  Bull. Chem. Soc. Jpn., 56, 1351—1356 (1983)

The Precipitation of Human Fibrinogen by Zinc Chloride

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(Received December 8, 1982)

The precipitation of human fibrinogen by $ZnCl_2$ was studied by measuring the turbidity and the protein concentration in the supernatant (referred as solubility). Different kinetic patterns, either fast or slow, were observed in both solubility and turbidity depending on whether $ZnCl_2$ concentration (C_M) was larger or smaller than the critical concentration. In the slow pattern, the increase of the turbidity and the decrease of the solubility both occurred slowly and almost linearly with time. In the fast pattern, these changes occurred rapidly and stationary values were reached within 30 min for the turbidity and 2 h for the solubility. These two stationary values were found to be linearly related to each other. The stationary solubility decreased as C_M increased, while it increased with the total amount of the protein. The dissolution of the precipitates was very slow resulting in a practically irreversible precipitation. A gelation model was proposed to account for these characteristic features, according to which fibrinogen molecules were linked to each other through the 'bond' mediated by a zinc atom.

The interaction of zinc ions with human plasma proteins was studied by Cohn *et al.* in relation to their system for the separation of the components of human blood.[1] Complete flocculation of human γ-globulin occurred under the presence of $ZnSO_4$.[2] The binding constant of zinc ions to human serum albumin was shown to be close to that to imidazole.[3] Recently, gelation of bovine fibrinogen was found when various metal ions including Zn^{2+} were added.[4]

Recently, we have also found that the precipitation of human fibrinogen occurs on addition of various divalent metal chlorides.[5] Among them $ZnCl_2$ and $CuCl_2$ were most effective. The interaction of fibrinogen with $ZnCl_2$ or $CuCl_2$ was studied by circular dichroism and fluorescence. However, the mechanism of the precipitation was not clear at all.

In the present study, the precipitation of human fibrinogen by $ZnCl_2$ was examined at a fixed pH of 7.4 in 0.15 M NaCl (1 M=1 mol dm^{-3}), by the measurement of the solubility and by the kinetics of precipitation. The interaction of $CuCl_2$ with tris(hydroxymethyl)aminomethane (Tris) buffer, found previously,[5] prevents us from the measurement of the solubility in the $CuCl_2$–fibrinogen system. Consequently, the present study was carried out on the $ZnCl_2$–fibrinogen system.

The results on the solubility and the kinetics of precipitation obtained in the present study were rather unusual from the standpoint of a phase equilibrium or a coagulation of hydrophobic colloids. Therefore, a considerable amount of efforts is made in the present study to find a reasonable and simple model for a possible mechanism of the present precipitation.

Experimental

Fibrinogen (Kabi, grade L) was dissolved into 0.15 M NaCl and dialyzed against 0.15 M NaCl for 24—36 h and then freeze-dried. Tris and $ZnCl_2$ were purchased from Nakarai chemicals Co. Ltd. (Kyoto). The freeze-dried material was dissolved into the solvent [Tris–HCl 1.0×10^{-2} M (pH 7.4)+NaCl 0.15 M]. Final NaCl concentrations were not larger than 0.17 M even at the highest protein concentration. The protein concentration (g dm^{-3}) was calculated from the absorbance at 280 nm for 1 cm light path, Abs, as Abs/1.60. $ZnCl_2$ concentration (C_M) was expressed in M (mol dm^{-3}).

The protein concentration of the solution phase under phase separation was determined as follows. A given amount (0.01—0.1 cm^3) of $ZnCl_2$ solution was added to a protein solution (4 cm^3, 0.22—0.23 g dm^{-3}) in a glass tube. After a planned incubation period, the glass tube was put into a rotor and centrifuged at ca 1000 g for 20 min. Temperature was kept at 22±2 °C. Then, the absorbance of the supernatant was measured and the concentration was evaluated. The value was assigned to the time when the centrifugation was *started*. Identical results were obtained under different conditions: 500 g for 20 min or 1000 g for 50 min.

