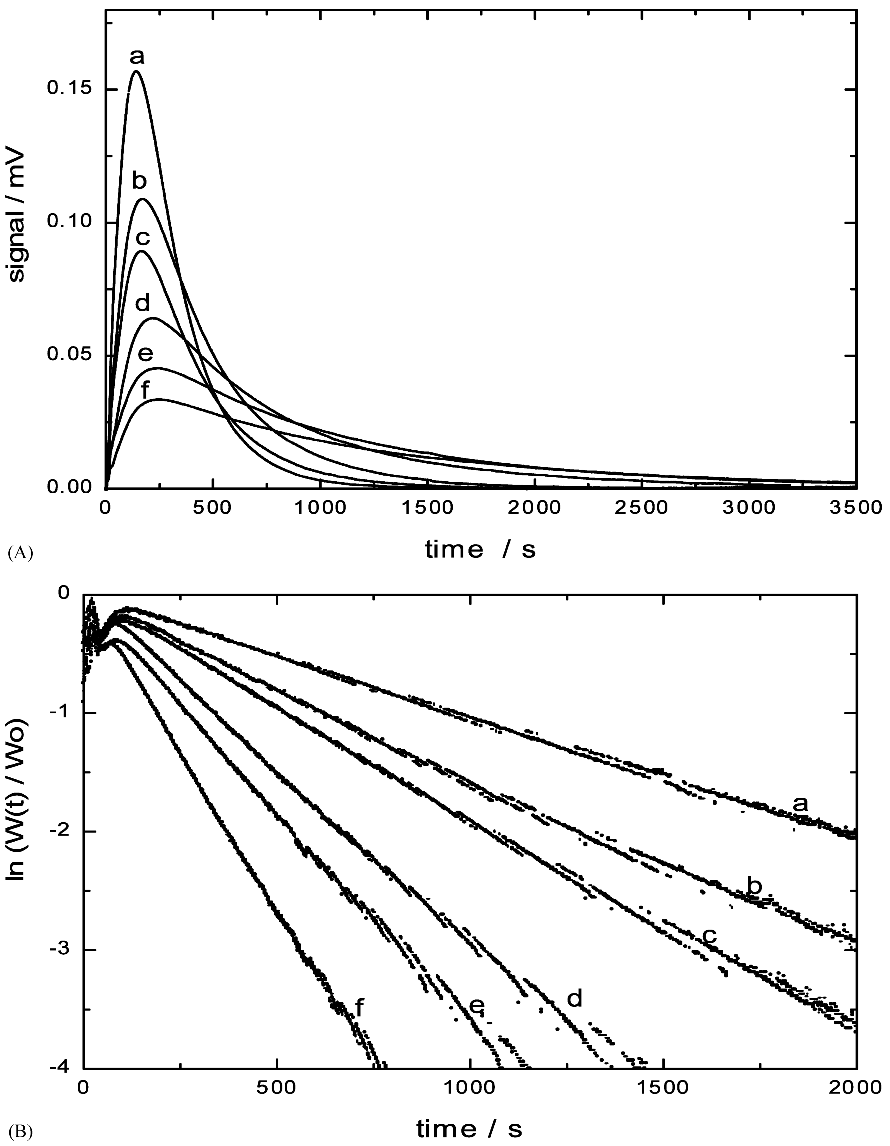

Fig. 2. (A) Calorimetric curves recorded for the reaction of periodate (6.25×10^{-4} M) with dextran D7 at dextran/periodate concentration ratios, $R = 160$ (a), 100 (b), 80 (c), 50 (d), 40 (e), 25 (f). (B) Kinetic curves (logarithm of the normalized power) vs. time of the curves of (A).

reported in Fig. 5) represent the instantaneous reaction rate of the kinetic process and clearly show the quantitative changes of reaction rates with time. Table 3 reports the data for dextran D7, showing that in the range of $R = 25$–160 the two methods provide reasonably comparable results. The analysis of calorimetric curves is mainly carried out in the time range of the reaction process, where the signal recorded (power) is high. Data at longer times are neglected since they are affected by errors resulting from both sets of approximations, and especially the low calorimetric signal. From the operative point of view, values less than 0.01 times the maximum of the calorimetric signal always show a splattering and curvature of the data points in the logarithmic plot as a function of time, irrespective of the time value.

