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A MECHANISM FOR THE CHYMOSIN-INDUCED FLOCCULATION OF CASEIN MICELLES

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The chymosin-induced flocculation of casein micelles of bovine milk can be explained and calculated in terms of three relationships, which are (i) the action of chymosin upon the κ-casein of the micelles; (ii) the probability that a micelle, with a given proportion of its κ-casein destroyed, will aggregate, and (iii) the aggregation of micelles by a Smoluchowski mechanism. Details of the calculations are given, and the theory is shown to be in good agreement with experimental observations of the dependence of the clotting time with variations in enzyme and substrate concentrations.

1. Introduction

A theory for the rates of enzyme-triggered coagulation reactions was recently provided by Payens and co-workers [1,2], with special emphasis on the chymosin-induced coagulation of the micelles of milk. According to this theory, the lag time between the initiation of the reaction and the observation of coagulation is the result of first, the time required for the enzyme to produce appreciable concentrations of coagulable material and second, the time required for this material to aggregate, by a diffusion-controlled mechanism after the model of von Smoluchowski [3]. This mechanism has been shown [4] to describe very well the coagulation of para-κ-casein produced from isolated κ-casein. However, in milk, the κ-casein exists distributed throughout casein micelles and the proteolysis of one κ-casein molecule within the micelle is not sufficient to bring about aggregation [5,6]. A description of the relationship between proteolysis of κ-casein and subsequent aggregation of micelles requires to be incorporated into the thory.

Experiments have shown that, prior to aggregation, between 80% and 90% of the κ-casein of milk has to be subjected to proteolysis before appreciable aggregation and clotting can occur, because of the low aggregating tendency of micelles which contain an appreciable amount of unmodified κ-casein [5,6]. Estimates of the weight-average molecular weight of casein micelles are of the order of 3×10^8, and number-average molecular weights are an order of magnitude lower [7, 8,9]. Therefore, the average micelle, having about 13% κ-casein [10], should contain some 200 κ-casein molecules, most of which are on the surface, but some of which may be contained within the micelle structure [11,12]. Chymosin acts on this κ-casein according to Michaelis-Menten kinetics [13], but it is clear that the aggregability of chymosin-treated micelles does not increase linearly with the proteolysis of κ-casein [6].

This paper describes a modification of the original mechanism of Payens to take account of the time-dependence of production of aggregable material produced by the action of chymosin on milk. A method of incorporating the extent of proteolysis into calculations is then developed, demonstrating that it is possible to describe the complete time-course of the reaction to accord with experimental evidence.

2. Experimental and results

Fig. 1 shows the results of an earlier study [6], made to define the relationship between aggregation and chymosin proteolysis of milk. In these experiments, controlled proteolysis of the κ-casein was achieved, in the absence of aggregation, by the use of immobilised chymosin at 4°C. A similar controlled proteolysis, using soluble chymosin in milk maintained at 0°C, gave

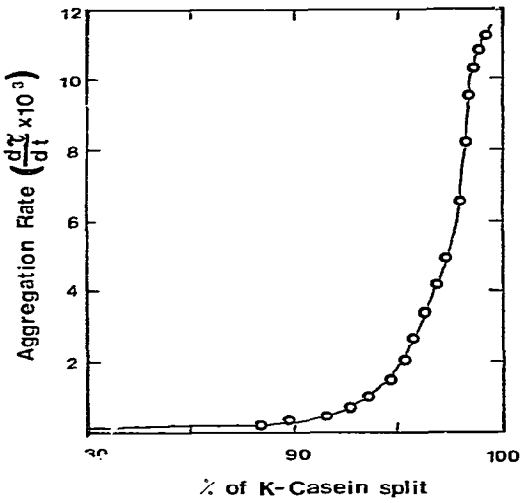

Fig. 1. The rate of aggregation (change of turbidity) of cooled renneted milk warmed by diluting 0.1 ml of milk into 3 ml synthetic serum at 25°C, plotted as a function of the extent of proteolysis at that reaction time, measured by quantitative electrophoresis. The small amount of residual aggregation at less than 80% conversion of κ-casein represents the reassociation of β-casein (see text).

