**RESEARCHES ON FORMOL-GELIFICATION OF VARIOUS
PROTEIN FRACTIONS CONTAINED IN SERUM, WITH
SPECIAL REFERENCE TO THEIR CHANGE OF VISCOSITY
IN THE WIDE RANGE OF pH***

BY SHO MIYAMOTO, MOSABURO KOBAYASHI,
HIKARU HIGUMA AND YASUO CHIBA

The Biochemical Laboratory, Tokyo Medico-Dental University

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INTRODUCION

The gelification of proteins has long been the subject of numerous researches. Regarding the structure of gelification, Barbu and Joly have recently shown that there exists a fundamental difference between the gelification of serumalbumin caused by heat and that by strong alkali, from the view-point of double refraction of flow. Moreover, according to Morel, Guintini and Grabar, one can see clearly on the electron microscope photographs the construction of fibres which constitute the gel of gelatin.

It seems to us, however, that it will be more interesting to make observations on various degrees of viscosity leading to the final gelification rather than to study the phenomena of gelification itself. It is true that the method of measuring the viscosity has often been applied to research works pertaining to proteins. For instance, we examine the viscosity of protein solution (e.g. serum) to know the ratio of A/G contained therein, because the viscosity reveals in what proportion various proteins are mixed in the solution. But even in this case, the viscosity of protein solution (serum) as it is, does not vary so remarkably in response to the change of the mixing ratio of different kinds of proteins in the solution. On the contrary, if we add beforehand some formalin or lactic acid to the solution of proteins (serum), it is likely that subsequent metamorphoses will present very vividly the ratio of one kind of protein to another mixed therein. From the foregoing, it is by no means accidental that reactions such as formol-gelification, acid-gelification and lactic-acid-

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gelification should have been discovered.

The authors, considering these points, pay their special attention to the fact that the quantity and the kind of protein fractions contained in serum have a profound influence on formol-gelification. Besides, they are interested in the method of observing the said reaction by following up the changes of viscosity till gelification manifests itself. Miyamoto and Suzuki^{3,4,5,6,7,8)}, based upon this method, and considering that pH affects the viscosity, have observed in the wide range of pH the serum of Kala-azar, and discovered and reported many a new fact about it. Later, Higuma has applied the same method to the serum of tuberculosis, and he has found that formol-gelification indicates clearly the state of progress of the malady, although Higuma's result has been obtained on the conditions somewhat different from those of Suzuki. According to these observations, the pH affects greatly the viscosity of the serums, which, when formalin is added, changes in all the range of pH as mentioned below: 1) the viscosity increases on the acidity side from pH 5.4. 2) a very particular augmentation of viscosity is recognizable in the extremely narrow limit around pH 6 to 8. The first phenomenon, however, appears whether formalin is added or not. Consequently, it may be regarded as an example of acid-gelification having no relation with the reaction against formalin. On the other hand, the second phenomenon is entirely due to formol-gelification. Such a remarkable increase of viscosity in the environs of pH 6 to 8 cannot be brought out if formalin is not added. Thus, the serum of Kala-azar and that of tuberculosis react almost in the same manner against formalin by changing their viscosity. But as for Kala-azar, this change occurs very well in the 20% diluted serum, while in order to obtain the similar result, the serum of tuberculosis must be diluted at the rate of 50%. It is interesting to look for the origin of this difference. And we imagine that perhaps this is on account of the proportion of the protein fractions mixed in the serums of the two maladies. Considering the experimental results obtained by Suzuki concerning the serum of Kala-azar with those obtained by Higuma concerning that of tuberculosis, we must take note that the value of pH at which the viscosity of the said serums attains the highest degree is not exactly the same, viz. the vertex of viscosity is to be seen around pH 6.4 in the case of Kala-azar, but around pH 7.4 in the case of tuberculosis. It is doubtful whether this divergence is fundamental or whether it is only attributable to some

technical defects, in view of the inferior quality of post-war paper for measuring the pH. But here we must again take into account the question whether the mixing ratio of protein fractions in the serums has something to do with the above-mentioned divergence. In order to clarify this question, the authors have considered it necessary to divide into each unit the protein fractions contained in the horse's serum and to observe how each one of them reacts against formalin.

In the present report, they treat, among other things relating to this subject, the attitude towards formol-gelification shown by each fraction of protein.