4.2.2. Dextrans as a function of molecular weight. By keeping the value of $R = 100$, the effect of the dextran molecular weight on the periodate oxidation was investigated. In all cases, the polymer concentration was 6.25×10^{-2} monomol L^{-1} and the periodate concentration 6.25×10^{-4} mol L^{-1}. Fig. 6 reports the kinetic curves (plotted as the natural logarithm of the power W normalized for the intercept value W_o) for the dextran samples with molecular weight from 1.2×10^3 to 500×10^3 Da. The increase in the slope of the curves, as the molecular weight decreases, indicates that, with an excess of oxidizable sites, the kinetics is faster for low-molecular-weight dextrans, and this behavior leads to the conclusion that the reaction is chain dimension-dependent. Therefore, the variation of the overall kinetics of the reaction is simply due to the combination of two different rates, since the higher reactivity of the diols belonging to the reducing terminal –OH groups determines the higher reaction rate for low molecular weight dextrans.

In order to analyze quantitatively the kinetics of the dextran substrates (reported in Table 4), the pseudo-first-order rate has been written as a linear combination of two different second order kinetics:

$$k'_1 = k_2 C_D = C_D(k_{2t}x_t + k_{2i}x_i)$$
$$= C_D\{k_{2i} + (k_{2t} - k_{2i})\mathrm{DP}_n\} \quad (6)$$

where the suffixes i and t denote the internal and the terminal glucose residues respectively, and the fraction of terminal group, x_t, is $1/\mathrm{DP}_n$. The result of this analysis is reported in Fig. 7, where the experimental overall values k_2 are plotted as a function of the logarithm of the degree of polymerization, together with the curve drawn by using $k_{2t} = 1.4$ L mol^{-1} s^{-1} and $k_{2i} = 0.054$ L mol^{-1} s^{-1} (obtained by linear regression). At any defined molecular weight, both reactions occur, although the contribution of the terminal groups become scarcely relevant when the chain length (i.e., DP_n) increases above 100. The straightforward consequence is the possibility of characterizing a dextran sample on the basis of the rate constant, and, therefore, by means of its number of oxidizable terminal sites.

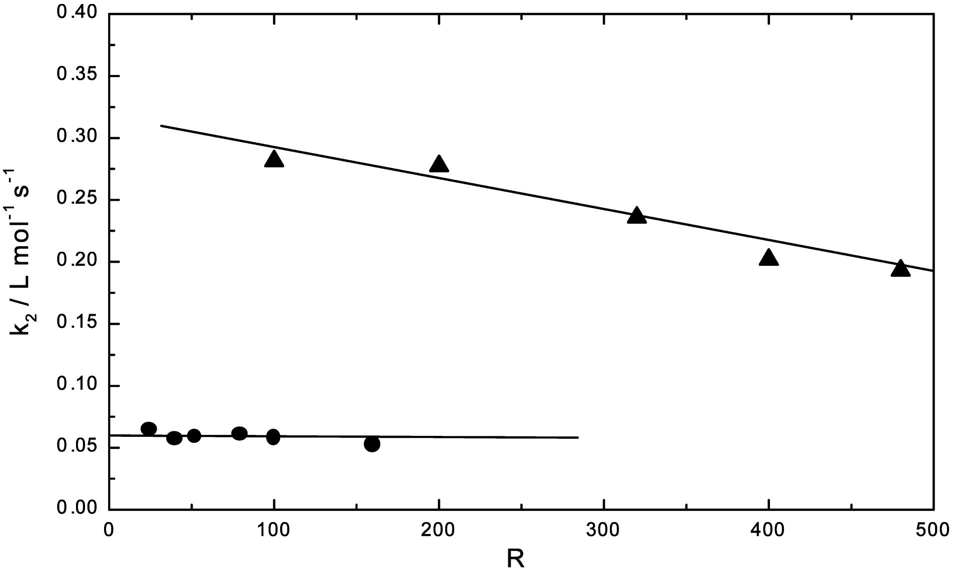

Fig. 3. Variations of the second-order reaction rate as a function of dextran/periodate concentration ratios (R) for samples D1 (▲) and D7 (●).

