STUDIES ON THE DENATURATION OF ANTIBODY

IV. THE INFLUENCE OF pH AND CERTAIN OTHER FACTORS ON THE RATE OF INACTIVATION OF STAPHYLOCOCCUS ANTITOXIN IN UREA SOLUTIONS

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In previous work on the denaturation of antibody, studies have been made of some of the factors influencing the inactivation of diphtheria antitoxin in urea solutions (1, 2). A quantitative formulation of a simple kinetic theory was found to fit satisfactorily the experimental data and to offer a reasonable explanation of the deviation of the inactivation from simple first order behavior (2). In the present work we have studied the inactivation of *Staphylococcus* antitoxin, investigating the influence of certain new factors on the rate and course of the over-all reaction, with a view toward gaining further insight into the mechanism of the reactions, and reinvestigating the influence of pH, a factor studied previously with diphtheria antitoxin, in order to test the applicability of the proposed kinetic mechanism to different antibodies.

Materials and Methods

The antitoxic globulin was obtained from crude horse plasma[1] by fractionation between 1.33 and 1.68 M ammonium sulfate. The final preparation contained about 9 per cent protein and 260 I.U. of antitoxin per ml. Only the neutralizing power for the *Staphylococcus* α-toxin, which hemolyzes rabbit red blood cells, was considered in the present work. The hemolytic toxin was obtained from a semisolid agar culture of *Staphylococcus aureus* in the usual manner; the toxin was preserved with merthiolate and used in its crude form. The antitoxin assays were carried out in the customary manner as described previously (3). The procedure consists essentially of determining the dilutions of the unknown and of the treated antitoxins required to reduce the toxicity of a constant amount of the toxin, containing about 150 minimal hemolytic units, to a point where only 50 per cent hemolysis of added rabbit red blood cells occurs after standard incubation. The ratio of the dilution of the partially inactivated antitoxin to the dilution of

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[1] Supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

the untreated antitoxin gives the proportion of antibody activity remaining; micro-Kjeldahl determinations (4) were necessary to correct for the random volume changes which occurred during the dialysis of the samples for the removal of the urea. The over-all determination appears to be accurate to within a few per cent.

RESULTS AND DISCUSSION

Influence of pH on Rate of Inactivation—In the previous work on the kinetics of the inactivation of diphtheria antitoxin in urea it was observed that the pH markedly influenced the course of the reaction. Kinetic experiments were performed with the *Staphylococcus* antitoxin at a comparable series of pH values and in a similar manner. 8 M urea and a protein con-

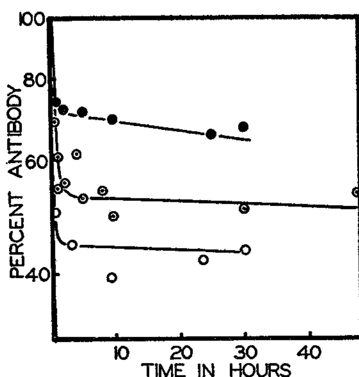


Fig. 1

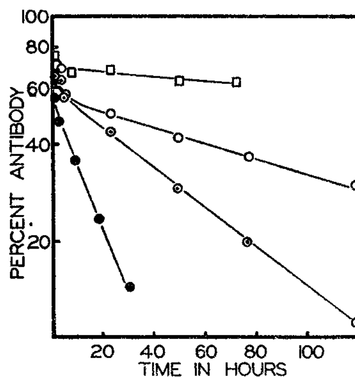


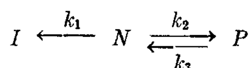
Fig. 2

Figs. 1 and 2. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 8 M urea at 25°, studied at various pH values. Fig. 1, ○ pH 4.97, ⊙ pH 5.38, ● pH 6.21; Fig. 2, □ pH 7.40, ○ pH 8.92, ⊙ pH 9.34, ● pH 9.81. The curves represent the equation $[T] = T_1e^{-\lambda_1 t} + T_2e^{-\lambda_2 t}$. For definitions of symbols see Wright and Schomaker (2).

centration of 0.3 per cent were used; the denaturation reactions were carried out in a thermostat at 25°. The buffer salts were held at an ionic strength of 0.1. Borate buffer was used at pH 9.81, 9.34, and 8.92, phosphate at pH 7.40, and acetate at pH 6.21, 5.38, and 4.97. Samples were removed at the required intervals, diluted with an equal volume of saline, and dialyzed in the cold to remove urea, and the per cent of the original antibody activity was then measured. The experimental results are given in Figs. 1 and 2.