In the kinetics of the precipitation, the change of the turbidity was recorded automatically after the complete mixing was established within 1 or 2 s. The details of the mixing procedure will be described in the accompanying paper.[6]

Absorption spectra and turbidity were measured with a Shimadzu UV-200 S spectrophotometer using a cell of 1 cm light path.

Results

The Protein Concentration in Solution Phase. The protein concentration in the solution phase was measured by absorption at 280 nm after centrifugation at 1000 *g* for 20 min. Throughout the present study, the supernatant concentration is conventionally called as 'solubility', though it does not represent a solubility as defined by the phase rule as shown later. The total protein concentration, was 0.22—0.23 g dm^{-3}. The time course of the protein concentration in the solution phase was followed by applying the above procedure at different times after the addition of $ZnCl_2$. Two distinct patterns were found as shown in Fig. 1 depending on the $ZnCl_2$ concentration C_M. At $C_M=1.9\times10^{-5}$ M or lower, the protein concentration decreased slowly with time. This behavior is typical to the slow coagulation phase of hydrophobic colloids. At $C_M=4.5\times10^{-5}$ M or higher, the protein concentration decreased rapidly and reached a stationary value after about 2 h. This behavior also resembles the fast coagulation of hydrophobic colloids. However, the stationary concentrations were different for different 'coagulant' concentration C_M, as shown in Fig. 1, which cannot be properly explained based on the picture of the coagulation of hydrophobic colloids.

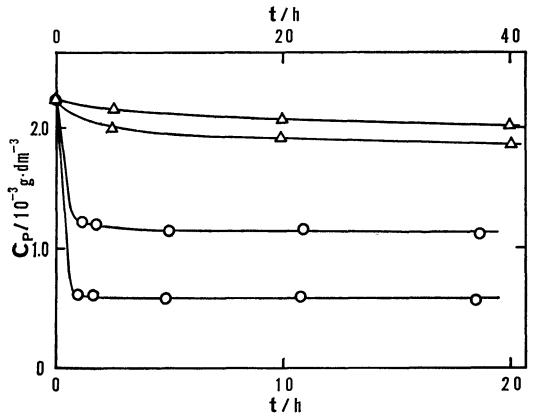

Fig. 1. Time course of the solubility.
$ZnCl_2$ concentrations(mol dm^{-3}) (from top to bottom): 9.02×10^{-6}, 1.80×10^{-5}, 4.49×10^{-5}, and 1.00×10^{-4}. Triangles refer to the upside abscissa.

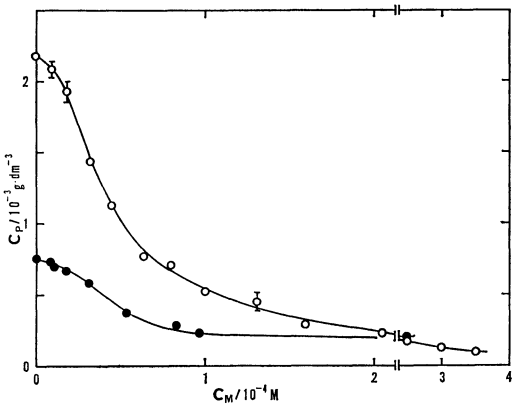

Fig. 2. Dependence of the stationary solubilities on $ZnCl_2$ concentration C_M.
The total protein concentrations (g dm^{-3}): 0.22 (○) and 0.077 (●).

The results in Fig. 1 suggest the presence of a kind of critical concentration in the range $(2—4)\times10^{-5}$ M. The rate of the concentration change of fibrinogen differed markedly depending on whether C_M was larger or smaller than the critical concentration.

In Fig. 2, the stationary values of the 'solubility' are plotted as functions of $ZnCl_2$ concentration C_M for two different total protein concentrations. The values at about 20 h were taken in place of the stationary values in the case of the low total protein concentration, at which the time course of the 'solubility' was not examined. The same approximate procedure was used for small values of C_M at which the protein concentration decreased slowly without exhibiting a stationary state. There are two characteristic features about the present precipitation as clearly shown in Fig. 2. The stationary values depend on $ZnCl_2$ concentration, as already noticed in Fig. 1. This dependence suggests that the chemical potential of $ZnCl_2$ plays an important role in the precipitation. It is likely, therefore, that the interaction of fibrinogen with Zn^{2+} ions leading to the precipitation is rather reversible. The other feature is that the stationary protein concentration depends not only on C_M but