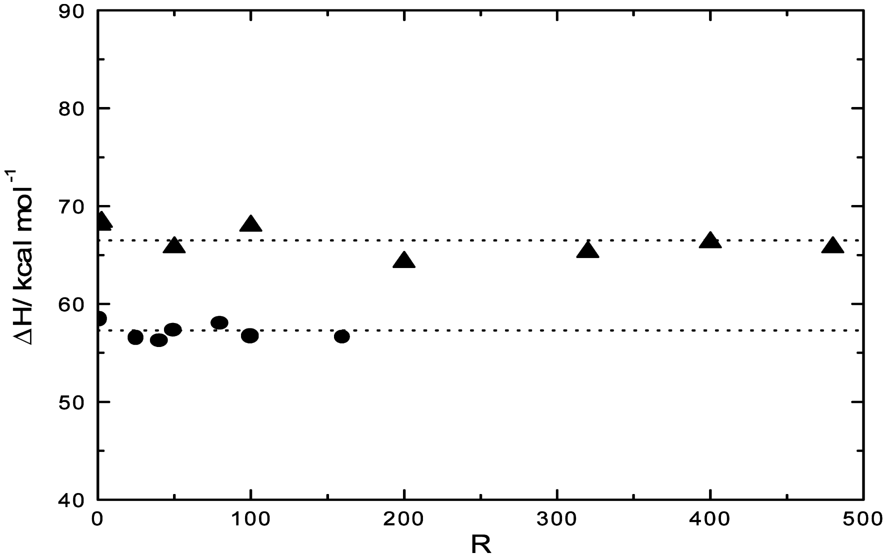

Fig. 4. Variations of the enthalpy of reaction, ΔH_{cal}, as a function of dextran/periodate concentration ratios (R) for samples D1 (▲) and D7 (●).

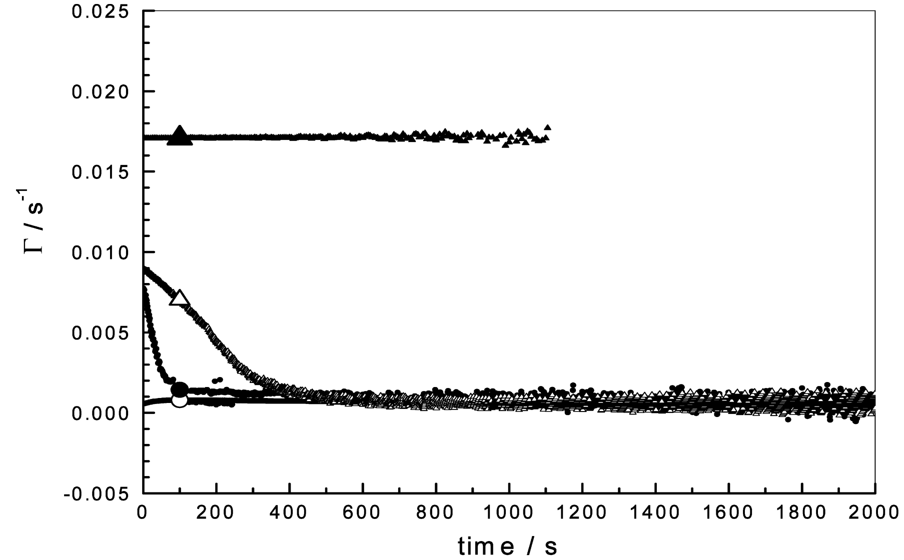

Fig. 5. Variations of the instantaneous reaction rate Γ as a function of time for samples D1 (▲, $R = 100$; △, $R = 1.2$) and D7 (●, $R = 320$; ○, $R = 1.2$).

