Biophysical Chemistry 6 (1977) 253–261

ON ENZYMATIC CLOTTING PROCESSES I. KINETICS OF ENZYME-TRIGGERED COAGULATION REACTIONS

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Received 20 July 1976

The kinetics of enzymatic clotting reactions such as the clotting of blood or milk, is analyzed. The appearance of a lag phase in the clotting is shown to be due to the difference in reaction order of enzymatic production and flocculation. The weight-average particle weight of the product formed is found to be a quadratic function of the reaction time. The condition for the clotting time is $t\sqrt{k_S V/2} = C$, where t is the clotting time, k_S the flocculation rate constant, V the maximum rate of enzymatic product formation and C a constant. In agreement with this result double-logarithmic plots of t versus enzyme dilution are always observed to be linear over a wide range of enzyme concentrations. No statistical evidence could be produced for the widely held belief that the clotting time should be inversely proportional to the enzyme concentration. Varying exponents of the latter in its relation to the clotting time are discussed in terms of von Smoluchowski's theory of the slow coagulation of colloidal particles.

1. Introduction

Enzymatic clotting processes are well known in pure and applied biochemistry. The clotting of blood [1] and the curdling of milk [2] are certainly the most spectacular examples, but also the conversion of ovalbumin into plakalbumin by subtilisin [3] and the formation of collagen fibers from procollagen by procollagen peptidase [4] fall into the same category of reactions.

Jollès recently [5] pointed out the close resemblance of the processes of blood and milk coagulation. In both instances the clotting is initiated by a limited, specific proteolysis of the substrate. Thus with blood clotting the final reaction consists in the conversion of fibrinogen into fibrin [1,5] through the splitting off of some low-molecular weight peptides by the enzyme thrombin (E.C. 3.4.21.5). In the case of milk the enzyme generally used is chymosin (E.C. 3.4.23.4), which cleaves a macropeptide with a molecular weight of about 6700 from κ-casein [5,6]. Thrombin and chymosin are proteases with a trypsin- and a pepsin-like activity respectively. They may successfully be replaced by less specific enzymes, such as ficin, papain or microbial proteases [1,2,5].

The clotting time of milk and blood is widely used for assay of the clotting enzyme [1,2,6,7]. The usefulness of the method rests on the observation that with both processes the clotting time, i.e. the lag period between the addition of the enzyme and the moment at which the clot becomes visible, is sometimes found to be roughly proportional to the reciprocal of the enzyme concentration. In the case of the clotting of fibrin, it is stated that over a limited range of thrombin concentrations

$$te^{\gamma} = C, \tag{1}$$

where t is clotting time, e enzyme concentration and γ and C are constants [1,7,8]. With the milk clotting reaction Segelcke and Storch [9] claimed more than a century ago that the exponent $\gamma = 1$, but numerous exceptions to this simple rule have been reported

[6,9]. On the basis of eq. (1) it is recommended [7,8] that the plot of log t versus log (e) is used in enzyme determinations. It is the aim of the present paper to investigate on what theoretical grounds eq. (1) is based, and to provide a qualitative picture of the exceptions to be expected.

The occurrence of a lag period in the clotting of blood and milk has given rise to much speculation about the existence of a primary (or enzymatic) and a secondary (or coagulation) stage in the clotting process. The underlying idea is that first the substrate must completely be modified before clotting can start. It has never been established unequivocally, however, that the two stages do proceed separately. Through the analysis given below, we shall demonstrate that actually both steps start simultaneously and that the lag period is merely the result of the different orders of the enzymatic and coagulation reactions. A preliminary account of this study has been presented before [10].

2. Kinetic theory

Consider the following sequence of reactions:

$$E + S \underset{k_1}{\overset{-k_1}{\rightleftharpoons}} ES \xrightarrow[k_3]{} E + P_1 + M \qquad (2)$$

and

$$i\,P_1 \xrightarrow[k_s]{} P_i \qquad (i = 2,3,\ldots.), \qquad (3)$$

where E stands for enzyme, S for substrate and P_1 and M for the clotting product and the peptide split off. The coagulation of P_1 is schematized in eq. (3). At this level of the theory we shall not discuss whether the coagulation of P_1 will lead to the formation of floccules, linear aggregates or a gel, but only mention that according to von Smoluchowski's early theory [11,12] coagulation reactions are bimolecular, the rate constant, k_s, being diffusion-controlled in the simplest case.