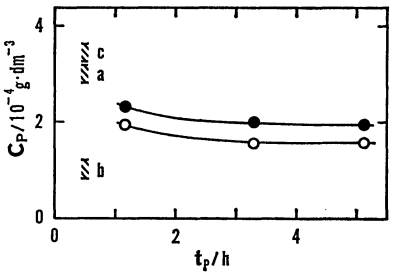

Fig. 3. Dependence of the solubility after dilution on the incubation time for precipitation t_p.
Measuring time of the solubility after dilution: 8 (○) and 20 (●) h. About three levels a, b, and c, see text.

also on the total amount of the protein. A simple phase equilibrium is hence ruled out, while phase equilibria of a multicomponent system can account for this dependence. In a study of bovine fibrinogen, the protein did not behave as a single component as defined by the phase rule.[7]

Irreversible Nature of the Precipitation. The following two kinds of experiments were carried out to examine the reversibility of the precipitation of fibrinogen by the addition of $ZnCl_2$.

Experiment A: A solution of $ZnCl_2$ was added to a protein solution (4 cm^3, 0.22 g dm^{-3}) to give a metal concentration of 1.70×10^{-4} M. Identical solutions were prepared in multiplicate. About one hour after the addition of $ZnCl_2$, one of the members was subject to centrifugation and the 'solubility' was determined as 0.030 g dm^{-3} (level a in Fig. 3), which was consistent with that given in Fig. 2. The other members of the solutions, which were not centrifuged, were diluted with 8 cm^3 of the solvent (containing no $ZnCl_2$) at the time about 70 or 200 or 310 min after the addition of $ZnCl_2$.

The 'solubilities' of these diluted solutions were measured at about 8 and 20 h after the dilution was made. If the precipitation is reversible, the expected 'solubility' can be read from the 'solubility curve' in Fig. 2 corresponding to the low total protein concentration (defined by filled circles) at $C_M=5.66\times10^{-5}$ M, which is about 0.035 g dm^{-3} (level c in Fig. 3). At about 8 h after the dilution (open circles), the 'solubility' was significantly larger than what would be expected for the completely irreversible situation (level b in Fig. 3). Undoubtedly, the precipitates can dissolve, even though slowly. The results as well as these expected values for the completely reversible and irreversible situations are indicated in Fig. 3. At about 20 h after the dilution (filled circles), the 'solubility' increased appreciably from that found at 8 h after the dilution (open circles). The increment of the 'solubility' was nearly independent of the *incubation time for the precipitation*. However, if compared at the same *incubation time for the dissolution*, the 'solubility' is larger if the *incubation time for the precipitation* is shorter. The dependence on the *incubation time for the precipitation* disappeared when it was longer than about 3 h. This dependence is closely related to the time course of the 'solubility' shown in Fig. 1.

In Fig. 3, the 'solubility' never reaches the value expected for the reversible situation (level c) even at 20 h after the dilution. Consequently, the present precipitation is rather irreversible in the sense that the rate of dissolution is very slow.

Experiment B: A small volume of $ZnCl_2$ solution was added to a protein solution (4 cm^3) of a concentration of 0.22 g dm^{-3} to make the metal concentration C_M to be 1.29×10^{-4} M. The 'solubility' was measured at about 5 h after the addition of $ZnCl_2$ to confirm the stationary value. The measured 'solubility' was 0.039 g dm^{-3}, consistent with the results in Fig. 2. Then 2 cm^3 of a protein solution (0.22 g dm^{-3}) containing no $ZnCl_2$ was added and kept for 8 and 20 h. The final metal concentration was the two thirds of the initial value, *i.e.*, 8.57×10^{-5} M. The 'solubilities' measured at 8 and 20 hours were nearly the same and 0.056 g dm^{-3}.