Table 3
Kinetic and thermodynamic data of periodate oxidation on dextran D7

Dextran concentration	R	W_o (kcal s^{-1})	k'_1 (s^{-1})	k_2^{35} (L mol^{-1} s^{-1})	ΔH_{cal} (kcal mol^{-1})	Γ_{av} (s^{-1})
1×10^{-1}	160	3.04×10^{-1}	5.24×10^{-3}	5.27×10^{-2}	56.6	5.84×10^{-3}
6.25×10^{-2}	100	1.54×10^{-1}	3.58×10^{-3}	5.79×10^{-2}	56.7	3.60×10^{-3}
5×10^{-2}	80	1.76×10^{-1}	2.98×10^{-3}	6.01×10^{-2}	58	3.15×10^{-3}
3.125×10^{-2}	50	7.87×10^{-2}	1.87×10^{-3}	6.00×10^{-2}	57.3	2.11×10^{-3}
2.5×10^{-2}	40	7.66×10^{-2}	1.38×10^{-3}	5.66×10^{-2}	56.2	1.66×10^{-3}
1.56×10^{-2}	25	6.21×10^{-2}	9.71×10^{-4}	6.50×10^{-2}	56.5	11.3×10^{-4}

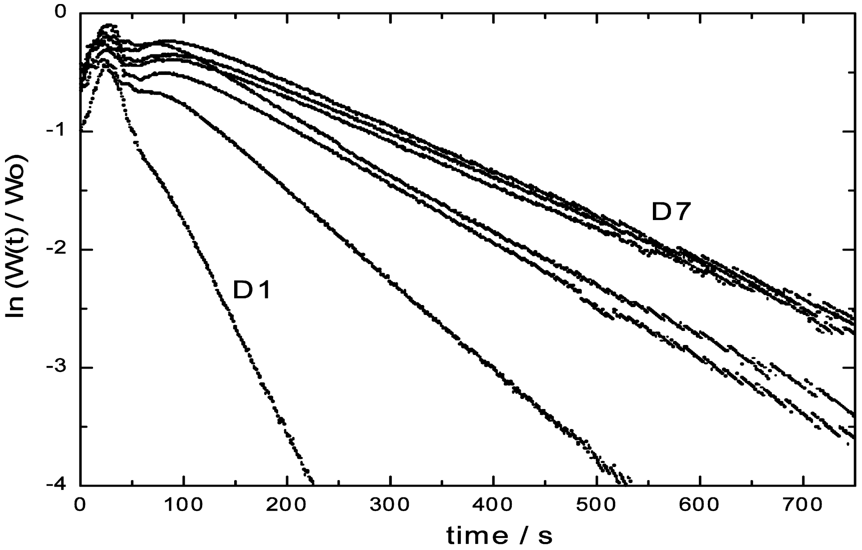

Fig. 6. Kinetic curves (logarithm of the normalized power) vs. time for the dextran samples at different molecular weight D1–D7 (see Table 1).

Table 4
Kinetic and thermodynamic data of periodate oxidation on dextran substrates

Dextran	W_o (kcal s^{-1})	k'_1 (s^{-1})	k_2^{35} (L mol^{-1} s^{-1})	ΔH_{cal} (kcal mol^{-1})	Γ_{av} (s^{-1})
D1	1.43	1.75×10^{-2}	2.86×10^{-1}	68	1.74×10^{-2}
D2	5.12×10^{-1}	7.43×10^{-3}	1.20×10^{-1}	62	7.88×10^{-3}
D3	3.22×10^{-1}	4.82×10^{-3}	7.81×10^{-2}	59	4.88×10^{-3}
D4	2.52×10^{-1}	4.51×10^{-3}	7.27×10^{-2}	58	4.43×10^{-3}
D5	1.49×10^{-1}	3.03×10^{-3}	4.91×10^{-2}	57	3.38×10^{-3}
D6	1.73×10^{-1}	3.18×10^{-3}	5.14×10^{-2}	57	3.59×10^{-3}
D7	1.54×10^{-1}	3.58×10^{-3}	5.79×10^{-2}	56.7	3.60×10^{-3}