Also we shall not discuss whether the enzymatic production of P_1 is slowed down by exhaustion of the substrate or the presence of competitive inhibitors. The rationale for this neglect is that we are interested only in the initial stages of the clotting process, where substrate and inhibitor concentrations are assumed to be constant. With that restriction the various rate laws for the enzymatic production of P_1 reduce to [13]:

$$(dP_1/dt)_{\text{enzyme}} = k\,(e) = V, \qquad (4)$$

with k a constant [‡].

The total rate of change of the concentration of P_1 now becomes the sum of its enzymatic production and the decrease of particle number by the bimolecular flocculation reaction, therefore

$$dP_1/dt = V - k_s P_1 \sum_{j=1}^{\infty} P_j. \qquad (5)$$

The production of multiple, aggregated particles is completely described by von Smoluchowski's rate theory [11,12]:

$$\frac{dP_i}{dt} = \tfrac{1}{2} k_s \sum_{\substack{j=1 \\ k=i-j}}^{k=i-1} P_j P_k - k_s P_i \sum_{j=1}^{\infty} P_j, \qquad (6)$$

where we have tacitly introduced von Smoluchowski's approximation of a rate constant, k_s, independent of particle size [11,12]. Summation then yields for the rate of change of the total particle number

$$\frac{d\,\Sigma_j P_j}{dt} = V - \tfrac{1}{2} k_s \left(\sum_j P_j\right)^2. \qquad (7)$$

Integration of the latter equation, subject to the condition that $\Sigma_j P_j = 0$ at $t = 0$, yields for the total particle number:

$$\sum_j P_j = \sqrt{2V/k_s}\,\tanh\,(t/\tau), \qquad (8)$$

where τ is defined as

$$\tau = 1/\sqrt{k_s\,V/2}. \qquad (9)$$

The parameter τ will be called the enzymatic clotting time hereafter.

With the same condition of zero particle numbers at zero time, we find for the numbers P_1 to P_5 (see Appendix I):

[‡] In case the substrate concentration is much larger than the Michaelis–Menten constant K_m, $k = k_3$, otherwise $k = k_3(S)/(K_m + (S))$.

$$P_1 = \sqrt{2V/k_s}\,\{\tanh(t/\tau) + (t/\tau)/\cosh^2(t/\tau)\}/2, \tag{10}$$

$$P_2 = \sqrt{2V/k_s}\,[\tanh(t/\tau) - \{(t/\tau) - 2(t/\tau)^2 \tanh(t/\tau)\}/\cosh^2(t/\tau)]/8, \tag{11}$$

$$P_3 = \sqrt{2V/k_s}\,[\tanh(t/\tau) - \{(t/\tau) + 2(t/\tau)^3/3 - (t/\tau)^3 \tanh^2(t/\tau)\}/\cosh^2(t/\tau)]/16, \tag{12}$$

$$P_4 = \sqrt{2V/k_s}\,[5\tanh(t/\tau)/4 - \{5(t/\tau)/4 + \tfrac{1}{2}(t/\tau)^2 \tanh(t/\tau) + (t/\tau)^3/3 - (t/\tau)^3 \tanh^2(t/\tau) - 2(t/\tau)^4 \tanh^3(t/\tau) + 4(t/\tau)^4 \tanh(t/\tau)/3\}/\cosh^2(t/\tau)]/32, \tag{13}$$

and

$$P_5 = \sqrt{2V/k_s}\,[7\tanh(t/\tau)/4 - \{7(t/\tau)/4 + (t/\tau)^2 \tanh(t/\tau) + (t/\tau)^3/6 - (t/\tau)^3 \tanh^2(t/\tau)/2 - 2(t/\tau)^4 \tanh^3(t/\tau) + 4(t/\tau)^4 \tanh(t/\tau)/3 - 4(t/\tau)^5/15 + 2(t/\tau)^5 \tanh^2(t/\tau) - 2(t/\tau)^5 \tanh^4(t/\tau)\}/\cosh^2(t/\tau)]/64. \tag{14}$$

The relative particle numbers $\Sigma_j P_j/\sqrt{2V/k_s}$ and $P_j/\sqrt{2V/k_s}$ are plotted in fig. 1 as a function of the dimensionless ratio t/τ.