Consider the situation immediately after the above mixing. The protein concentration in the solution phase is 0.1 g dm^{-3} $(0.039\times2/3+0.22\times1/3)$. The 'solubility' expected at $C_M=1.29\times10^{-4}$ M for this total protein concentration is between the two 'solubility' curves in Fig. 2. If the solubility is assumed to be proportional to the total protein concentration, then the 'solubility' is 0.031 g dm^{-3}. On the other hand, the 'solubility' expected for a completely reversible situation is 0.063 g dm^{-3}: the 'solubility' at $C_M=8.57\times10^{-5}$ M for the total protein concentration of 0.22 g cm^{-3}. Since the observed 'solubility' (0.056 g dm^{-3}) was close to 0.063 g dm^{-3} rather than 0.031 g dm^{-3}, it is likely that the precipitation of newly added protein occurred under the control of the amount of the precipitates present in advance to the mixing.

In this experiment, the amount of the precipitates increased after mixing the two solutions. The time dependence of the 'solubility' was not found, therefore, between 8 and 20 h, in contrast to the case shown in Fig. 3, where the dissolution of the precipitates was followed.

A conclusion derived from Experiment A is an extremely slow rate of dissolution of the precipitates. On the other hand, Experiment B suggests that the 'solubility' is controlled by the amount of the precipitates, indicating a microscopic reversibility between the precipitates and the solution phase. The idea of the phase equilibria of a multicomponent system cannot reconcile one of these two results with the other.

Kinetics of Precipitation. The time courses of the turbidity are shown in Figs. 4(A) and (B) for the protein concentration of 0.22—0.23 g dm^{-3}: the same concentration as used in the study of solubility. At $C_M=2.97\times10^{-5}$ M, the turbidity increased linearly but only slightly. However, at $C_M=3.95\times10^{-5}$ M, a sigmoidal increase began to appear. Typical sigmoidal patterns were obtained at $C_M=4.92\times10^{-5}$ M[Fig. 4 (A)], 5.88 and 6.84×10^{-5} M[Fig. 4(B)]. At $C_M\approx(9.7—9.8)\times10^{-4}$ M, a sigmoidal shape could not be recognized any more, probably because the lag time was too short to be detected in the time scale of the present experiment.

The present kinetics can be summarized in the following two points. First, there is a critical $ZnCl_2$ concentration C_M*, below which the turbidity increases linearly but very slowly (slow phase) and above which it increases rapidly in a sigmoidal way (fast phase). The critical concentration was $(3—4)\times10^{-5}$ M for the protein concentration of 0.23 g dm^{-3}. In the second place, the turbidity reaches a stationary value after 25—30 min in the present kinetics as shown by horizontal bars in Fig. 4(A) if $C_M\geq C_M$*. The similar kinetic behavior was recently observed in the gelation of bovine fibrinogen by metal ions, where a stationary turbidity was reached after 2—3 min.[4] This kind of behavior has been rarely observed in the studies on the coagulation of colloids. Since the details of the kinetics of the present precipitation will be described in the following paper,[6] it is sufficient in the present study to point out the close relationship between the 'solubility' and the turbidity as shown below.

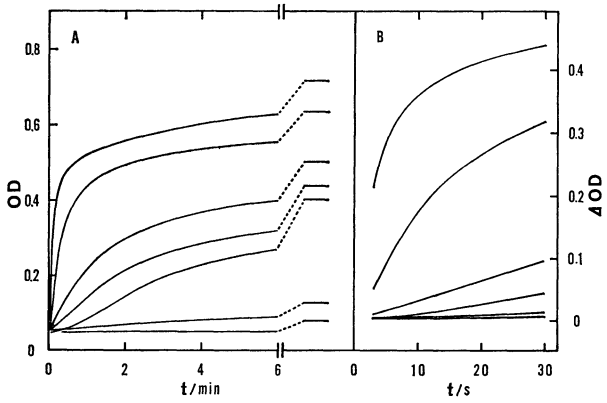

Fig. 4. Time courses of the turbidity at 350 nm. Protein concentration: 0.22—0.23 g dm^{-3}. $ZnCl_2$ concentrations(mol dm^{-3}) (from top to bottom): 1.88×10^{-4}, 9.68×10^{-5}, 6.84×10^{-5}, 5.88×10^{-5}, 4.92×10^{-5}, 3.95×10^{-5}, and 2.97×10^{-5}. In (B) $\Delta OD=OD(t)—OD(t=0)$ is given.

