Kinetics and polydispersity changes during acid hydrolysis of *streptococcus salivarius* levan

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The kinetics of the acid degradation of *Streptococcus salivarius* levan was studied by observing changes in the number-average molecular weight, $\bar{M}_n$, using end-group analysis. Rate constants for the hydrolysis are presented and a tentative mechanism is forwarded. The data has been analysed according to a statistical model for a polymer with trifunctional branching and it is shown that the hydrolysis of levan proceeds through a mechanism quite different to that reported for the polysaccharide dextran.

INTRODUCTION

Levan is a polysaccharide consisting of D-fructofuranose units[1], joined by $\beta(2 \rightarrow 6)$ bonds[2] and branched through $\beta(2 \rightarrow 1)$ bonds. It is found in plants as a storage polysaccharide[3] and it is also produced by bacteria that are capable of synthesizing polysaccharides from sucrose or fructose[4,5].

The levan of interest in this work is that produced by bacteria of dental plaque, which has been implicated in the etiology of dental caries and periodontal diseases. The levan synthesized by *Streptococcus salivarius* has a molecular weight of $20\text{--}60 \times 10^6$ daltons, is highly branched through the $\beta(2 \rightarrow 1)$ linkages, and has a compact spherical structure[6]. Some solution properties of this levan have been reported by Stivala and coworkers[7–9]. Recent work has indicated that the molecular weight may be dependent upon the environmental conditions during synthesis, e.g. pH[10].

The bacteria of dental plaque are capable of hydrolysing levan. Thus, levan can serve as a carbohydrate source for bacteria which continually release acid as a by-product of the hydrolysis[11]. Levan is readily hydrolysed by acids[12] and more so than dextran which serves mainly as an adhesive between the plaque and the tooth enamel[13].

In an earlier publication, the authors examined the acid hydrolysis of *S. salivarius* levan by observing changes in the weight-average molecular weight, $\bar{M}_w$, as a function of time, temperature, pH and levan concentration[14]. Two simultaneous first-order reactions were observed; an initial rapid reaction and a slower reaction. The former was attributed to the breaking of $\beta(2 \rightarrow 1)$ branch point bonds while the slower reaction was attributed to scission of the $\beta(2 \rightarrow 6)$ non-branch point bonds in the main chain and branches. First-order rate constants k_1 and k_2 were calculated for the fast and slow reaction from Guggenheim plots and thence the corresponding activation energies of 24.4 kcal/mol and 24.1 kcal/mol respectively were obtained from Arrhenius plots.

The purpose of this paper is to analyse the hydrolysis of levans using changes in the number-average molecular weights, $\bar{M}_n$. The effect of hydrolysis on the polydispersity of the levan solutions was also studied.

MATERIALS AND PROCEDURE

Materials

All experiments were conducted on *Streptococcus salivarius* (ATCC 13419) levan, synthesized and purified according the procedure previously described[14]. The native levan had $\bar{M}_n = 2.57 \times 10^5$.

Experimental

The kinetics were followed by observing changes in $\bar{M}_n$. The $\bar{M}_w$ data, obtained from light scattering measurements, were presented in an earlier publication[14]. The hydrolyses were all performed by adding 15 ml of HCl to 20 ml of a levan solution at a given concentration to produce a solution of pH 2.0. Number-average molecular weights were obtained as a function of time from aliquots neutralized with $NaHCO_3$ to approximately pH 6.8. The $NaHCO_3$ was found not to affect the absorbance of the coloured complex at 750 nm. All measurements were run in duplicate and standard fructose solutions were run with each experiment.

To calculate the number-average molecular weight of levan aliquots from the colorimetric data, it was assumed that each molecule contains only one reducing end group. The concentration of reducing sugar in a given levan aliquot was obtained from a Beer's law plot between absorbance and concentration for fructose used as standard. If C is the concentration of levan per ml of solution and R the concentration of reducing sugar in that solution, then the number-average degree of polymerization, $\bar{x}_n$, for the sample is given by