Since we are likewise interested in the turbidity or viscosity changes brought about by the clotting enzyme, we have also investigated the time dependence of the weight-average molecular weight of the clotting product:

$$\bar{M}_w = M_1 \sum_j j^2 P_j \Big/ \sum_j j P_j, \tag{15}$$

where M_1 is the molecular weight of the species P_1. Since the computation of P_j becomes increasingly laborious with increasing degree of flocculation, and since it is not known beforehand how many P_j-terms are needed for a representative summation by eq. (15),

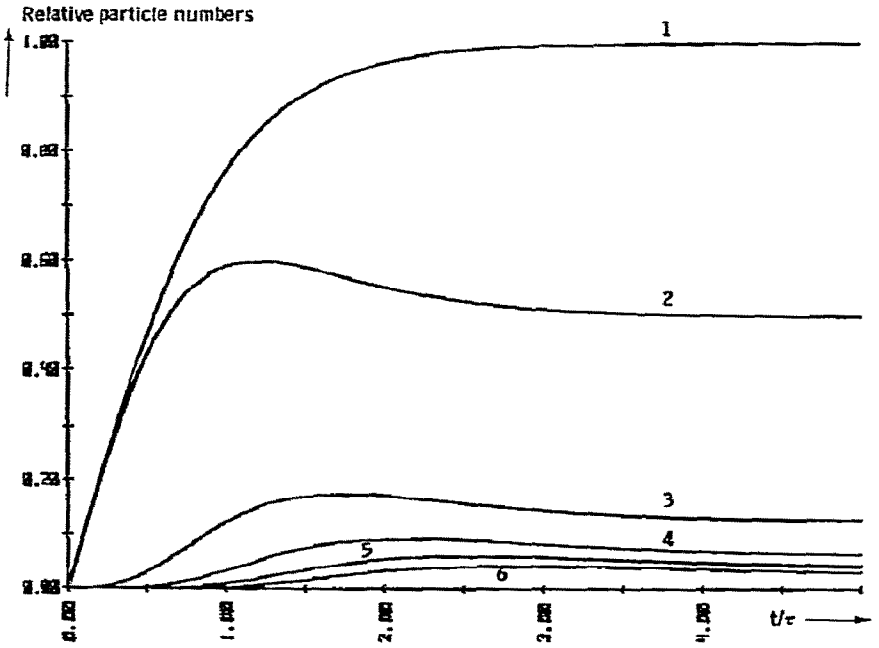

Fig. 1. Showing relative particle numbers as a function of time. (1) $\Sigma_j P_j/\sqrt{2V/k_s}$; (2) $P_1/\sqrt{2V/k_s}$; (3) $P_2/\sqrt{2V/k_s}$; (4) $P_3/\sqrt{2V/k_s}$; (5) $P_4/\sqrt{2V/k_s}$; (6) $P_5/\sqrt{2V/k_s}$. Calculations performed on the Hewlett-Packard 9830 A calculator, equipped with the 9862 A calculator plotter.