It is clear that the general features of the influence of pH on the inactivation of *Staphylococcus* antitoxin and diphtheria antitoxin are closely similar. Application to the data of the theoretical treatment proposed previously for the inactivation of diphtheria antitoxin in urea solutions gave the curves presented in Figs. 1 and 2; the rate constants derived from the theoretical

curves are given in Table I. This treatment and the proposed kinetic mechanism of which it is the quantitative expression have been developed and discussed in detail previously (2). Briefly, however, the proposed mechanism may be represented by the diagram



where N represents the native, undenatured antibody, I the inactivated antibody, P a hypothetical "protected antibody" which is fully active (or becomes so on removal of urea) but is not susceptible to inactivation, and k_1, k_2, and k_3 represent first order rate constants for the reactions as indicated. Examination of Figs. 1 and 2 indicates that the present data are well represented by this treatment, since the experimental points are, in general, closely fitted by the curves. The values of the rate constants derived from the theoretical curves and given in Table I, moreover, appear to be identical with those obtained in the experiments with diphtheria antitoxin (2), within the rather large probable errors associated with this sort of curve fitting when the number of experimental points is small.

TABLE I

Variation with pH of Rate Constants Obtained from Quantitative Treatment Described in Text

pH	k_1	k_2	k_3
	hrs.$^{-1}$	*hrs.*$^{-1}$	*hrs.*$^{-1}$
4.97	2.54	2.04	0.0023
5.38	0.83	0.94	0.0020
6.21	0.64	1.62	0.013
7.40	0.46	1.02	0.0049
8.92	0.24	0.30	0.013
9.34	0.30	0.41	0.035
9.81	1.31	1.49	0.10

The satisfactory fit of the experimental data to the theoretical treatment outlined above appears to be additional evidence of the validity of this explanation. If the proposed kinetic explanation is in fact substantially correct, the striking similarity of the values of the calculated rate constants for the two antitoxins suggests that corresponding regions of the protein molecules, with essentially identical major stabilizing bonds, may be involved in the structures of the combining groups of the antibodies for different antigens.

Influence of Neutral Salts on Rate of Inactivation—An interesting aspect of the denaturing action of urea and related substances is the influence of electrolytes on the reaction. The denaturing activity of guanidine salts

was observed to be markedly influenced by the nature of the anion (5). Burk (6) studied the effect of a large number of salts on the concentration of urea necessary for the liberation of sulfhydryl groups of several proteins, and observed very striking differences, the effects varying from strong inhibition of denaturation to striking augmentation. The rate of denaturation of tobacco mosaic virus in urea was also observed to be influenced by the kind and quantity of electrolyte in the solution (7).

Experiments were carried out to determine whether the inactivation of antitoxin is similarly influenced by salts. It was considered possible that the different component reactions of the over-all inactivation might be influenced in different ways, a result which might lead to a separation of the constituent reactions and a better understanding of their nature. Three

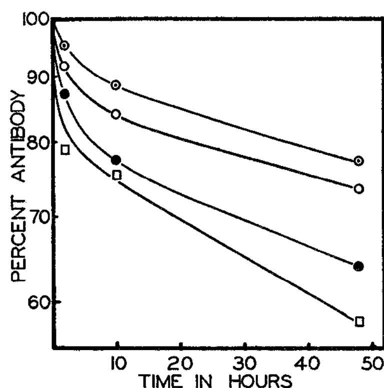


FIG. 3. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 6 M urea at 25°, in the presence of 0.25 M concentrations of various salts, as follows: ○ none, ⊙ sodium sulfate, ● sodium chloride, □ calcium chloride. The curves are empirical.

representative salts were selected from the large group studied by Burk (6). Calcium chloride was chosen as a denaturation-favoring salt, sodium sulfate as a denaturation-inhibiting salt, and sodium chloride as an intermediate salt. The urea concentration was 6 M. Because of the large influence of pH on the rate and course of the over-all reaction, it was necessary to control this factor by the addition of acetate buffer at a final concentration of 0.05 M. The experiments were set up in the usual manner, the different salt solutions being added to measured portions of a solution containing the urea and the buffer, to give a final concentration of 0.25 M. After the solutions had reached temperature equilibrium in the 25° thermostat, the experiment was started by adding the antibody globulin. The samples were removed at intervals, immediately dialyzed, and assayed for antibody activity as before. The results are given in Fig. 3.