$$\bar{x}_n = \frac{\bar{M}_n}{162} = \frac{C}{R} \qquad (1)$$

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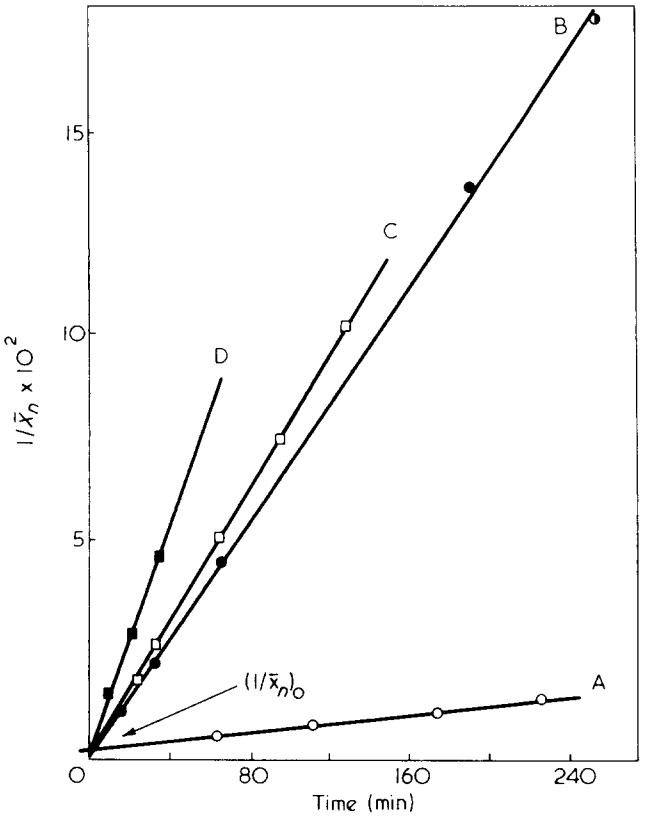

Figure 1 $1/\bar{x}_n$ *vs.* time, hydrolysis at pH 2.0 at A, 25°; B, 35°; C, 40° and D, 45°C

Table 1 Number-average molecular weight of native levan

Levan conc. (g/ml)	μg/2ml Reducing sugar	$\bar{M}_n$
0.006975	8.60	262 700
	8.62	262 200
	8.62	262 200
0.00465	5.67	266 000
	6.26	241 000
	6.04	249 000
	Average:	257 200

Average deviation from mean = 3.2%; Molecular weight reducing sugar = 162

where 162 is the molecular weight of the anhydrofructose repeat unit. Clearly, then,

$$\bar{M}_n = \frac{C \times 162}{R} \qquad (2)$$

where both C and R were measured in μg/ml.

A series of hydrolyses at different levan concentrations was performed at 35°C. Experiments were also conducted at a levan concentration of 1794 μg/ml at 25, 35, 40, and 45°C.

RESULTS

All plots of $1/\bar{x}_n$ versus time were found to be linear obeying the relationship

$$1/\bar{x}_n = 1/(\bar{x}_n)_0 + k_3 t \qquad (3)$$

where k_3 is the specific rate constant and $\bar{x}_n$ and $(\bar{x}_n)_0$ are the number-average degrees of polymerization at time t and at time zero. *Figure 1* is a plot of $1/\bar{x}_n$ versus time for the hydrolyses at 25, 35, 40 and 45°C, for levan concentration of 1794 μg/ml.

Table 2 shows the specific rate constants, k_3, for levan hydrolysis as a function of initial levan concentration. The fact that the reaction rate is independent of the initial levan concentration between 3592 to 476 μg levan per ml suggests a zero order reaction rate in this range. *Table 3* contains values of k_3 obtained from hydrolyses at 25, 35, 40, and 45°C for 1794 μg levan per ml, and an Arrhenius activation energy, E_3, of 29.5 kcal/mole may be obtained from this data.

DISCUSSION

The variation of weight-average molecular weight, $\bar{M}_w$, with time during acid hydrolysis of levan was presented in an earlier paper[14]. Presented below is a mathematical treatment in an attempt to obtain for that reaction, an equation for the time dependence of molecular weight. This derived equation will later be used to analyse changes in polydispersity in the reaction.

The type of curves obtained in the earlier study[14] for $\bar{M}_w$ with time can best be explained assuming two parallel first-order reactions, one faster than the other, having rate constants k_1 and k_2. For such a complex reaction, one can derive[16]:

$$\bar{M}_w(t) = Ae^{-k_1 t} + Be^{-k_2 t} + (\bar{M}_w)_\infty \qquad (4)$$

where A and B are the molecular weights obtained by extrapolation to zero time for the two reactions, (*Figure 2*), and $(\bar{M}_w)_\infty$ is the molecular weight at infinite hydrolysis time[14]. *Table 4* shows the values of A and B for levan hydrolysis at pH 2 at five different temperatures. Over this wide temperature range, the values of A and B remain virtually constant. It is not clear whether A and B play any significant role in the mechanism of the hydrolysis.