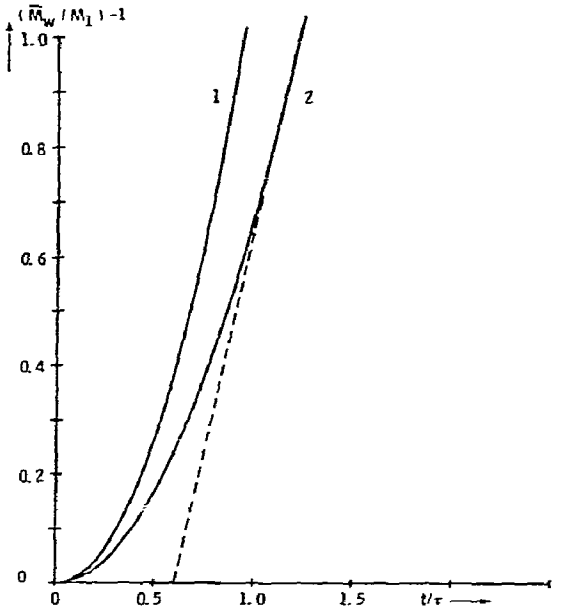

Fig. 2. Time-dependence of the reduced weight-average particle weight, $(\bar{M}_w/M_1)-1$, plotted against t/τ. (1) The incomplete result obtained by summation of the first 5 terms in $\Sigma_j j^2 P_j/\Sigma_j j P_j$. (2) According to the complete expression (16). Dashed line: extrapolation according to T. Sanner and G. Kovács-Proszt (ref. [16]). Calculations performed on the Hewlett-Packard 9830 A calculator, equipped with the 9862A calculator plotter.

an exact expression for $\bar{M}_w$ was derived by means of a generating function. It is shown in Appendix II that this leads to

$$\bar{M}_w = M_1\{1 + \tfrac{2}{3}(t/\tau)^2\}. \qquad (16)$$

A graphical representation of the reduced weight-average molecular weight, $\bar{M}_w/M_1$ is given in fig. 2.

3. Discussion

The most interesting result of the present theory is the appearance of a lag period in the formation of multiple particles and in the concomittant increase of the weight-average particle weight (cf. figs. 1 and 2). Such lag periods have been observed a great many times [1,2,8,9] and are in striking contrast with the familiar kinetics of the flocculation of colloidal particles, which shows a linear increase of the average particle weight [11,12]. The lag period arises as a consequence of the second order of the flocculation reaction (cf. eq. (7)), which will not get going as long as the concentrations of the flocculating species, P_j, remain low. Indeed, the concentrations of the latter are triggered by the enzymatic, zeroth order production of P_1. By expansion of eq. (8) we find for the total particle number:

$$\sum_j P_j = Vt - k_s V^2 t^3/6 + k_s^2 V^3 t^5/30 - \ldots, \qquad (17)$$

which shows that initially the total particle number is only determined by the enzymatic production of P_1 and not influenced by flocculation. The same conclusion is reached when inspecting the plots of the multiple particles P_2 to P_5 versus time in fig. 1.

Due to the counteracting effects of the enzymatic production of P_1 and the decrease of the total particle number through flocculations $\Sigma_j P_j$ becomes stationary at, say, $t/\tau \geqslant 3$:

$$\lim_{t/\tau \geqslant 3} \sum_j P_j = \sqrt{2V/k_s}. \qquad (18)$$

All particle numbers can be related to this limiting total particle number. The significance of the enzymatic clotting time, τ, can now be visualized as the time needed for the enzyme to produce this limiting particle number in the absence of any flocculation.

In the example of fig. 2 we have chosen to explore the fate of the weight-average molecular weight. Indeed, this is the ultimate physical quantity determining the light scattering and turbidity, and, with proper restrictions [14] also the intrinsic viscosity of the system. These quantities have indeed been used mostly to monitor the lag phase in the clotting of blood or milk [15–18].

Strictly speaking it is the weight-average of the whole solute that is relevant in these matters and the disappearance of the substrate and the appearance of the peptides split off should therefore also be considered. It is clear, however, that as long as the molecular weight of the latter is much smaller than that of the substrate, this correction can safely be omitted. With fibrinogen as a substrate this may largely be the case [5,19]. With micellar or κ-casein, however, the macropeptide split off has the considerable molecular weight of about 6700 [5,20]. The cleavage may lead

in these cases to a small, but distinct decrease of the weight-average molecular weight in the beginning of the clotting process. The appropriate correction will be dealt with in the next paper of this series.