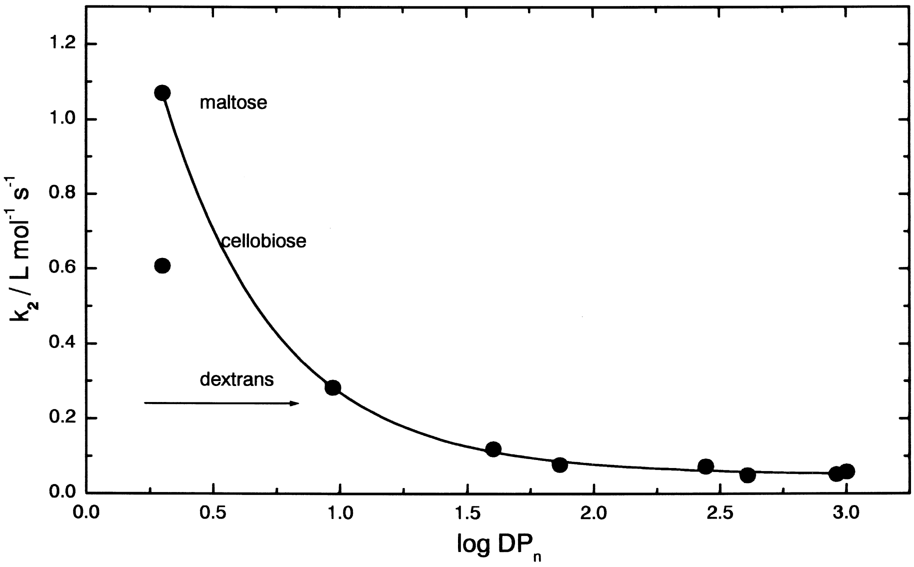

Fig. 7. Variations of the second-order reaction rate (refereed to monomole of glucose ring) as a function of the logarithm of the number average degree of polymerization, DP$_n$. Data for cellobiose and maltose are also reported. Curve represents the theoretical trend calculated with Eq. (6).

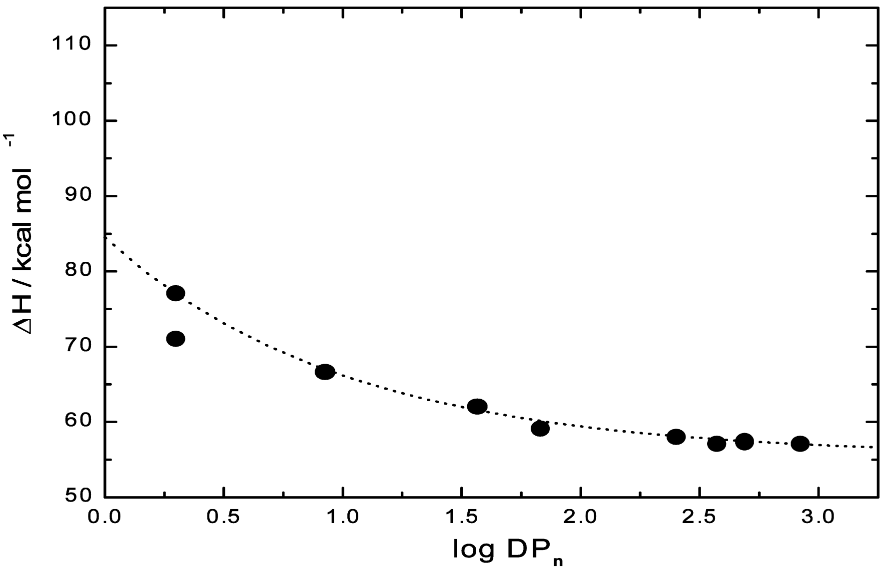

Fig. 8. Variations of the enthalpy of reaction, ΔH, as a function of the logarithm of the number average degree of polymerization, DP$_n$.