A and B are best regarded as parameters which allow the correct form of the change in $\bar{M}_w$ with time to be predicted for the hydrolysis of levan with acid. The value of A plus B approaches 24×10^6, the molecular weight of the levan before hydrolysis.

Table 2 Rate dependence upon levan concentration at 35°C, pH 2.0

Levan concentration (μg/ml)	k_3, (sec^{-1})
3 592	1.30×10^{-5}
1 794	1.10×10^{-5}
946	7.95×10^{-6}
476	9.07×10^{-6}

Table 3 k_3 for levan hydrolysis (1 794 μg levan/ml) at different temperatures

Temperature °C	k_3, sec^{-1}
25	1.09×10^{-6}
35	1.10×10^{-5}
40	1.43×10^{-5}
45	2.48×10^{-5}

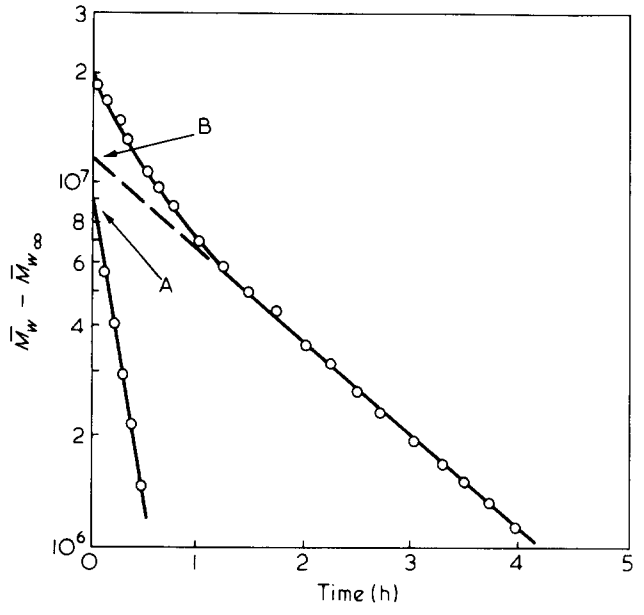

Figure 2 First order Guggenheim plot for hydrolysis at pH 2.0, 30° C showing *A* and *B*

Table 4 Values of molecular weights, *A* and *B*

Temperature °C	*A*	*B*	A + B
30	8.5×10^6	13.0×10^6	21.5×10^6
35	9.4×10^6	13.8×10^6	23.2×10^6
40	9.3×10^6	14.0×10^6	23.3×10^6
45	8.8×10^6	14.8×10^6	23.6×10^6
50	9.0×10^6	14.2×10^6	23.2×10^6
Average	9.0×10^6	14.0×10^6	23.0×10^6

The time dependence of the dispersity, $D(t)$, may be given by

$$D(t) = \bar{M}_w(t)/\bar{M}_n(t) \tag{5}$$

As a consequence of Equation 3,

$$\bar{M}_n(t) = \frac{(\bar{M}_n)_0}{1 + (\bar{x}_n)_0 k_3 t} \tag{6}$$

From Equation 4 the time dependence of the weight-average molecular weight, $\bar{M}_w(t)$, is known. Equations 4 and 6 may be substituted into equation 5 to give:

$$D(t) = \{(Ae^{-k_1 t} + Be^{-k_2 t} + (\bar{M}_w)_\infty)/(\bar{M}_n)_0\}\,[1 + (\bar{x}_n)_0 k_3 t] \tag{7}$$

Using the average values of *A* and *B* from *Table 4* and $(\bar{M}_n)_0 = 2.57 \times 10^5$, this leads to

$$D(t) = \left[35e^{-k_1 t} + 45e^{-k_2 t} + \frac{(\bar{M}_w)_\infty}{(\bar{M}_n)_0}\right][1 + 1590 k_3 t] \tag{8}$$

where $(\bar{M}_w)_\infty$ values were reported in ref 14. Equation 8 faithfully reproduces the experimental data as seen in *Figure 3*, in the range of time periods studied. Slight deviations indicate experimental error.