As mentioned in the Introduction, the determination of the clotting time is widely used for the assay of the clotting enzyme. To this end Sanner and Kovács-Proszt [16] extrapolated the seemingly linear part of the absorbance increase in stopped-flow experiments as indicated in fig. 2. The present analysis (cf. Appendix II) demonstrates, however, that the absorbance is not a linear function of the reaction time, and that consequently a linear extrapolation might introduce a considerable error in the determination of the clotting time. If the clotting time is found by visual inspection of the samples turbidity or by a rheological technique, one actually measured the (t/τ)-value corresponding to a particular molecular weight. It is obvious from fig. 2, eq. (16) and the definition of τ given in eq. (9) that the condition for the clotting time thus is:

$$t\sqrt{k_s V/2} = C, \qquad (19)$$

with C a constant depending on the practice chosen to determine t.

The importance of the relation (19) lies in the observation that it clearly indicates that the clotting time is determined by the flocculation rate constant, k_s, to the same extent as by the enzymatic velocity. Clotting time measurements therefore do not appear an unambiguous method for the assay of the clotting enzyme as long as the constancy of k_s is not guaranteed. For enzyme determinations the release of the peptide split off or the use of synthetic substrates [21–23] are therefore to be preferred. On the other hand it is seen that under carefully controlled enzymatic conditions the measurement of the clotting time may yield useful information about the flocculation rate constant k_s. Examples of such application will be given in subsequent papers of this series.

Since V is proportional to enzyme concentration, the relationship (19) predicts that the plot of $\log(t)$ versus $\log(e)^{-1}$ should be linear with a slope 0.5. Indeed, an investigation of the numerous data obtained by different authors with the clotting of blood (or fibrinogen) and of milk (or casein) reveals linear double-logarithmic regression lines over a wide range of enzyme concentrations. Two typical examples are shown in fig. 3. The expectation of the slope of such plots being 0.5 is, however, seldom fulfilled as the statistical analysis of the results of a good number of authors in table I demonstrates. Only Fischer [25], studying the clotting of blood plasma by chicken embryonic extract, has observed that the clotting time was roughly proportional to $1/\sqrt{(e)}$. The exceptions found by him, were ascribed to association-dissociation reactions of accompanying heparin, which is a notorious blood anticoagulant.

Table I further shows, that there exists no statistical evidence that the clotting time should be inversely proportional to the enzyme concentration proper, as has often been suggested [2,15–17].

Fischer's experiments [25] do indeed prove that the presence of varying inhibitor-concentrations may interfere with the establishment of the correct dependence of the clotting time on the enzyme concentration. In experiments, in which the enzyme concentration is varied by simple dilution, care should therefore be taken to keep the concentration of accompanying inhibitors (or activators) constant.

In general, however, there is no reason to question the validity of eq. (4), and most of the deviating slopes in table I must therefore be explained by a flocculation rate constant increasing with the concentration of the enzyme. A likely explanation, which will be explored further in the next paper, is that not every collision between P_f-particles leads to permanent contact, but only those encounters, in which the sites of attack of the enzyme are involved. In the case of fibrin there are at least 4 of such sites [19]; with micellar or κ-casein this number is much higher due to the strong polymerization of the caseins [30,31]. Clotting, however, is known to start already before the maximal number of peptides is split off by the enzyme [19]. The fraction of sites available for permanent contact and the flocculation rate constant will therefore increase with the concentration of the clotting enzyme. To explain the values of the enzyme-exponents actually observed, would require a careful study of the experimental conditions, under which the clotting was studied and of the structure of the floccules formed in each separate case. This will be attempted in the next papers on this subject.