similar results. After proteolysis for given times, small samples were taken from the reaction mixture, and were rapidly diluted and warmed to 25°C. The rate of aggregation of these samples was estimated by the rate of change of turbidity following warming. The extent of κ-casein proteolysis was estimated for each sample by quantitative electrophoresis on cellulose acetate supports. Both types of experiment gave similar results, namely, that no aggregating tendency of the proteolysed milk was found until a considerable extent (about 88%) of the total κ-casein present had been destroyed. At higher conversions of κ-casein, the aggregation rate increased sharply, rising to a maximum when virtually all of the κ-casein had been destroyed. This aggregation appeared to be almost completely caused by the action of chymosin: since it is known that there is a tendency for β-casein to dissociate from casein micelles at low temperatures, tests were made to check that the turbidity changes which were observed on warming the cooled samples were not simply caused by reassociation of β-casein. By duplicate experiments similar to the above, but lacking chymosin, it was shown that the turbidity changes from this cause were small, being less than 2% of the maximal aggregation rate of chymosin-treated micelles.

These results were interpreted [6] in terms of a model which defined that individual micelles which had less than approximately 97% of their κ-casein proteolysed do not aggregate. From the average composition of casein micelles, a simple statistical formulation allowed the calculation of the proportion of micelles which could aggregate at any given extent of proteolysis.

To test the results of any calculation method by reference to experimental observations, there are two definable variations which can be readily studied, namely the variation of coagulation time (t_c) with enzyme concentration, at a fixed concentration of milk, and secondly, the variation of t_c with the concentration of milk, at fixed chymosin concentration. The first of these, is it well-established, gives a relation such that t_c is approximately inversely related to the enzyme concentration [2]. The second, however, is not well-defined, and a brief series of experiments was carried out to study the phenomenon.

Milk from herd-bulk samples was pasteurized (63°C, 30 min), and was then concentrated by ultrafiltration until the protein-containing moiety was concentrated by a factor of 3. Both permeate and retentate were collected. Visual measurements of t_c at 30°C were then made, on the 3X concentrated milk, and on successive dilutions of this (using the ultrafiltrate as diluent) down to a concentration of 0.025 of the concentrate. The reactions were started by adding to 5 ml of the milk solution, 10 μl of a solution of chymosin (5 mg/ml), prepared according to Bunn et al. [15]. The results are shown in fig. 2. The coagulation time remained relatively constant as concentration decreased, until somewhat below the concentration of the original milk, after which there was a sharp rise in t_c when the concentration of milk was further decreased. Since it was possible that the dilution of the milk caused alterations in micelle structure and hence alterations in t_c, two sets of experiments were compared. In the first set, the milk was treated with chymosin immediately after dilution, and t_c was measured. A second set of diluted milks were prepared and held for 6 hours at 30°C before chymosin treatment. These gave identical t_c to the first set, and it was therefore concluded that dilution did not cause

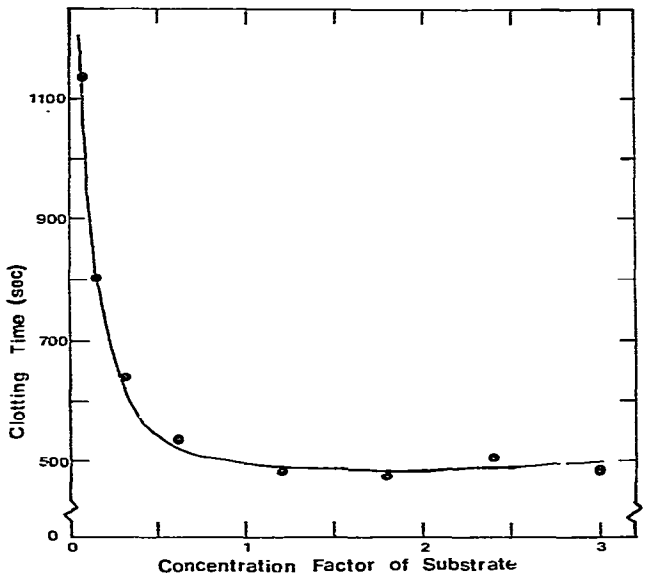

Fig. 2. Coagulation times of chymosin-treated milks as a function of milk concentration. Measurements were made on 3× concentrated milk, and successive dilutions, using ultrafiltrate as diluent. Chymosin concentration was constant. The full line represents the results of calculation, with $K_m = 4 \times 10^{-4}$ mole l^{-1}, $V_{max} = 3.7 \times 10^{-6}$ mole^{-1} s^{-1}, and $k_s = 3.7 \times 10^4$ mole^{-1} l s^{-1}. It was assumed for the calculation that micelle. were monodisperse, with $M = 1.8 \times 10^7$ (containing 100 κ-casein molecules).

appreciable changes in the mechanism which could be detected in this time-span.