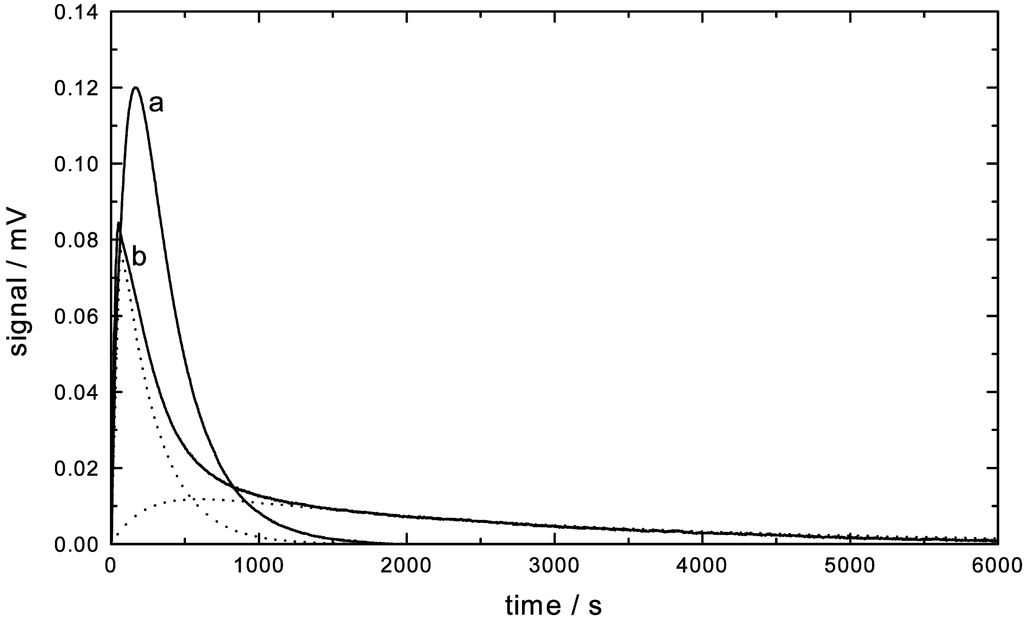

Fig. 9. Calorimetric curves recorded for the reaction of periodate (6.25×10^{-4} M) with dextran D5 (curve a, $C = 6.25 \times 10^{-2}$ M) and with HA (Curve b, $C = 3.12 \times 10^{-2}$ M). Deconvolution of curve b into two kinetic responses is also shown (dotted curves b_1 and b_2). Measurements were carried out at 35 °C in 0.1 M $NaClO_4$.

Analogously, the values of the enthalpy of the reaction follow the same rule (see Fig. 8).

To the best of our knowledge, the effect of the molecular weight of dextrans or of other polymeric carbohydrates on the kinetics and the thermodynamics of periodate oxidation has not been investigated. Therefore, these results cannot be compared with other literature data, but can only be judged on the basis of the reasonable agreement between the scheme proposed and the values for similar more simple substrates.

A heuristic value of the above analysis may derive from the possibility of simulating the overall degree of advancement of the reaction for the different conditions reported in Fig. 5. It is quite clear that the decrease in the reaction rate with time (for times less than 500 s) is a slowdown due to the consumption of the sites displaying the faster oxidation rate. The upper and lower limits are consistent with the range of values obtained by the application of Eq. (6).

4.3. Hyaluronan

We report here a preliminary analysis of the results obtained with HA. Experiments were carried out under several experimental conditions of ionic strengths and temperatures, with both HA and partially modified samples. However, we focus on the measurements carried out at 35 °C (salt 0.1 M) under conditions of a pseudo-first-order reaction, with a concentration of HA = 0.0312 monomol L^{-1} and a ratio $R = 50$. Given the molecular weight of HA and the excess of substrate with respect to the periodate, a comparison can be made with the dextran sample D5. The two calorimetric signals for HA and D5 are shown in Fig. 9. Although the sugar stereochemistry is similar (equatorial OH group), the reaction with dextran is overall faster than that with HA. However, it is also clear that the kinetics of HA presents both fast and slow components, a fact that has to be analyzed in terms of the reactivity of both terminal and internal sites. The calorimetric curve has been deconvoluted for the calorimetric response and then analyzed with both the pseudo-first-order formalism and the Γ parameter; the former plot (not shown) presents an almost continuous curvature which reaches a constant slope only at $t > 2000$ s. The presence of a fast component and a slow tail in the calorimetric curve clearly emerges from the deconvolution of the calorimetric curve and from the plot of the Γ parameter in Fig. 10. The instantaneous reaction rate decreases to reach a value of about 4.3×10^{-4} for $t > 1500$ s. Since this last kinetics is still occurring under the excess of substrate, a second-order reaction rate can, therefore, be simply calculated. The resulting value of k_2 is 1.37×10^{-2} L mol^{-1} s^{-1}, a value which is only slightly smaller than that calculated for dextrans with high molecular weight. The faster reaction of terminal groups will have to take into account that the fraction of α-anomeric carbons is about 55% (from the NMR data on oligomers[20]).