For random scission of all bonds, the polydispersity falls continuously as a function of time. This has been shown in the case of dextran, a branched polyglucose[17]. The polydispersity change during acid hydrolysis of dextran at 71°C, pH = 1 is shown in *Figure 3* for comparison. In this respect, also, acid hydrolysis of levan is distinct from that of dextran and other polysaccharides. The $\bar{M}_n$ values used in constructing the curve in *Figure 3* were also obtained from end-group analysis by Antonini *et al.*[17]. These workers noted that any error in calculating $\bar{M}_n$ by this method may be large owing to the very small numbers of reducing end-groups at extremely high molecular weights[18]. However, they confirmed their values by extrapolation of the values obtained on native dextran at different hydrolysis times. Based on this observation, the values of $\bar{x}_n$ (*Table 5*) obtained for levan samples in this study should be reliable, particularly since the $\bar{M}_w$ for the native levan was around 24×10^6 compared with 11×10^7 daltons for the native dextran. Any error associated with reducing groups in extremely high molecular weight samples would be common to both the levan and dextran, and it is still noted in *Figure 3* that the polydispersity curve for the levan is distinctly different from that of the dextran.

It is apparent, from an analysis of the polydispersity changes during hydrolysis of levan, that this hydrolysis proceeds in a manner quite different to that of the hydrolysis of dextran, amylopectin and glycogen. The weight-average molecular weight data on dextran also points to a complex reaction[17] and the molecular weight does not decay as a first-order function of time. The polydispersity, however, decays in a manner which may be attributed to the random

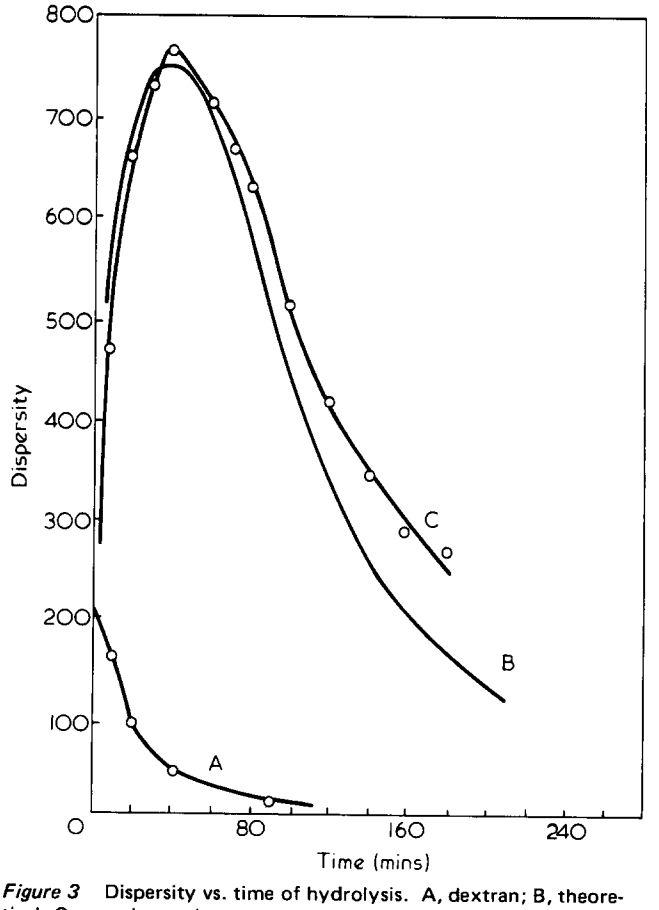

Figure 3 Dispersity vs. time of hydrolysis. A, dextran; B, theoretical; C, experimental

Table 5 $\bar{M}_n$, $\bar{x}_n$, $\bar{x}_w$ and D as a function of time for levan hydrolysis different temperatures

Conditions	Time (min)	$\bar{M}_n$	$\bar{x}_n$[a]	D[b]
25° C, pH = 2	0	257 200	1590	92
	10	120 000	740	162
	30	60 000	370	234
	60	35 000	216	288
	107	21 000	130	318
	215	11 000	68	299
35°, pH = 2	0	257 200	1590	92
	3	85 300	527	240
	5	49 500	306	379
	8	32 100	198	505
	15	15 500	96	707
	30	7100	44	1052
	60	3400	21	1058
	180	1180	7	397
	240	915	6	267
40° C, pH = 2	0	257 200	1590	92
	10	20 000	124	503
	20	9000	56	678
	30	6250	39	594
	60	3080	19	315
	90	2130	13	176
	120	1560	10	143
45° C, pH = 2	0	257 200	1590	92
	1	76 100	470	271
	3	33 900	209	464
	5	19 500	120	612
	10	10 300	64	743
	20	5500	34	556
	30	3400	21	444

[a] $\bar{x}_n = \bar{M}_n/162$ [b] $D = \bar{x}_w/\bar{x}_n$ ($\bar{x}_w$ from reference 14)

splitting of a statistically branched polymer[17]. This is a first indication that levan may degrade, in acid, *via* a non-random or non-statistical path, i.e., preferential cleavage of a given bond. This is not withstanding the fact that the activation energies associated with the fast and slow reactions do not differ appreciably, an observation also in keeping with dextran hydrolysis[17].