It should be recalled that the above equations have been developed for the case that the enzymatic production of P_1 is constant. If exhaustion of the sub-

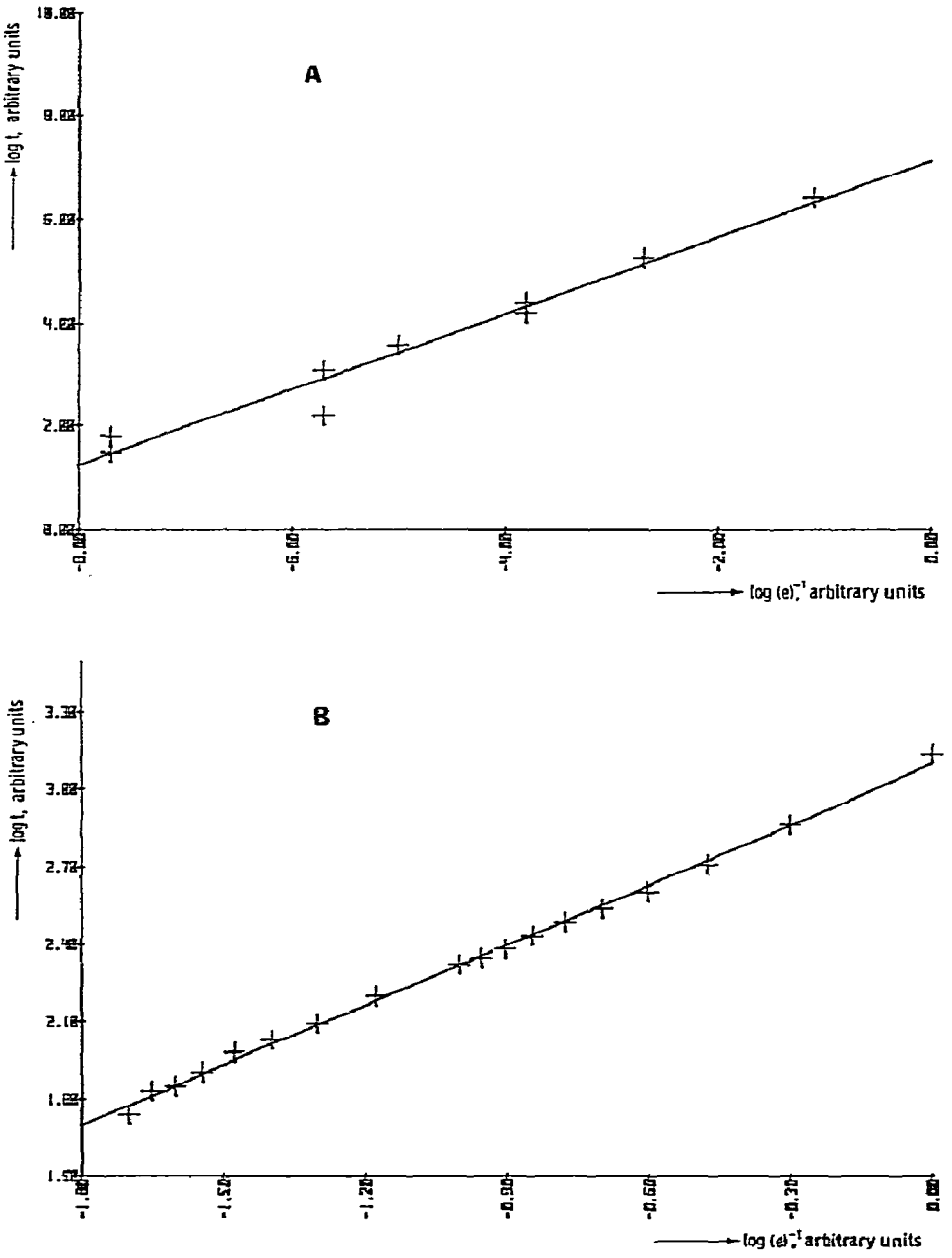

Fig. 3. Typical examples of the regression of log clotting time on log enzyme dilution, (A) Data collected by L.B. Jacques [27] with the clotting of citrated horse plasma by thrombin. (B) Data collected by W. Grimmer and M. Krüger [28] with the clotting of milk by rennet. Data digested according to J.H. Nairn, Plotter Pack Program for Polynomial Regression, Hewlett-Packard Co., ed., Loveland, Colorado.