Some variation in pH did occur in the experiment, since the pH readings of the solutions were as follows: control 5.66, calcium chloride 5.33, sodium sulfate 5.54, sodium chloride 5.52. These differences appear to be considerably too small to account for the observed effects on the rate of inactivation, however.

It is clear that the influence of the three salts on the inactivation of antibody parallels their influence on the liberation of sulfhydryl groups in other proteins. Thus the addition of calcium chloride promoted the inactivation, sodium sulfate inhibited it, and sodium chloride was intermediate in its effect. There is, however, a slight difference in the behavior of sodium chloride, since it promoted the inactivation slightly, whereas it slightly inhibited the liberation of sulfhydryl groups (6). Thus the results provide additional evidence of the general similarity of the inactivation of antibody to other reactions of proteins which occur in urea solutions and which have been considered classical manifestations of protein denaturation. Although the data are not sufficiently extensive to permit the application of the quantitative treatment of the reaction discussed above, it is clear that a very considerable change in the values of the rate constants in this treatment would be required to produce the large differences in initial slopes among the different experiments. Thus the effects of the salts on the reaction rates may be considerably greater than would appear from a casual inspection of the data for the over-all reaction. It seems as though the three rate constants are affected by the salts in approximately the same manner.

Influence of Hydrostatic Pressure on Rate of Inactivation—It has frequently been observed that the rate of denaturation of proteins is decreased by a moderate increase in hydrostatic pressure, indicating that there is an appreciable volume increase when a protein molecule goes from the normal to the activated state for this reaction. Studies of this sort on the denaturation of the bacterial luminescent system have been particularly complete (8), but evidence has accumulated that denaturation of many other proteins is also accompanied by an increase in volume (9). The rate of denaturation of *Staphylococcus* antitoxin at 65° in dilute salt solutions was observed to decrease with increasing hydrostatic pressure, thus providing additional evidence of the close relationship of antibody inactivation and protein denaturation (3). The influence of hydrostatic pressure on the denaturation of proteins in urea solutions does not appear to have been investigated.

An experiment was performed to determine the influence of hydrostatic pressure on the inactivation of the antitoxin in 6 M urea solutions at 25°. Sodium acetate-sodium chloride buffer was adjusted to give an ionic strength of 0.1 and pH 5.57. As soon as the globulin was added, four 5 ml. samples of the solution were placed in tubes, stoppered with rubber stoppers to leave no air space, placed in the pressure chamber, and a pressure of 10,000 pounds per sq. in. applied. The pressure chamber and the remain-

der of the solution at atmospheric pressure were placed in the 25° thermostat. Samples were taken at intervals from both the atmospheric pressure and high pressure portions, dialyzed, and assayed as before. The results are given in Fig. 4.

Within the experimental error of the method there appears to be no influence of hydrostatic pressure on the rate of the inactivation. In view of the considerable influence of pressure on the rate of inactivation at 65° in the absence of urea, this result was rather unexpected. It seems possible, however, that a reasonable explanation of this difference in behavior may be proposed. The action of urea is generally believed to involve the weakening of the numerous secondary bonds which hold the polypeptide chain in its native configuration (10). High temperature, on the other hand, presumably does not weaken the intramolecular bonds significantly, but breaks

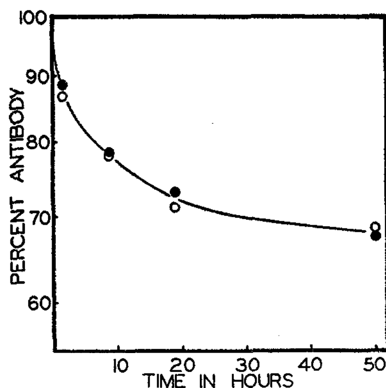


FIG. 4. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 6 M urea at 25° at atmospheric pressure (●) and at 10,000 pounds per sq. in. (○). The curve is empirical.

them by means of locally accumulated kinetic energy. The activated state for this latter process might well involve a general volume increase of the protein, whereas the urea-saturated protein, its stabilizing bonds largely neutralized, could unfold without appreciable volume change.