Erlander and French[19,20] proposed a model for a statistical polymer with random trifunctional branching. According to the model,

$$\bar{x}_w = (1 - p_a^2 - p_b^2)/(1 - p_a - p_b)^2 \quad (9)$$

and

$$\bar{x}_n = 1/(1 - p_a - p_b) \quad (10)$$

where $\bar{x}_w$ and $\bar{x}_n$ are the weight- and number-average degrees of polymerization of the polymer and p_a and p_b are the probabilities of reaction of hydroxyl groups on carbon atoms a and b with an aldehydic group to form the branched polymer. The model may be used to test the randomness of splitting of bonds during hydrolysis. In such a case, the relative probability of the occurrence of a branch point along the chain remains constant as shown by Antonini *et al.*[17]. *Figure 4* shows the theoretical relations obtained from the model as solid straight lines. Data on dextran from *Reference 17* is also shown (open circles). Dextran does conform to the model and hence random-splitting may be concluded[17]. Experimental data on levan, however, is at variance with the model throughout the range studied. This is a second indication that levan may degrade through a non-random splitting mechanism.

Since levan contains two types of linkages, $\beta(2 \rightarrow 1)$ and $\beta(2 \rightarrow 6)$, it may be assumed that these two bonds split at different rates, and the preferential splitting of one type may be leading to the non-random splitting behaviour observed above. In an earlier paper[14] the faster reaction was identified as branch point, $\beta(2 \rightarrow 1)$, scission and the slower one as main chain, $\beta(2 \rightarrow 6)$, scission. An alternative explanation may be that the terminal groups in levan split at a much faster rate than either the branch points or the main chain, which may have equal acid susceptibilities[21]. In either of the two cases, after a certain time of hydrolysis an effectively linear or unbranched chain will be left, since the average branch length is much smaller than the backbone length[22]. This does not necessarily imply that the chain is completely linear but that it will behave hydrodynamically as if it is linear..

Such a contention can easily be checked from a Mark–Houwink plot of intrinsic viscosity, $[\eta]$, and $\bar{M}_w$. The exponent in the equation

$$[\eta] = k(\bar{M}_w)^{\alpha} \quad (11)$$

is related to particle shape. For linear random coils, the exponent is in the range of 0.5 to 0.9 whereas for branched polymers the value generally lies in the range of 0.2 to 0.4 but values below 0.2 have also been reported. For the native levan in this work, the exponent has the value of 0.17[8,23], which is well in the branched region.

To obtain a Mark–Houwink plot, the specific viscosity, η_{sp}, was measured continuously with time for a levan concentration of 1g/dl in a solution of pH 2 at 35°C. ($\eta_{sp} = t/t_0 - 1$, where t and t_0 refer to efflux times for solution and solvent respectively). For uncharged molecules[24]:

$$\frac{\eta_{sp}}{C} = [\eta] + k[\eta]^2 C \quad (12)$$

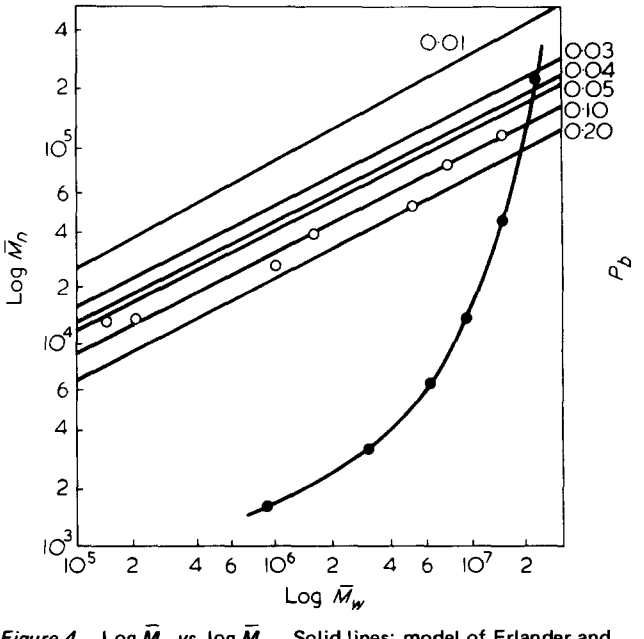

Figure 4 Log $\bar{M}_n$ *vs.* log $\bar{M}_w$. Solid lines: model of Erlander and French[19,20]; open circles: Dextran hydrolysis[17]; filled circles: levan hydrolysis at pH 2.0, 25° C. P_b is the probability of the occurrence of a $\beta(2 \rightarrow 1)$ linkage