3. Calculation of the time-course of the reaction

It is to be assumed that the attack of chymosin on micellar κ-casein is random, For, if this were not so, e.g. if the chymosin were to bind to a micelle and to completely denude it of κ-casein before dissociating, Michaelis-Menten kinetics would be unlikely to apply, and also, the relationship between aggregation and extent of reaction (fig. 1) would not hold. For such random attack, at any extent of overall proteolysis, α, it is possible to define the proportion of micelles which have particular extents of their κ-casein split by chymosin. If the micelles originally each contain n molecules of κ-casein, the proportion of micelles which have i molecules of κ-casein destroyed at overall extent of proteolysis α is:

$$P_{n,i,\alpha} = \frac{n!}{i!(n-i)!}(\alpha)^i(1-\alpha)^{n-i} . \quad (1)$$

If there is a limiting fraction (f) of the original n molecules of κ-casein of the micelle which must be proteolysed before aggregation can occur, the fraction of micelles which can aggregate must simply be

$$g_n = \sum_{i=fn}^{n} P_{n,i,\alpha} \sum_{i=fn}^{n} \frac{n!}{i!(n-i)!}(\alpha)^i(1-\alpha)^{n-i} . \quad (2)$$

In milk, the micelles are heterogeneous in size [7–9], and in composition: that is, there exists a range of values of n. Thus, the total aggregable fraction must represent the sum of g_n:

$$f_{agg} = \sum_{j=a}^{b} \sum_{i=fj}^{j} n_j P_{j,i,\alpha} , \quad (3)$$

where n_j is the number fraction of micelles which possess j molecules of κ-casein, and a and b are the minimum and maximum number of κ-casein molecules in a micelle. It is at present not possible to fully define the distribution of κ-casein in micelles of different sizes. However, it will be shown below that this is likely to have little effect upon t_c.

This relationship can explain the observed behaviour of the aggregability with respect to the extent of proteolysis [6]. A good fit of the experimental results is obtained for $f = 0.97$ (fig. 1), with constant κ-casein content for micelles assumed. This implies that any micelle requires to have about 97% of its κ-casein destroyed prior to aggregation.

Thus, the fraction of micelles which can aggregate at any particular extent of proteolysis can be calculated. The extent of proteolysis (α) can be simply determined from the integrated form of the Michaelis-Menten equation [16].

$$K_m \ln\left(\frac{1}{1-\alpha}\right) + \alpha s_0 = V_{max} t , \quad (4)$$

where K_m and V_{max} are the Michaelis parameters, t is the reaction time, and s_0 is the concentration of substrate (i.e. κ-casein) at $t = 0$. Digital solutions for α as functions of time can be obtained for given K_m and V_{max}. α, once calculated, can be used to give the fraction of micelles which can aggregate, according to eq. (3).