A simple evaluation of the concentration of terminal units in the HA solution (assuming $M_n = 7.05 \times 10^4$) gives $C_t = 1.8 \times 10^{-4}$ M, which means that about 30% of the periodate is consumed in the fast reaction with the terminal units (if no other reaction occurs at this end).

The rate constant evaluated for the oxidation of about 1% of internal sites, leaving the rest of the HA chain unchanged, is therefore much faster than that reported in the literature[9] (taking into account the temperature difference, by using the Arrhenius slope). This difference could be ascribed either to the different experimental conditions or to the different methodology used. Although the result itself is not discussed for the further implications, which have been ascribed to the HA conformation, let us here simply consider the question of the experimental procedures and the analysis of the results. A number of experiments carried out on several substrates by calorimetry have always provided reliable results over a considerable number of data points, and often excellent curve simulation has smoothed out the electronic noise of the data collected. The more reliable calorimetric curves have always been those spanning over times larger than 1000 s. We therefore believe that the present results add substantial knowledge on the kinetic parameters for the 'early' stage of the reaction. This has to be emphasized since it would correspond to a macromolecular substrate which has not already been modified by the periodate. When the periodate and substrate are at equal concentration, the reaction rate is measured, while the chemistry and the stereochemistry of the polymeric substrate are continuously changing.

4.4. Discussion on the limitations of the calorimetric method

The calorimetric investigation on periodate reaction kinetics presented in this paper and in the previous account[1] has the great advantage of a high sensitivity due the heat exchanged in the microcalorimeter. For a reaction with a ΔH of 60 kcal mol^{-1}, the limit of detection of the microcalorimeter is of 1.3×10^{-9} mol, since the microcalorimeter sensitivity is 0.1 mcal. However, since 'heat does not come in different colours',[21] the measured quantity does not provide any fingerprint information about the structure of the molecules reacted or produced in the calorimeter, and has to be associated with a known reaction. On the other hand, calorimetric data, when produced in a reliable and reproducible way, are of great value by themselves and may be subjected to interpretation by using other methods, e.g., molecular spectroscopies and models.

It has also to be said that in a well-defined simple reaction there is a one-to-one correspondence between calorimetric results and data obtained from other methods.[15,16,22] With multiple reactions, the overall heat is obtained by calorimetry; data have been used for comparison purposes in order to study the difference in the thermal or kinetic quantities, with the assumption that differences can be attributed to known structural differences of the substrates.[1] An excess of substrate (that is a pseudo-first-order condition) has been chosen here, also on the basis of the previous experience, in order to simplify the mathematical procedures. For dextrans with very different molecular weights, the presence of multiple sites in variable amounts introduces too many variables, and the system becomes mathematically untreatable.

An issue has been raised by one of the referees about the effect of reactive impurities on the results here presented, specifically in the conditions of 'first-order reaction rate'. Admittedly, with a large excess of substrate, a reactive impurity may significantly affect the