RPREPARATION OF PROTEIN FRACTIONS

The proteins used for experiment are serumalbumin, euglobulin and pseudoglobulin I & II extracted from horse's serum. Each fraction has been prepared in the following way:

1) *Horse's serumglobulin*. Complying with Howe's method^{10, 11, 12)} modified by Kobayashi¹³⁾, powdered Na_2SO_4 is added to horse's plasma at the rate of 10.6, 13.6, 17.5 and 21.5 g/dl, and fibrinogen, euglobulin and pseudoglobulin I & II are isolated. The sediment thus obtained of each fraction is dissolved in distilled water, the quantity of which corresponds to 1/5 of that of the original plasma. Then filtering the solutions and adding to each of them the same quantity of powdered Na_2SO_4 as has been used to fractionate the plasma, we precipitate the fractions. Repeating this treatment thrice, we filter and dissolve in the distilled water the sediment of each fraction. After dialysing for five days each solution contained in a cellophane bag, we add thereto NaCl at the rate of 0.9%. N in the solution is measured after micro-Kjeldahl's method. All the solutions are used for experiment immediately after preparation.

2) *Horse's serumalbumin*. In accordance with Kekwick's method¹⁴⁾ modified by Kobayashi¹³⁾ we crystallize horse's serumalbumin as follows: Adding to horse's plasma powdered Na_2SO_4 at the rate of 20 g/dl, we precipitate serumglobulin and leave the thus processed plasma for 24 hours at 30°C. Then filtering the solution, we crystallize serumalbumin at pH 4.8 by adding n/5 H_2SO_4 and powdered Na_2SO_4 . The crystallized fraction is dissolved in the distilled water corresponding to about 1/5 of the original plasma and kept at 30°C for about 10 hours. After that, filtering the solution and adding thereto n/5 H_2SO_4 and powdered Na_2SO_4 , we re-

crystallize serumalbumin. The process of recrystallization is repeated thrice. We use for our experiment the serumalbumin thus extracted after having treated it in the same manner as in the case of serumglobulin.

METHOD OF EXPERIMENT

1) *Arrangement of pH value.* For our experiment we use the solution of each protein fraction diluted at $3/4$ times, arranging its pH value as shown in Table 1. Owing to buffer action proper to each of the solutions, their pH value does not coincide even if the same quantity of acid or alkali is added to them. But in the present case, the identical pH value for each solution is not needed, inasmuch as pH is indicated on the abscissa. It is only required to measure the precise pH value of each solution in the test-tube. Immediately upon completion of the pH arrangement, we measure the viscosity of each solution, and adding thereto one drop of formalin (about $1/33$ cc), we measure the viscosity again. Then we keep the test-tubes at 30°C in the incubator for 24 hours, and after the lapse of 24 hours we measure the viscosity for the third time. But this time before measuring the viscosity, we examine the pH of each solution in the test-tube with test-paper (manufactured by Toyo Filter-paper Co.). Furthermore, as the commercial formalin contains formic acid, we add thereto precipitated calcium carbonate in order to neutralize its high acidity. Filtering the neutralized formalin 24 hours later, we use it for our experiment. (By so doing, the pH of the formalin always indicates the value of 5.4. For the purpose of control, we prepare other protein solutions to which one drop of distilled water is added instead of formalin.

2) *Measurement of viscosity.* Using Hess's viscosimeter, we measure the relative viscosity of protein solutions in contrast with the viscosity of distilled water at the same temperature. Since it is not the absolute viscosity that matters, our experiment is performed at the room temperature, regardless of the influence of temperature. As for the rapidity of measuring the viscosity, we adjust the viscosimeter so that it takes regularly 4 to 5 seconds for water to advance from the graduation 0 to 1.0, with a view to minimizing the differences originated by the structural viscosity, etc.

EXPERIMENTAL RESULTS

1) *Influence of formalin on albumin.* Fig. 1A shows the experimental result obtained with 1.3 g/dl of albumin solution. The curve marked with $\times$

indicates the viscosity measured before addition of formalin and immediately after mixture of the solution with acid and alkali, and the curve marked with $\bigcirc$ the viscosity measured 24 hours after the mixture without addition of formalin. The curve marked with $\triangle$ denotes the viscosity measured immediately after addition of formalin, and the curve marked with $\bigcirc$ the viscosity measured 24 hours after addition of formalin. (These signs are common to the viscosity curves of all the undermentioned fractions.)