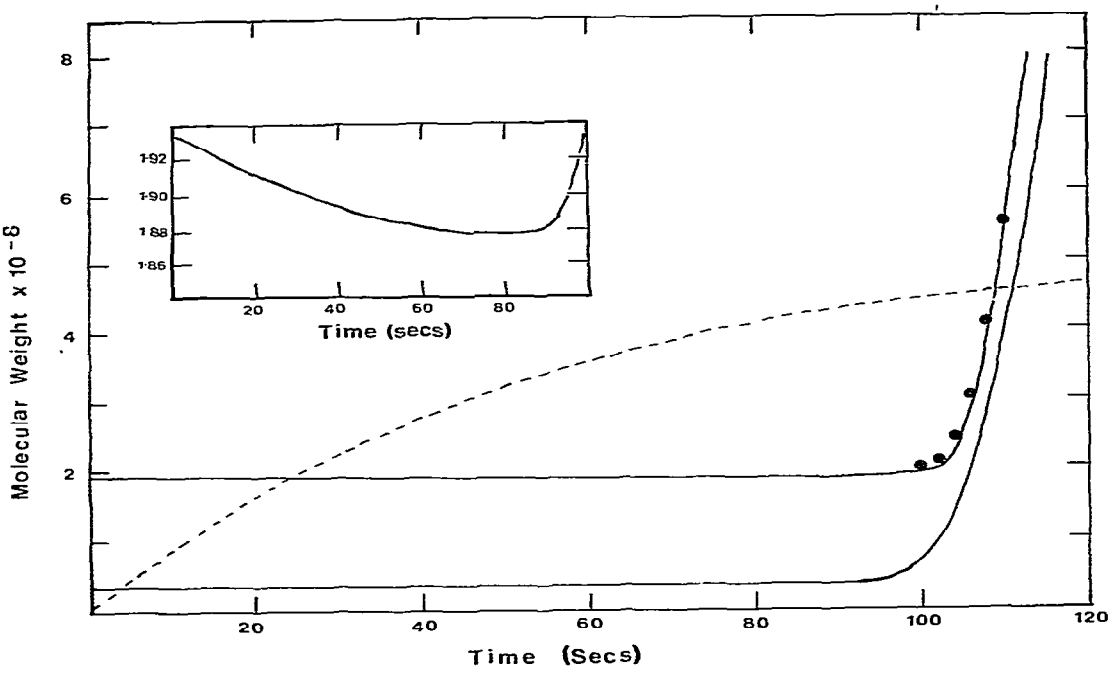

Fig. 3. Calculated time-courses of chymosin-induced coagulation: (i) lower solid curve, taking monodisperse micelles with $M = 3.6 \times 10^7$; (ii) upper solid curve, taking a distribution of micellar sizes from ref. [7], $M_w = 1.94 \times 10^8$, with κ-casein taken as 13% of the total casein content; (iii) Insert shows on larger scale the initial decrease of molecular weight for (ii); (iv) The points on the upper curve demonstrate the effect of κ-casein distribution, when it was assumed that the micelles in (ii) had, in the largest size class, 9%, and in the smallest, 17% κ-casein; (v) the broken line shows the extent of κ-casein splitting.

Thus the function f_{agg} can be calculated for any time during the reaction, and this must then be inserted into the Smoluchowski relationship which governs the aggregation [2] where all aggregating species association with a rate constant $2k_s$. Appendix 1 demonstrates that, for a Smoluchowski reaction, in which the amount of material which can aggregate is not constant, but varies with time (as f_{agg} does) the weight-average molecular weight of the aggregating material is

$$M_w(t) = M_0 \left\{ \frac{\int(\Sigma_i i^2 dn_i/dt)dt}{\int(\Sigma_i i dn_i/dt)dt} + \frac{2k_s}{\int(\Sigma i dn_i/dt)dt} \int \left\{ \int(\Sigma_i i dn_i/dt)dt \right\}^2 dt \right\} \quad (5)$$

where M_0 is the molecular weight of a monomer (i.e. an individual casein molecule) and i is the number of these monomers in the particles which are added to the reaction mixture at rates dn_i/dt.

The equations used by Payens [1,2] and Hyslop et al. [4] are a specific case of eq. (5), where there is only one species present (constant i) and where dn/dt is linear (Appendix 1).

Eq. (5) may be used to calculate the molecular weight for any aggregation reaction provided that dn_i/dt can be expressed as an analytical function or calculated directly. In the case under consideration, analytical solution is not possible for full Michaelis-Menten kinetics, followed by the probability function, so that digital means were employed.

Eq. (5) gives the time dependence of the molecular weight of aggregating material only. The full average molecular weight of the milk undergoing chymosin action is expressed by [1]:

$$\bar{M}_{total} = W_{GMP}\bar{M}_{GMP} + W_{micelles}\bar{M}_{micelles} + W_{agg}\bar{M}_{agg},$$

where the W factors represent the weight fractions of glycomacropeptide released, of micelles which cannot aggregate, and of micelles which have been sufficiently attacked to that aggregation is possible.