When the kinetic results shown in Figs. 4(A) and (B) are compared with the time course of the 'solubility' in Fig. 1, a good correspondence is found. At first, the critical $ZnCl_2$ concentration found in Fig. 1 lies between 2 and 4×10^{-5} M, which coincides with C_M* ($(3—4)\times10^{-5}$ M) found in the kinetics. When $C_M<C_M$*, both turbidity and 'solubility' change slowly with time in an approximately linear way. When $C_M\geq C_M$*, the turbidity increases and reaches a stationary value, as the 'solubility' decreases and reaches a stationary value. Accordingly, we can conclude that these two kinds of experiments are observing the same process but in terms of different quantities in different time ranges. We can reasonably assume that the change of the protein concentration occurred mostly within about 30 min if $C_M\geq C_M$*, as judged from the kinetic data.

To examine the correlation between the stationary turbidity and the stationary 'solubility', they are plotted against each other in Fig. 5. To construct this plot, the 'solubilities' at the $ZnCl_2$ concentrations where the kinetic measurements were carried out were read from a curve shown in Fig. 2. A good correlation is found between the two quantities as shown in Fig. 5. In other words, the turbidity in the present study

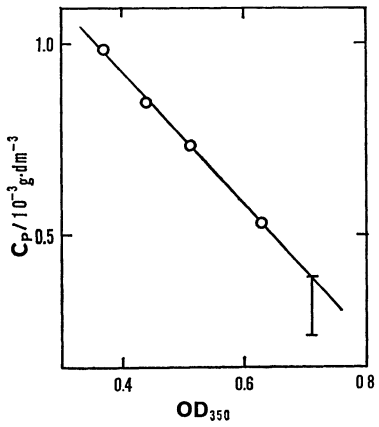

Fig. 5. Correlation between the stationary solubility C_p and the stationary turbidity at 350 nm.
The total protein concentration: 0.22—0.23 g dm^{-3}.

is approximately linearly related to the 'solubility'.

Summary of the Present Results. The 'solubility' behavior examined in the present study is considerably complex. It is pertinent, therefore, to summarize main results concerning a process characterizing the fast kinetic pattern. As to the slow pattern, we cannot make any discussion at present. The main results can be summarized into the four points.

(1) A critical $ZnCl_2$ concentration C_M* exists which defines different time courses of the 'solubility'.

(2) Stationary values of the 'solubility' exist and they decrease as $ZnCl_2$ concentration C_M increases.

(3) The 'solubility' increases with the total amount of the protein.

(4) The precipitates dissolve very slowly, so the precipitation is irreversible to a considerable extent.

The results (1)—(4) cannot be understood consistently by a phase equilibrium even the polydispersity is taken into account, since it fails to explain the irreversible nature. The coagulation of colloids cannot explain the dependence on the 'coagulant' concentration [result (2)], even though it is assumed that a thermodynamically stable fibrinogen solution is converted to a colloidal dispersion of kinetic stability by the addition of $ZnCl_2$.

If we regard the present precipitation as a kind of gelation, on the other hand, these results seem to be understood rather reasonably. The critical concentration is related to the gel point. The dependence of the solubility (proportional to the amount of the sol) on the total amount is consistent with gelation. Irreversible bond formation is also rather frequently encountered in gelation. On the other hand, it is difficult to explain the existence of a finite solubility still after the gelation is completed. However, the 'bond' between fibrinogen molecules is mediated by a zinc atom in the present case. Therefore, there is a room for models describing the coupling between the reversible binding of Zn^{2+} ions and the highly irreversible precipitation. An attempt will be given in the next section.

A Simplified Model. Gelation of Trifunctional Units: We will present a model with some assumptions, which can account for all the results (1)—(4) and for several

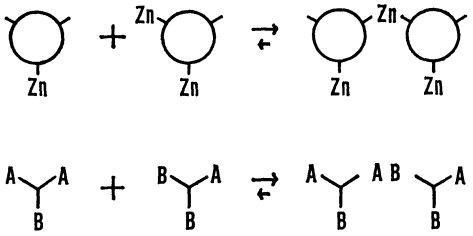

Fig. 6. Schematic representation of the model for gelation.
Free and bound sites on a fibrinogen molecule (represented as a circle) are denoted as A and B, respectively. The bond between two fibrinogen molecules is mediated by a zinc atom. In the model the bond is formed as a reaction between two 'functional groups' A and B.

kinetic results as well, although qualitatively.