As shown in Fig. 1A, we cannot see a remarkable influence of formalin on albumin at this stage of concentration. And the curve of its viscosity does not ascend 24 hours later, whether formalin is added or not. As Barbu and Macheboeuf report, acid-gelification we see in globulin does not take place in the case of albumin. We recognize only a slight opaqueness in the solution between pH 4.8 and 5.0 24 hours after addition of formalin. When the solution is of high concentration, we see an increase of viscosity in the extremely narrow limit around pH 4.8. The authors will explain later the formol-gelification of more highly concentrated albumin.

It must be noted that the albumin solution to which formalin is not added does not display any remarkable change in all the range of pH even 24 hours after addition of acid and alkali. On the other hand, immediately after addition of formalin to the solution, a change of pH is recognizable on the alkali side beginning from pH 4.8–5.0, and this change becomes more evident in the course of 24 hours. Contrary to this, hardly any change of pH is observable on the acidity side from pH 4.8–5.0 even 24 hours after addition of formalin. Taking these experimental results into consideration, we understand that it is impossible to prove the reaction of albumin against formalin by means of measuring the viscosity, but that formalin combines with amine groups of protien only on the alkali side from the iso-electric point.

2) *Influence of formalin on pseudoglobulin II.* Fig. 1B indicates the experimental result obtained with 1.3 g/dl of pseudoglobulin II. The viscosity curve shown 24 hours after addition of formalin (marked with $\bigcirc$) rises a little on the acidity side as it goes away from the iso-electric point. And opaline opaqueness is seen in the solution in parallel with this rise of the viscosity curve. A phenomenon nearly alike is recognized after the lapse of 24 hours in the solution not containing formalin, as the viscosity

curve marked with ○ indicates. It is therefore due to acid-gelification.

On the other hand, neither a notable rise of viscosity curve nor a remarkable opaqueness is seen on the alkali side from the iso-electric point. Moreover, the value of pH after addition of formalin shifts only on the alkali side from the iso-electric point.

In brief, we may conclude that formalin does not greatly affect pseudoglobulin II.

3) *Influence of formalin on pseudoglobulin I.* In Fig. 1C is shown the experimental result relating to 1.3 g/dl of the solutions of pseudoglobulin I. In this case, the viscosity curve rises a little higher than that of pseudoglobulin II on the acidity side as it goes away from the iso-electric point. This rise is almost the same, whether formalin is added or not. Accordingly it may be regarded as a phenomenon of acid-gelification as in the case of pseudoglobulin II. The characteristic of pseudoglobulin I to be pointed out is that the viscosity curve rises on the alkali side, which is not perceivable either in albumin or in pseudoglobulin II. Namely, the viscosity curve which finds itself at the lowest point at pH 5.2 begins to go up again when it enters the alkali side, and around pH 7.8 the highest point is reached. After that, the curve falls again as it goes farther on the alkali side. This increase of viscosity around pH 7.8 may be considered as formalin-gelification, if we contrast the curve of the solution containing formalin with that of the one not containing formalin (marked with ●). Opaline opaqueness is to be seen at and around pH 7.8 only when formalin is added. The opaqueness increases or diminishes with the rise or the fall of the viscosity curve.

The variation of pH is recognized only on the alkali side from the iso-electric point, after addition of formalin to the solution.

4) *Influence of formalin on euglobulin.* Fig. 1D represents four viscosity curves shown by 1.3 g/dl of euglobulin solution. They reveal a special attitude of euglobulin towards formol-gelification. Without formalin, no particular augmentation of viscosity takes place on the alkali side from the iso-electric point, even 24 hours after addition of acid and alkali. On the contrary, soon after addition of formalin, the viscosity curve ascends moderately between pH 6.6 and 7.8, and 24 hours after the process, we notice a very particular increase of viscosity within the narrow range surrounding pH 7.2 where the vertex of viscosity is situated. Observing the curve drawn by the viscosity 24

hours after addition of formalin, we find that a concavity exists at pH 5.2 and that the curve goes up on both sides of this concavity. In other words, on the acidity side the curve rises gradually as in the case of pseudoglobulin I, while on the alkali side it begins to ascend abruptly around pH 6.4, reaching the highest point at pH 7.2. Later, it goes down again rapidly. A phenomenon of formol-gelification is to be seen around pH 7.2 if the solution is highly concentrated. (The authors will explain it later on.) Simultaneously with the attainment of the highest point by the viscosity curve, opaqueness like an opal appears in the solution. But on the acidity side beginning from pH 5.2, the rise of the curve is not so remarkable as on the alkali side, whether formalin is added or not.