Rate of Change of Viscosity at Various pH Values—The increase in viscosity of serum proteins when subjected to the denaturing action of urea is well known (11). The rate at which this increase occurs, however, does not seem to have been investigated to any considerable extent, although recently the viscosity change has been observed to consist of an initial rapid rise followed by a gradual increase (12). Experiments were undertaken to determine whether the rate of the change in viscosity of the antibody globulin in urea could be correlated in any way with the kinetics of the antibody inactivation.

Solutions at a series of pH values in 8 M urea were studied. These were prepared in the same way as in the antibody inactivation experiments. The antibody globulin was added last, after which the solutions were quickly filtered to remove any trace of lint, and samples were accurately measured into Ostwald viscometers in a thermostat at 25° ± 0.01°. Measurements of the flow time were made frequently at first and then at increasing intervals as the viscosity change became slower. Between measurements the viscometers were stoppered to prevent evaporation of the solutions. After completion of each experiment the viscometer was cleaned and dried and the flow time of the solvent was determined on a solution made up in the same way as the denaturation mixture (usually an aliquot of the same solution) except that an equal volume of water was substituted for the antibody

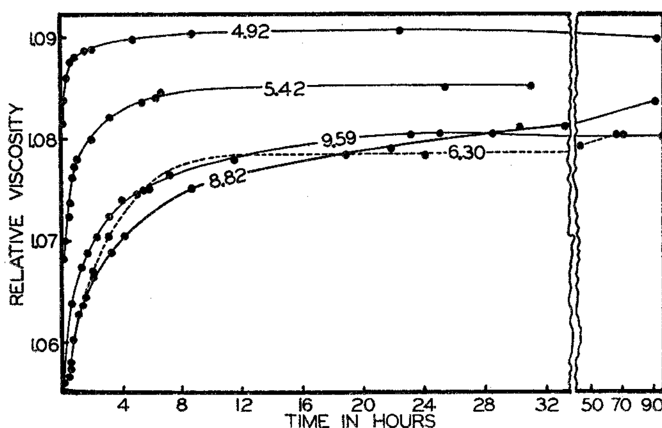


FIG. 5. The relative viscosity of serum globulin as a function of time of denaturation in 8 M urea at 25°, studied at various pH values as indicated. The curves are empirical.

globulin. The relative viscosity was calculated from the two flow times, since density difference between the two solutions was assumed to be negligible. The results of five experiments at a range of pH values are given in Fig. 5. The early points in each experiment represent single measurements of the time of flow, but later measurements are the average of several measurements.

It is clear that the behavior of the viscosity of the solutions varied greatly with the pH and that the results show certain similarities to those of the antibody inactivation experiments. In acid solution the viscosity reached essentially its final value rapidly. At pH 6.30 the initial change was slower but did seem to approach a limiting value. At pH 8.82 the viscosity continued to increase for the duration of the experiment. At pH 9.59 a similar behavior was noted until about 20 hours, when the viscosity leveled off and

finally began to decrease. This suggests that at this high pH some reaction other than the denaturation under consideration, for example a hydrolysis of the peptide bonds, contributed to the change of viscosity. Thus the results are similar to those obtained in the antibody inactivation experiments in certain respects and certainly confirm the existence of relatively slow changes in the protein when exposed to the denaturing action of urea. An adequate test of the applicability to the viscosity changes of the theory proposed for the antibody inactivation (2) would be very difficult to make, however, because it would be necessary to know the viscosities of the different molecular species in the various buffer-urea solutions and to have information about the manner in which their contributions add to produce the observed viscosity.

SUMMARY

A study has been made of the influence of pH, of certain neutral electrolytes, and of hydrostatic pressure on the rate of inactivation of equine *Staphylococcus* antitoxin in urea solutions and of the viscosity changes which take place during the denaturation. Deviations from simple first order behavior similar to those encountered in the inactivation of diphtheria antitoxin were observed, and the course of the reaction could be similarly explained by the simple kinetic mechanism proposed previously, involving two competing reactions of the native protein. The influence of certain electrolytes on the inactivation of the antitoxin was observed to be generally similar to their influence on the liberation by urea of sulfhydryl groups in other proteins. Hydrostatic pressure was found to have little or no effect on the rate or course of the reaction. The rate of the viscosity changes of the protein in the urea solution was also observed to vary with the pH, in a manner which was suggestive of the variation of the inactivation reaction, and confirmed the occurrence of relatively slow changes of the protein in the urea solution. The meaning of the results was discussed.

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