Each fibrinogen molecule consists of a pair of three chains, $(A\alpha)_2(B\beta)_2\gamma_2$[8] and presumably carries a number of binding sites for Zn^{2+} ions of different affinities. The number of binding sites should be an even number because of the symmetric structure. However, a simple model of three equivalent binding sites is assumed here, hoping that the essential features of more realistic but more complicated models are fully retained in it (Assumption 1).

The binding equilibrium can be written in terms of free and occupied sites A and B (Assumption 2).

$$A + Zn^{2+} \overset{K}{\rightleftharpoons} B. \quad (1)$$

The association constant K is written in terms of site concentrations [A] and [B].

$$[B]/[A] = KC_M. \quad (2)$$

Here and throughout, free Zn^{2+} ion concentration is approximated to the total metal concentration C_M. At a given protein concentration, relative populations of four possible species A_3, A_2B, AB_2, and B_3 are 1: $2KC_M$: $2(KC_M)^2$: $(KC_M)^3$. As discussed later, values of KC_M in the present study are considered to be much smaller than unity. The contribution from species B_3 is therefore neglected in the following analysis. The binding equilibrium described by Eq. 1 is established instantly compared with the time scale of the present precipitation.

It is assumed that the reaction between site A and site B occurs after the binding equilibrium is established (Assumption 3). An example of the reaction leading to aggregation is schematically depicted in Fig. 6. For species A_2B or AB_2, this kind of association occurs with any other species as well as with themselves, while species A_3 or B_3 cannot associate with themselves. Flory has shown that the self-association of species A_2B or AB_2 cannot produce a gel, though three-dimensional branched polymers are formed.[9] Accordingly, any one of the four species alone cannot produce a gel.

Next, we examine the reactions in binary mixtures of A_3+A_2B or A_2B+AB_2. At low $ZnCl_2$ concentrations, the system can be well approximated to a binary mixture A_3+A_2B. In this mixture, association occurs between A_3 and A_2B as well as between two A_2B mole-

cules. However, gelation does not occur in this mixture. At larger $ZnCl_2$ concentrations where the population of AB_2 is significant, a ternary mixture has to be considered. Since an essential reaction leading to gelation occurs between A_2B and AB_2, we will examine, for simplicity, a binary mixture of A_2B and AB_2. We introduce the extent of reaction p_A (p_B) for a 'functional group' A(B), after the standard theory of gelation.[10] As shown in Appendix, a critical extent of reaction for gelation exists in this mixture. The range of gelation is given as follows.

$$\begin{aligned} &2(2-X_1)/(4+X_1) \ll P_A \ll 1, \text{ for } X_1 \leqq 0.5. \\ &2(1+X_1)/(5-X_1) \ll p_B \ll 1, \text{ for } X_1 \geqq 0.5. \end{aligned} \quad (3)$$

Here X_1 denotes the mole fraction of A_2B. Since $A_2B:AB_2 \simeq 1:KC_M$, and since KC_M is considered to be much smaller than unity, X_1 is supposed to be much larger than 0.5.

In Flory's model, the extent of reaction goes to unity as gelation is completed, since crosslinkings are allowed in gels (but not in soluble aggregates).[10,11] In this extreme case, no free B groups are present any more in gels, while free A groups are still present in gels since p_A is smaller than unity ($p_A=(2-X_1)/(1+X_1)<1$). In this situation, the 'solubility' is expected to be negligibly small.

According to Stockmayer's model, on the other hand, the extent of reaction does not exceed a certain limit, since no crosslinkings are allowed even in gels.[11] A finite solubility can be expected in this case. The limiting value of p_B is calculated in Appendix for the binary mixture A_2B+AB_2 and given as $(2-X_1)^{-1}$. This value is nearly equal to but always smaller than $2(1+X_1)/(5-X_1)$, the critical extent of reaction for $X_1 \geqq 0.5$. Since X_1 is expected to be much larger than 0.5, gelation cannot occur in the binary mixture A_2B+AB_2 if crosslinkings are forbidden in gels.