These calculations can be applied to the chymosin-induced flocculation of milk, and specimen calculations are shown in fig. 3. These calculations replicate several features of the chymosin treatment of milk. There is, during the lag time after addition of enzyme, a small decrease in average molecular weight, arising from the proteolysis of the κ-casein as found by Payens [2], and at the end of the lag stage there is a progressively increasing gradient, where the molecular weight increases rapidly. No unambiguous definition of "clotting time" can, however, be made by these calculations, which predict a continuous, rather than singular, process.

We may check the applicability of the calculation method by computing coagulation times for a variety of enzyme and substrate concentrations. For the calculations, it was necessary to define a criterion of 'clotting time' this was done by defining t_c as the time at which the weight-average molecular weight was 10, taking account of the electron-microscopic studies of Green et al. [5]. The calculated dependence of t_c against enzyme concentration is shown in fig. 4, where it is seen that, over a range of enzyme concentration, the plot of ln (t_c) against ln (E) is not completely linear, but has a slight curvature. Nevertheless, the curve illustrates that the approximate inverse relationship between t_c and E is obtained, in conformity with experiment. Experimental determinations of the exact value of proportionality between t_c and $1/E$ vary, with values from 0.99 to 0.77 being quoted [1]. The calculations are well within this range. Fig. 4 also shows that the inverse relationship can, however, by only approximate, since it varies with enzyme and substrate concentrations.

Similarly, the calculations also describe very well the variation in the coagulation time with the concentration of substrate as experimentally measured (fig. 2), showing that the calculation replicates the behaviour of the experimental system in terms of both enzyme and substrate concentrations.

It was pointed out earlier that the value of n (the number of κ-casein molecules contained in a casein micelle) is important in defining f_{agg} (eq. (3)). Detailed calculations were made of the effect of this, and

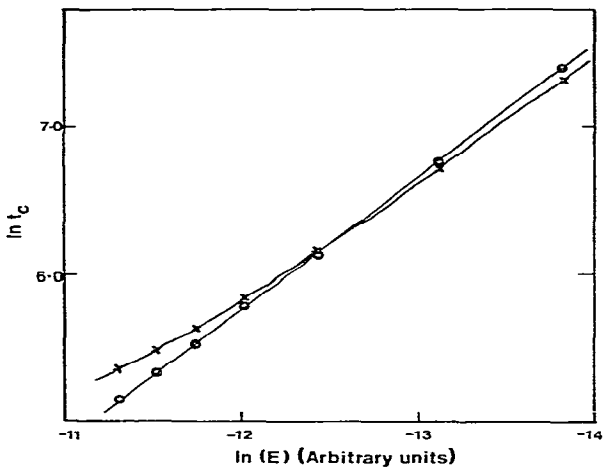

Fig. 4. Calculated coagulation times as functions of enzyme concentration, with $K_m = 4 \times 10^{-4}$ mol l^{-1} and $k_s = 3.7 \times 10^4$ l mole^{-1} s^{-1}, at selected values of V_{max} between 1.24×10^{-6} mole l^{-1} s^{-1} and 1.24×10^{-5} mole l^{-1} s^{-1}. Two milk concentrations are shown, namely natural milk with total casein concentration of 25 mg ml^{-1} (×) and 3× concentrated milk (o).

also of the effect of polydispersity. The results of calculation (fig. 3) show that the effect of both variations is small. This results from the probability function g_n (eq. (2)) being relatively insensitive to n unless n is small. As described above, the average micelle will contain some 200 molecules of κ-casein, and at this point the value of n is sufficiently large that changes in n do not affect the calculation greatly. If n is smaller, (as perhaps in so-called submicelles), the probability distribution of g_n is much broader, and aggregation will occur at a lower α. Since the largest micelles have the greatest effect on molecular weight growth (since we are dealing with a weight-average phenomenon), the aggregation of small micelles or submicelles has a relatively small effect both experimentally and in terms of the calculation of molecular weight growth and t_c.