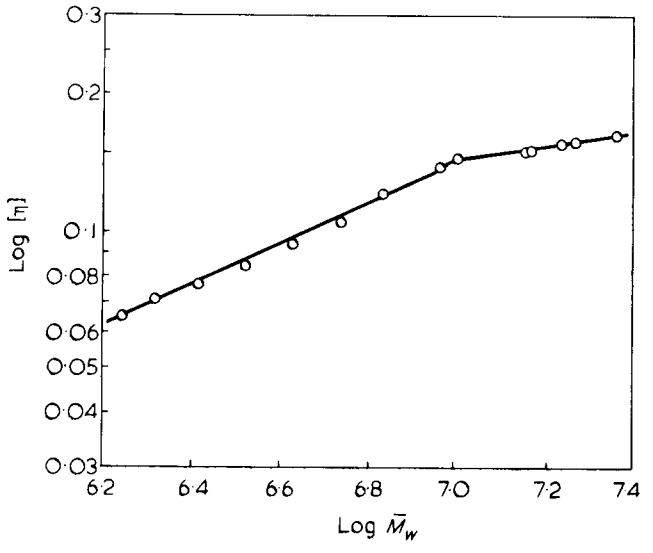

Figure 5 Log $[\eta]$ *vs.* log $\bar{M}_w$ for levan hydrolysis at pH 2.0, 35°C

so that

$$\eta_{sp} = [\eta] + k[\eta]^2 \qquad (13)$$

where $[\eta]$ is in dl/g. The constant k was found through an independent experiment to be 1.90[25]. Equation 13 may be solved quadratically for $[\eta]$, the positive root giving the desired value. This data was plotted according to Equation 11, against $\bar{M}_w$ obtained from a smoothed-out curve of the variation of $\bar{M}_w$ versus time at 35°C, pH = 2 (see *Figure 5*). In the high molecular weight range, $\bar{M}_w > 6 \times 10^6$ daltons, the exponent α is 0.15; while below $\bar{M}_w = 6 \times 10^6$, the value increases to 0.49. This suggests that the branched structure of levan is maintained down to 6×10^6 daltons with further decreasing values of molecular weight indicating that the structure is hydrodynamically approaching the random coil. The low molecular weight levans may still be somewhat branched. It should be noted that the sensitivity of some hydrodynamic measurements decreases with decreasing length of the branches, at the limit exhibiting no difference between linear and short branched structures.

A similar effect was noted during the acid degradation of *Bacillus subtilis* levan by acid[26]. That study, however, involved hydrolysing the material, fractionating the hydrolysate and characterizing the individual fractions obtained by light scattering, viscosity, sedimentation data and also end-group-analysis.

In contrast Antonini *et al.*[17] obtained a single straight line in the Mark–Houwink plot with an exponent of about 0.25 and Senti *et al.*[27] observed a straight line down to about $\bar{M}_w = 70\,000$ with the exponent in the branched region. This implies that all the bonds are equally susceptible to rupture under conditions examined by these workers and the hydrolysis is random except for very low molecular weight dextrans.

The polydispersity data may also be reconciled with the conclusions reached above. If initially a large number of small fragments (monomers from terminal groups or whole short branches 8 to 10 units long) appear in the reaction mixture, the polydispersity should increase drastically since the number-average molecular weight will fall faster than the weight-average molecular weight. This is so since the $\bar{M}_w$ is more sensitive to larger particles but the $\bar{M}_n$ is sensitive to the number of particles. After the chain becomes effectively linear, the shape of the decay in polydispersity is reminiscent of a random splitting polymer, *Figure 3*. The time required to reach the peak in the polydispersity curve is the same as time t_a mentioned in an earlier paper[14] and for 35°C, pH = 2, $\bar{M}_w = 6.8 \times 10^6$ at $t = t_a$. This value of $\bar{M}_w$ is close to the value where the exponent in Equation 11 changes to reflect an effectively linear conformation.

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