It is likely that the actual situation lies between these two extreme cases; the limiting value of p_B, $p_B(\infty)$, lies between $(2-X_1)^{-1}$ and unity. Consequently, we can expect that there are not a few free A and B groups in gels even when the gelation is completed ($t \to \infty$). Returning to the original ternary mixture, $A_3+A_2B+AB_2$, we find that a certain amount of soluble substances can exist in equilibrium with these free A and B groups in gels. In soluble fraction, these free A and B groups are present in species A_3, A_2B, and possibly in soluble aggregates. The 'solubility' is hence guaranteed if both free A and B groups are present in gels to a considerable amount even when the gelation is completed. In the above argument, we have to assume that the participation of species A_3 into gelation does not alter the essential features of the gelation of the binary mixture A_2B+AB_2 (Assumption 4).

The suggested 'solubility' is larger when $ZnCl_2$ concentration C_M is lower, if compared at the same protein content, as a consequence of the assumed mass action law:Eq. 2. Since the concentration of species A_3 increases with the total amount of the protein, the 'solubility' also increases with the latter. The critical concentration C_M^* can be identified with the gel point. From these considerations, it is clear that the present model can account for the results (1)—(3). One obvious way to account for result(4), the slow rate of dissolution of the precipitates, is to assume that the rate of dissolution is greatly reduced if crosslinkings occur in gels (Assumption 5). It is to be noted that the validity of the above arguments on the existence of the solubility and its dependence on C_M does not depend on the rate of dissolution of the precipitates.

In this way, the present model can explain the four main results by identifying the present precipitation with a gelation. Also, the time course of the turbidity can be understood based on the present model. Since the turbidity does not develop until the extent of reaction reaches a critical value for the gelation, a lag time will be observed in the kinetics.

Discussion

In the last section, we present a model to account for the present results on the precipitation of human fibrinogen with $ZnCl_2$. It is frequently inferred in deriving various properties of the model that the occupied fraction of binding sites for Zn^{2+} ions is very small. This point is discussed here.

As a rough estimation of the value of the association constant K defined as Eq. 2, we can take $1\times10^3\ M^{-1}$, since the association constant of imidazole with Zn^{2+} ions was about $6\times10^2\ M^{-1}$.[12] The range of KC_M in the present study is then below 0.2 (corresponding to $C_M=2\times10^{-4}$ M) or smaller. When KC_M is 0.1, the fractions of various species are 0.82(A_3), 0.16(A_2B), and 0.016(AB_2) and species B_3 can be safely neglected as assumed. Since X_1 can be roughly equated to the ratio $A_2B/(A_2B+AB_2)$, it is about 0.91. At $C_M=C_M^*$ ($\approx3\times10^{-5}$ M), $KC_M^*\approx3\times10^{-2}$ and $X_1\approx0.97$. Practically, gelation does not occur below C_M^*, for X_1 is nearly unity. It is found that the conditions used in the preceding section, $X_1 \gg 0.5$ and $KC_M \ll 1$, are reasonable.

It has been reported that fibrinogen is a heterogeneous mixture.[7,13-16] However, this point is not taken into account in developing the present model for the sake of simplicity. The introduction of the heterogeneity into the model does not violate its validity, since it only makes the model more versatile.

The most important feature of the present model resides in the respect that it can account for the finite solubility (dependent on $ZnCl_2$ concentration) in the situation that gelation is completed and is highly irreversible. However, the problem is treated only approximately in the present study. Moreover, the treatment is based on the classical theory of gelation, according to which the most probable distribution is considered for an infinitely large system and the mean field approximation is often used. Although the mean field approximation is criticized from the standpoint of the scaling theory,[17] and although the ensemble averages should be used in place of the most probable distribution as was recently carried out by computer simulation,[18,19] it is hoped that the present simple treatment gives an essentially correct picture of the present results.