4. Discussion

This method of calculation for the chymosin-induced aggregation of casein micelles is similar to that of Payens [1,2], but incorporates certain impor-

tant differences. The most fundamental of these are the use of full Michaelis-Menten kinetics, and the definition of aggregable material by invoking the probability functions. The conclusions which are deduced are, however, rather different from those in the earlier work [2]. The calculations described here allow quantitative relationships between clotting time and the concentrations of enzyme and substrate to be derived, by digital calculation, although it is not possible to provide a full analytical description. The calculations are in effect a generalisation of which the formulation given by Payens [1,2] is a special case, and lead to the same result, given the same assumptions. Detailed calculations (fig. 3) demonstrate that, although a part of the lag stage is produced by the slow aggregation of material, a major part of the delay before observable aggregation is caused by the time required for the enzyme to produce the extensive proteolysis of the κ-casein which is required to produce aggregable particles. The calculations are in good agreement with the experimental evidence, in terms of variation of enzyme and substrate concentration.

The requirement that over 90% of the κ-casein must be destroyed before a micelle may aggregate demonstrates the very high stabilizing ability of κ-casein, and it appears that a particularly critical factor is involved in the aggregation of the micelles. It has been suggested that coagulation of chymosin-treated micelles may arise from the reduction in the overall negative charge on the surface of the micelles by the removal of the glycomacropeptide [17], and also that the hydrophobic nature of the residual para-κ-casein will also contribute to the aggregation tendency [18]. Without detailed knowledge of the struture of the micelle, before and after chymosin action, it is difficult to define in detail what it is which promotes the sudden onset of coagulation. The use of Smoluchowski theory does not help in this respect, since it does not allow detailed definition of the flocculating particles. It does not appear however, that the aggregating units behave as polyfunctional entities, since in studies of partially-proteolysed micelles, aggregation appeared to be of the Smoluchowski type rather than that predicted for polyfunctional particles by Gordon et al. [19] and Parker and Dalgleish [20].

From measurements of the rate of reaction of micelles in experimental systems, and comparison with calculation a value of k_s of about 4×10^4 l mole^{-1} s^{-1} (in terms of micelles) is found at 30°C. This is slow compared with the possible rate, calculated on the basis of collision frequency, although somewhat higher than the estimate given by Payens [2]. This reflects the fact that there is still an inhibitory factor acting in micellar interactions, even after the destruction of their κ-casein.

The calculations outlined above may also be used for the coagulation of the isolated substrate, κ-casein, which produces aggregates of para-κ-casein on hydrolysis. Studies of this by Hyslop et al. [4] have measured the time-dependence of aggregation, and calculations using the method described in this paper (but omitting the probability function since $n = 1$) for pure κ-casein) can be used to describe the results (Appendix 1).

Potentially, this approach may be used for any enzymically-induced coagulation reaction, allowing for differences in the production of coagulable material. Thus the proteolysis and aggregation of κ-casein alone do not require the probability function. The mechanism of fibrin coagulation will also require definition before calculation can be achieved, but should also be susceptible to detailed analysis in this manner.

Appendix 1

The behaviour of a Smoluchowski aggregation, into which aggregatable material is added as a function of time

The basic Smoluchowski system, in which a given weight of material is aggregating, with all possible aggregations having a constant of $2k_s$, is described by a set of equation.

$$\frac{dv_x}{dt} = -2k_s v_x \sum_{i=1}^{\infty} v_i + k_s \sum_{i=1}^{x-1} v_i v_{x-i} , \qquad \text{(A1)}$$

where v_i are the concentrations of the i-mer species in the aggregation. From this, it is simple to show that

$$\nu_i = \nu_0(k_s\nu_0 t)^{i-1}/(1 + k_s\nu_0 t)^{i+1} \tag{A2}$$

and the weight-average molecular weight is given by

$$M_w(t) = (1 + 2k_s\nu_0 t)M_0 \,. \tag{A3}$$

The original formulation assumes that the starting "monomer" is monidisperse. If, however, the reaction starts with ν_{10} monomers, ν_{20} dimers ..... ν_{i0} i-mers, then solution of a set of equations as in (A1), but with initial values of the monomer and polymer concentrations inserted, can be performed.