The variation of pH is recognizable on the alkali side from the iso-electric point only when formalin is added. When the solution is not highly concentrated, the said variation is of light nature.

SUMMARY

1) In order to examine the attitude towards formol-gelification taken by respective protein fractions contained in serum, the authors, using Hess's viscosimeter, have made observations on formol-gelification by following up the changes of viscosity in the wide range of pH till the gelification manifests itself.

2) The authors have presumed that the viscosity of protein solutions will be greatly affected by pH. It is why they have made their experiment in the wide range of pH after "Miyamoto-Suzuki Method", always paying special attention to the pH of the solutions.

3) The authors have measured in the wide range of pH the viscosity of respective protein fractions contained in serum immediately and 24 hours after addition of acid and alkali, and they have obtained the following results: As for albumin, its viscosity has scarcely increased with the lapse of time in all the range of pH. On the contrary, with regard to euglobulin, pseudoglobulin I & II, no remarkable increase of viscosity is recognized on the alkali side from the iso-electric point, while on the acidity side, the viscosity curve rises as it goes away from the iso-electric point, and opaline opaqueness is to be seen in parallel with the rise of the curve. This tendency is particularly noticeable in the case of euglobulin.

4) The authors have observed the changes that take place in the vis-

cosity of respective protein fractions when formalin is added, and have obtained the following results: Formalin has almost no influence on albumin and pseudoglobulin II with respect to their viscosity. Pseudoglobulin I, too, is not greatly affected by formalin, except for a slight increase of its viscosity around pH 7.8.

On the contrary, formalin exerts a great influence on euglobulin. A very particular increase of viscosity in the extremely narrow limit around pH 6 to 8 is quite characteristic of the influence of formalin on this fraction. From the foregoing, it has been concluded that euglobulin takes the most important part in the formol-gelification of serum.

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REFERENCES

- 1) Barbu, E. et Joly, M.: Mécanisme de gélification de la sérumalbumine. Etude par la biréfringence d'écoulement. *Bull. Soc. Chim. Biol.*, **32**, 116-122, 1950.
- 2) Barbu, E. et Joly, M.: Biréfringence d'écoulement des solutions alcalines de sérumalbumine de cheval. Etude du mécanisme de l'hydrolyse. *Bull. Soc. Chim. Biol.*, **32**, 123-129, 1950.
- 3) Suzuki, N.: Researches on the physical and chemical quality of the serum of Kala-azar.
(I) *Nisshin Igaku*, **32**, 424-434, 1943 (in Japanese).
- 4) Suzuki, N.: —ditto—
(II) *Nisshin Igaku*, **32**, 526-537, 1943 (in Japanese).
- 5) Suzuki, N.: —ditto—
(III) *Nisshin Igaku*, **32**, 702-719, 1943 (in Japanese).
- 6) Suzuki, N.: —ditto—
(IV) *Nisshin Igaku*, **32**, 867-877, 1943 (in Japanese).
- 7) Suzuki, N.: —ditto—
(V) *Hokuetsu Igakukai Zasshi*, **59**, 825-843, 1944 (in Japanese).
- 8) Suzuki, N.:
(VI) *Hokuetsu Igakukai Zasshi*, **59**, 859-863, 1944 (in Japanese).
- 9) Higuma, H.: Application of formol-gel reaction (Miyamoto-Suzuki Method) in diagnosing the clinical course of tuberculosis. *Ochanomizu Gakukaishi*, **3**, 15-20, 1951 (in Japanese).
- 10) Howe, P.E.: The use of sodium sulfate as the globulin precipitant in the determination of protein in blood. *J. Biol. Chem.*, **49**, 93-107, 1921.
- 11) Howe, P.E.: The determination of fibrinogen by precipitation with sodium sulfate compared with the precipitation of fibrin by the addition of calcium chloride. *J. Biol. Chem.*, **57**, 235-240 1923.
- 12) Bálint, P. und Bálint, M.: Über die chemische Zusammensetzung der men-

- schlichen Bluteiweisskörper. *Biochem. Z.*, 306, 296-315, 1940.
- 13) Kobayashi, M.: Studies on the denaturation of proteins. I and II. *Nichidai Igaku Zasshi*, 8, 70-80, 1949 (in Japanese).
- 14) Kekwick, R.A.: Observation on the crystallizable albumin fraction of horseserum. *Biochem. J.*, 32, 552-560, 1938.