Table 1
Double-logarithmic regression of clotting time versus enzyme dilution observed with the clotting of blood (or fibrinogen) and milk (or casein)

Substrate	Enzyme prep.	Slope	R^{2} a)	Number of-exps.	Ref.
blood plasma	lung tissue extract	0.29	0.995	18	24
blood plasma	chicken embryon. extract	0.41	–	–	25
blood plasma	chicken embryon. extract	0.50	–	–	25
blood plasma	chicken embryon. extract	0.68	–	–	25
blood plasma	thromboplastin	1.08	0.999	4	26
citrated horse plasma	thrombin	0.73	0.967	9	27
human fibrinogen	human thrombin	1.0	–	–	15
milk	calf rennet	0.77	0.998	18	28
milk	calf rennet	0.95	0.999	16	17
micellar casein	calf rennet	0.99	0.976	5	b)
κ-casein	calf chymosin	0.87	0.987	4	29
κ-casein	M. Pusillus protease	0.83	0.992	4	29

a) Data digested according to J.H. Nairn, Plotter Pack Program for Polynomial Regression, Hewlett-Packard Co., ed., Loveland, Colorado.
b) Unpublished results from this laboratory.

strate would begin to occur, some modification of the theory is necessary to account for the decreasing production. Since, however, the lag phase coincides with the very beginning of the enzymatic reaction, it is clear that exhaustion cannot severely affect the conclusions given above.

Finally we note that the model proposed could find wider application in colloid chemistry. It could, for instance, also be used to describe the kinetics of the interfacial flocculation of adsorbed particles, the surface concentration of which is triggered by a slow adsorption step.

Appendix I Solving the rate equations for individual particle numbers

Substitution of the expression (8) for $\Sigma_j P_j$ in eq. (5) yields

$$dP_1/dt = V - A(t)\,P_1, \tag{A.1}$$

where $A(t)$ is defined as

$$A(t) = (2/\tau)\tanh(t/\tau). \tag{A.2}$$

The solution to eq. (A.1) is

$$P_1 = e^{-\int A(t)dt}\left(C + \int V e^{+\int A(t)dt}\,dt\right),$$

which after introducing the proper boundary conditions, reduces to

$$P_1 = V e^{-\int A(t)dt}\int e^{+\int A(t)dt}\,dt. \tag{A.3}$$

After working out the exponentials in this expression and integrating one finds P_1 as given in eq. (10).

With the expressions (8) and (10) for $\Sigma_j P_j$ and P_1, one has for the rate of change of double particles:

$$dP_2/dt = \tfrac{1}{2}k_s P_1^2 - k_s P_2 \sum_j P_j$$

$$= B_2(t) - A(t)\cdot P_2, \tag{A.4}$$

with

$$B_2(t) = \tfrac{1}{2}k_s\left(V e^{-\int A(t)dt}\int e^{+\int A(t)dt}\,dt\right). \tag{A.5}$$

The solution to (A.4) is

$$P_2 = e^{-\int A(t)dt}\int B_2(t) e^{+\int A(t)dt}\,dt, \tag{A.6}$$

which after working out the exponentials and integration yields eq. (11).

It is easily verified that since

$$dP_3/dt = k_s P_1 P_2 - k_s P_3 \sum_j P_j,$$

that

$$B_3(t) = k_s V e^{-2\int A(t)dt} \times \int B_2(t) e^{+\int A(t)dt} dt \int e^{+\int A(t)dt} dt, \qquad (A.7)$$

and that therefore the solution of P_3 – as that of particles of higher index – proceeds along the same line as with P_2. We have not succeeded in finding a generalized expression for P_i with the particle numbers integrated thus far.