Summation of all equations (A1) for all x gives

$$\left(\sum \nu_i\right)_t = \sum \nu_{i0} \Big/ \left(1 + \sum \nu_{i0} k_s t\right). \tag{A4}$$

For momomer flux

$$d\nu_1/dt = -2k_s\nu_1 \sum \nu_i = -2k_s\nu_1 \sum \nu_{i0} \Big/ \left(1 + k_s t \sum \nu_{i0}\right) \,.$$

Thus $\int_{\nu_{10}}^{\nu_1} \frac{d\nu_1}{\nu_1} = -2k_s \int_0^t \frac{\sum \nu_{i0}}{1 + k_s t \sum \nu_{i0}}\, dt$.

Therefore $\nu_1 = \nu_{10}/(1 + k_s t \sum \nu_{i0})^2$. (A5)

For dimers, $d\nu_2/dt = k_s\nu_1^2 - 2k_s\nu_2 \sum \nu_i$

and substitution for ν_1 and $\sum \nu_i$, followed by integration, gives

$$\nu_2 = \nu_{20}/(1 + k_s t \sum \nu_{i0})^2 + \nu_{10}^2 k_s t/(1 + k_s t \sum \nu_{i0})^3 \,. \tag{A6}$$

If for brevity, we write $x = (1 + k_s t \sum \nu_{i0})^{-1}$, then the calculation can be continued to give

$$\nu_3 = \nu_{30}x^2 + 2\nu_{10}\nu_{20}k_s t x^3 + \nu_{10}^3(k_s t)^2 x^4 \,, \tag{A7}$$

$$\nu_4 = \nu_{40}x^2 + (2\nu_{10}\nu_{30} + \nu_{20}^2)k_s t x^3 + 3\nu_{10}^2\nu_{20}(k_s t)^2 x^4 + \nu_{10}^4(k_s t)^3 x^5 \,. \tag{A8}$$

And so on, for all ν_i.

Number and weight-average molecular weights demand the knowledge of the sums $(\sum \nu_i)_t$, $(\sum i\nu_i)_t$ and $(\sum i^2\nu_i)_t$. The sum $\sum \nu_i$ has already been calculated (eq. (A4)). Since the total weight of material is constant,

$$\left(\sum i\nu_i\right)_t = \left(\sum i\nu_{i0}\right)_{t=0} .$$

The sum $\sum i^2\nu_i$ is calculated from eqs. (A5)–(A8) and continuing the summation over all i, which may be achieved by considering terms in x^2, x^3, x^4 .... .

$$S(x^2) = x^2(\nu_{10} + 4\nu_{20} + 9\nu_{30} + 16\nu_{40} + ...) = x^2 \sum i^2\nu_{i0} \,,$$

$$S(x^3) = x^3 k_s t(4\nu_{10}^2 + 18\nu_{10}\nu_{20} + 32\nu_{10}\nu_{30} + 16\nu_{20}^2 + 50\nu_{10}\nu_{40} ...) = 2x^3 k_s t \left(\sum i^2\nu_{i0}\right)\left(\sum \nu_{i0}\right) + \left(\sum i\nu_{i0}\right)^2 .$$

Similarly

$$S(x^4) = 3x^4(k_s t)^2\left(\sum i^2 v_{i0}\right)\left(\sum i v_{i0}\right)^2 + 2\left(\sum i v_{i0}\right)^2\left(\sum v_{i0}\right),$$
$$S(x^5) = 4x^5(k_s t)^3\left(\sum i^2 v_{i0}\right)\left(\sum i v_{i0}\right)^3 + 3\left(\sum i v_{i0}\right)^2\left(\sum v_{i0}\right)^2$$

or, in general

$$S(x^n) = (n-1)x^n(k_s t)^{n-2}\left(\sum i^2 v_{i0}\right)^{n-2} + (n-2)\left(\sum i v_{i0}\right)^2\left(\sum v_{i0}\right)^{n-3}.$$

The sum is then

$$\sum_{i=1}^{\infty} i^2 v_i = \sum_{n=2}^{\infty} S(x^n) = \sum_{j=1}^{\infty}\left\{ j\left(\sum i^2 v_{i0}\right)\left(\sum v_{i0}\right)^{(j-1)} + (j-1)\left(\sum i v_{i0}\right)^2\left(\sum v_{i0}\right)^{(j-2)} (k_s t)^{(j-1)} x^{(j+1)}\right\},$$

which reduces to, on summing over all j,

$$\sum i^2 v_i = \left(\sum i^2 v_{i0}\right) + 2k_s t\left(\sum i v_{i0}\right)^2. \tag{A9}$$