Appendix

Gelation of a binary mixture of trifunctional units, A_2B and AB_2, is considered here based on the most probable distribution for an infinitely large system.

The mole fractions of A_2B and AB_2 are denoted as X_1 and X_2, respectively: $X_1+X_2=1$.

The extents of reaction of A and B groups are denoted as p_A and p_B. They are related to each other by Eq. A1.

$$p_A(1+X_1) = p_B(1+X_2). \tag{A1}$$

The expected number of units(irrespective of A_2B and AB_2) bound to A_2B is $2p_A+p_B$. Similarly, $2p_B+p_A$ is the expected number of units bound to AB_2. Gelation occurs when the expected numbers of units bound to both kinds of unit are equal to or larger than 2, *i.e.*,

$$2p_A + p_B \geqq 2, \tag{A2}$$

and

$$2p_B + p_A \geqq 2. \tag{A3}$$

Other obvious restrictions on p_A and p_B are

$$p_A \leqq 1 \text{ and } p_B \leqq 1. \tag{A4}$$

When these conditions are rearranged and coupled, we find that gelation occurs if the following conditions are satisfied.

$$\begin{aligned} &2(1+X_1)/(4+X_1) \leqq p_B \leqq (1+X_1)/(2-X_1) \text{ for } X_1 \leqq 0.5. \\ &2(1+X_1)/(5-X_1) \leqq p_B \leqq 1 \text{ for } X_1 \geqq 0.5. \end{aligned} \tag{A5}$$

Inequalities (A5) are given in the text as inequalities (3). For pure species, either $X_1=1$ (pure A_2B) or $X_1=0$ (pure AB_2), gelation occurs only at the end of reaction, either $p_B=1$ or $p_A=1$ ($p_B=0.5$) depending on whether X_1 is larger or smaller than 0.5. This is Flory's result.[9]

Next, the limiting extents of reaction of A and B groups when the gelation is completed are evaluated according to Stockmayer's model, in which no crosslinkings are allowed not only in soluble aggregates (as in most theories) but also in gels (contrary to Flory's model).[10,11] As a first step ($n=1$), only one unit, either A_2B or AB_2, is chosen. The number of unreacted A or B groups, $A_f(1)$ or $B_f(1)$, is given on the average as $A_f(1)=2X_1+X_2$ or $B_f(1)=2X_2+X_1$. The number of reacted groups, $A_b(1)$ or $B_b(1)$, is zero. As the second step ($n=2$), all three groups of a unit chosen in the first step are reacted to make a tetramer [$N(2)=4$]. As the third step ($n=3$), all the unreacted groups on the tetramer are reacted and an aggregate consisting of 10 units [$N(3)=10$] is produced. We define in this way $A_f(n)$, $B_f(n)$, $A_b(n)$, $B_b(n)$, and $N(n)$ as the numbers of A and B groups unreacted, reacted and the total number of units when the n-th step is finished.

The following recurrence formulas are obtained for $A_f(n)$ and $B_f(n)$.

$$\begin{aligned} A_f(n) &= (1+X_1)A_f(n-1) + X_1B_f(n-1), \\ B_f(n) &= (1+X_2)B_f(n-1) + X_2A_f(n-1). \end{aligned} \tag{A6}$$

$A_b(n)$, $B_b(n)$, and $N(n)$ are given as follows.

$$A_b(n) = B_b(n) = \sum_{i=1}^{n-1} [A_f(i)+B_f(i)] = 3(2^{n-1}-1). \tag{A7}$$

$$N(n) = 1 + 3(2^{n-1}-1). \tag{A8}$$

From these results, we obtain

$$\begin{aligned} p_A(n) &= 3(2^{n-1}-1)/[1+X_1+3(1+X_1)(2^{n-1}-1)]. \\ p_B(n) &= 3(2^{n-1}-1)/[1+X_2+3(1+X_2)(2^{n-1}-1)]. \end{aligned} \tag{A9}$$

In the limit that n goes to infinity,

$$p_A(\infty) = (1+X_1)^{-1} \text{ and } p_B(\infty) = (1+X_2)^{-1}. \tag{A10}$$

This result is given in the text.

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