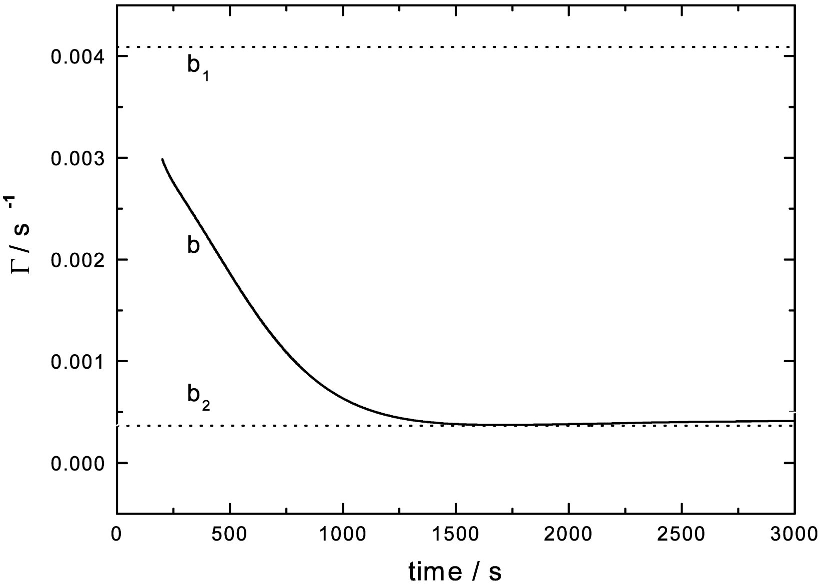

Fig. 10. Variations of the instantaneous reaction rate Γ as a function of time for HA (at 35 °C) and for the two deconvoluted curves b1 and b2 of Fig. 9.

course of the reaction. Although some routine analytical tests have been carried out to check sample purity, none of those methods could have revealed an amount of say 0.1% of impurity. A small amount of 0.1% of reactive impurity could indeed consume 10% of the periodate in the pseudo-first-order conditions chosen here. We have not been able to run analytical tests that could detect such a small amount of 'reactive' impurity in the dextran samples and in other carbohydrate substrates.

Although indirectly, the 'analytical calorimetric method' can be used to provide these pieces of information, since the results of the kinetics of periodate consumption were in agreement with other literature values. In a previous report[1] a number of substrates (11) were analyzed at different temperatures and a substantial agreement was found between the k_2 values obtained by calorimetry (Table 1 of Ref. 1) and those reported in the literature for the initial second-order rate of periodate consumption (respectively, k_2 of Ref. 11 and $(k_P)_0$ of Ref. 7 quoted in Ref. 1). For the polymeric substrates, a comparison can be made between the value of $k_2 = 0.06$ L mol^{-1} s^{-1} obtained for dextran sample D7 at 35 °C and the value of $k_2 = 0.025$ L mol^{-1} s^{-1} reported at 25 °C for a dextran with molecular weight of 2×10^6.[10] These two values are in line with the temperature dependence calculated for methyl xylosides,[1] taken as the most similar monomeric substrate for dextran. A small difference could have been expected because of a different number of branchings in dextrans with high molecular weight.[13] More importantly, the absence of reactive impurities can be deduced from data in Fig. 4 (samples D7 and D1), the latter showing even a decrease of k_2 with increasing substrate concentration, the opposite behavior that should have been observed in the presence of reactive impurities. This argument does not diminish the risks of a possible contamination and a careful consideration of the consequences, since it may simply indicate an apparent insensitivity of the method used.

As a final remark, a series of data on samples of dextrans with different molecular weight and in a different range of conditions has been analyzed in this work to investigate the molecular weight dependence of the rate of oxidation by periodate reaction. This dependence has been ascribed to the fraction of the terminal groups and interpreted in terms of their higher reactivity in comparison with the groups of internal residues. Whether this conclusion is quantitatively correct, or merely qualitatively (on the base of the errors affecting the use of the calorimetric method alone), is undoubtedly a matter of future consideration.

5. Conclusions

The general and important result of our study is that, from many points of view, the calorimetric analysis is sensitive enough to provide a satisfactory set of data under conditions that cannot be explored by other analytical methods.

From the results and the correlations presented here, calorimetry of the periodate oxidation seems to be a good method for determining the number-average molecular weight (M_n) of polysaccharides. The method is based on the different rate constants of the reaction involving terminal and internal diols in the conditions of a pseudo-first-order reaction. Furthermore, by limiting the reaction to the pseudo-first-order conditions, it is possible to extract rate constants that refer to polymeric substrates without major modifications from the original structure (provided that no highly reactive impurities are present, as it has been annotated by a referee).

In the presence of several sites with different reactivities, the overall reaction can be deconvoluted into the several parallel reactions which consume the periodate at different rates. A simplification of this more complex behavior may arise from knowledge of the composition of the polymeric substrate and of the individual contribution to the total kinetics.

In conclusion, the major goal of the results presented here is the evaluation of the rate constants at the early stage of reaction for polymeric substrates, dextrans and HA. A more detailed analysis will follow of the oxidation of HA under several experimental conditions.

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