The weight-average molecular weight, where M_1 is the monomer molecular weight is:

$$M_w(t) = \frac{\Sigma M_1^2 i^2 v_i}{\Sigma M_1 i v_i} = M_1 \frac{\Sigma i^2 v_{i0}}{\Sigma i v_{i0}} + 2k_s t M_1 \sum i v_{i0} = M_w(0) + 2k_s t M_1 \sum i v_{i0}. \tag{A10}$$

Eq. (A10) is similar to (A3), with the summation of original particle weights replacing the monomer weight. However, (A9) allows a time-dependent function to be derived which allows calculation of molecular weights in a reaction in which material is added at any rate. For simplicity we assume that at $t = 0$, there is no aggregating material present.

$$\sum i^2 v_{i0} = 0, \quad \sum i v_i = 0.$$

Consider now adding material in increments dn_1, dn_2, dn_3, ... in successive time intervals dt.
After one time interval

$$\sum i^2 v_1 = dn_1, \quad \sum i v_i = dn_1.$$

After two intervals

$$\sum i^2 v_i = dn_1 + dn_2 + 2k_s dt (dn_1)^2, \quad \sum i v_i = dn_1 + dn_2.$$

After three intervals

$$\sum i^2 v_i = dn_1 + dn_2 + dn_3 + 2k_s dt\,(dn_1)^2 + 2k_s dt (dn_1 + dn_2)^2, \quad \sum i v_i = dv_1 + dv_2 + dv_3.$$

And after n intervals

$$\sum i^2 v_i = \sum_{i=1}^{n} dn_i + 2k_s dt\left\{(dn_1)^2 + (dn_1 + dn_2)^2 + (dn_1 + dn_2 + dn_3)^2 + \ldots + \left(\sum_{i=1}^{n-1} dn_i\right)^2\right\}, \quad \sum i v_i = \sum dn_i.$$

So that, if we replace summations by integrals as follows

$$M_w = M_0 \frac{\Sigma i^2 v_i}{\Sigma i v_i} = M_0 \left\{ 1 + \frac{2k_s dt \{(dn_1)^2 + (dn_1 + dn_2)^2 + \ldots + (\Sigma_{i=1}^{n-1} dn_i)^2\}}{\int (dn/dt)\, dt} \right\} .$$

The numerator of the fractional term is

$$2k_s dt \left\{ (dn_1)^2 + (dn_1 + dn_2)^2 + \ldots + \left(\sum_{i=1}^{n-1} dn_i \right)^2 \right\} = 2k_s \int \left\{ \int (dn/dt)\, dt \right\}^2 dt .$$

Thus

$$M_w = M_0 \left\{ 1 + \frac{\int \{\int (dn/dt)\, dt\}^2 dt}{\int (dn/dt)\, dt} \right\} . \quad \text{(A11)}$$

Exactly similar reasoning allows the determination of M_w when material is added in non-monomeric form. The expression for M_w becomes:

$$M_w = M_0 \left\{ \frac{\int (\Sigma i^2 dn_i/dt)\, dt}{\int (\Sigma i dn_i/dt)\, dt} + \frac{2k_s}{\int (\Sigma i dn_i/dt)\, dt} \int \left\{ \int \left(\sum i dn_i/dt \right) dt \right\}^2 dt \right\} \quad \text{(A12)}$$

where M_0 is the monomer molecular weight, and dn_i/dt is the rate at which particles of degree of polymerisation i are added to the aggregating mixture.

This reduces to eq. (A3) if the amount of material is constant, and can also be used to derive the special case where material (monomer) is added at constant rate V. The summations vanish since $i = 1$, and (A12) becomes

$$M_w = M_0 \left\{ \frac{Vt}{Vt} + \frac{2k_s}{Vt} \int V^2 t^2 dt \right\} = M_0 (1 + \tfrac{1}{3} k_s V t^2) .$$

This is the equation derived by Payens [1] and used also by Hyslop et al. [4], bearing in mind that the aggregation rate constant ($2k_s$) used in this work is twice that used in refs. [1], [2] and [4].

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