Appendix II The time-dependence of the weight-average particle weight

The general expression for the rate of change of the number of i-fold particles is

$$dP_i/dx = -\sqrt{2k_s/V}\, P_i \sum_{j=1}^{\infty} P_j + \sqrt{k_s/2V} \sum_{j=1}^{i-1} P_j P_{i-j}, \qquad (A.8)$$

where $x = t/\tau$. Introducing $\Sigma_j P_j$ from eq. (8), eq. (A.8) becomes

$$dP_i/dx = -2P_i \tanh x + \sqrt{k_s/2V} \sum_{j=1}^{i-1} P_j P_{i-j} \qquad (i = 2,3,\ldots). \qquad (A.9)$$

The general solution of (A.9), subject to the condition that $P_i = 0$ at $x = 0$, is

$$P_i = \sqrt{k_s/2V} \int_0^x \cosh^2(s) \sum_{j=1}^{i-1} P_j P_{i-j}\, ds/\cosh^2 x \qquad (i = 2,3,\ldots). \qquad (A.10)$$

Consider the following generating function:

$$\psi(z,x) \equiv \sum_{i=1}^{\infty} z^i P_i(x),$$

which after introducing P_i from (A.10) and changing the sequence of summation and integration, becomes

$$\psi(z,x) = zP_1(x) + \sqrt{k_s/2V} \int_0^x \cosh^2(s) \left\{ \sum_{i=2}^{\infty} \sum_{j=1}^{i-1} P_j P_{i-j} z^i \right\} ds/\cosh^2(x). \qquad (A.11)$$

It is easily verified that the double sum in this expression equals ψ^2 and that therefore

$$\psi(z,x) = zP_1(x) + \sqrt{k_s/2V} \int_0^x \cosh^2(s)\, \psi^2(z,s)\, ds/\cosh^2(x). \qquad (A.12)$$

It follows from the definition of $\psi(z,x)$ given above that

$$\sum_{j=1}^{\infty} j^2 P_j = \sum_{j=1}^{\infty} j P_j + \left.\frac{\partial^2 \psi(z,x)}{\partial z^2}\right|_{z=1}. \qquad (A.13)$$

The first term on the right-hand side of this equation is the total number of subunits produced by the enzyme, $\sqrt{2V/k_s}\, x$. The second term equals $\frac{2}{3}\sqrt{2V/k_s}\, x^3$. This can be proved as follows. By straightforward differentiation of (A.12) we have:

$$\left.\frac{\partial^2 \psi(z,x)}{\partial z^2}\right|_{z=1} = \sqrt{k_s/2V} \int_0^x \cosh^2(s) \left\{ 2\left(\left.\frac{\partial \psi}{\partial z}\right|_{z=1}\right)^2 + 2\psi\Big|_{z=1} \left(\left.\frac{\partial^2 \psi}{\partial z^2}\right|_{z=1}\right) \right\} ds/\cosh^2 x. \qquad (A.14)$$

Since $\psi|_{z=1} = \Sigma_j P_j$ and $\partial\psi/\partial z|_{z=1} = \Sigma_j j P_j$, it follows from (A.14) that the function $h(x)$ defined as

$$h(x) \equiv \cosh^2(x) \left.\frac{\partial^2 \psi(z,x)}{\partial z^2}\right|_{z=1} = 2\sqrt{2V/k_s} \int_0^x s^2 \cosh^2(s)\, ds + 2\int_0^x \tanh(s)\, h(s)\, ds, \quad (A.15)$$

and that

$$dh(x)/dx = 2\sqrt{2V/k_s}\, x^2 \cosh^2(x) + 2 \tanh(x)\, h(x). \quad (A.16)$$

The solution to (A.16) is

$$h(x) = \tfrac{2}{3}\sqrt{2V/k_s}\, x^3 \cosh^2(x), \quad (A.17)$$

which shows that indeed

$$\left.\frac{\partial^2 \psi(z,x)}{\partial z^2}\right|_{z=1} = \tfrac{2}{3}\sqrt{2V/k_s}\, x^3. \quad (A.18)$$

Inserting this result into (A.13) and dividing both sides by $\Sigma_j j P_j$ finally yields

$$\sum_j j^2 P_j \Big/ \sum_j j P_j = 1 + \tfrac{2}{3} x^2. \quad (A.19)$$

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