STUDIES ON THE DENATURATION OF ANTIBODY

II. The Effect of Protein Concentration on the Rate of Denaturation of Diphtheria Antitoxin by Urea

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Although in a recent study (1) the susceptibility of diphtheria antitoxin to the denaturing action of urea was apparently established, the question of the fundamental nature of the reaction remains essentially unsolved. Since the action of urea probably involves a partial unfolding of the polypeptide chains of proteins, the lability of antibody in urea suggests that the antibody structure resides in a particular folding of these chains, as proposed by Pauling (2). It is possible, however, to suggest other mechanisms for the observed inactivation; in particular, an aggregation of the protein molecules as a result of the denaturation might cause blocking off of unaltered antibody groups. Moreover complexing has frequently been observed by physical or immunological methods following denaturation of serum proteins by such agents as heat (3–6), ultraviolet light (7), and visible light in the presence of a photosensitizer (8, 9). With mixtures of serum albumin and globulin, urea denaturation leads to complexing demonstrable by electrophoretic analysis (10).

It is probable that if the inactivation of antibody were due to complexing, obviously a polymolecular process, this fact should be apparent from a study of the kinetics of the inactivation. It is of course difficult to exclude by kinetic studies all possibility of a polymolecular step in the process, since it may be masked by a unimolecular rate-determining reaction. It has been suggested, however, that complexing of the antibody would affect its behavior in the quantitative precipitation reaction (11). Thus complexing of solutions containing antibody and normal globulin might increase the amount of precipitable protein, since normal protein linked to the antibody would precipitate with it. The extent of this reaction should give some estimate of the degree of complexing accompanying the denaturation.

In a previous paper (1) the rate of inactivation of antitoxin in urea was found to decrease more rapidly as the reaction progressed than would be required by the first order law; preliminary experiments, however, suggested that the rate of inactivation was independent of the initial antibody concentration. The present paper is concerned with further experiments on the effect of protein con-

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centration on the denaturation of diphtheria antitoxin by urea, as measured by the Römer intracutaneous neutralization method and by the quantitative precipitation titration.

The diphtheria antitoxin used in all of the work was a pseudoglobulin preparation prepared from horse plasma by fractionation between 1.38 and 1.65 molar ammonium sulfate, followed by removal of water-insoluble globulin. It corresponds with fraction GII of Neurath *et al.* (12). Electrophoretic analysis indicated that most of the protein consisted of T component (13) plus a small amount of gamma globulin and traces of other components. The solution contained about 4000 units per ml. and 16 per cent protein. The antitoxic plasma and toxins were obtained from Sharp and Dohme, Glenolden, Pennsylvania, through the courtesy of Dr. Arnold Welch. Urea was twice recrystallized from 70 per cent alcohol (14). The pH measurements were made with a Beckman glass electrode meter, and are recorded as measured. All the denaturation reactions were carried out in a thermostat at 25° C.

The toxin neutralization titrations on the urea-denatured antitoxins were carried out by the Römer intracutaneous technique in rabbits (15). For each sample an appropriate series of dilutions in 1.0 ml. volume was prepared, each dilution usually being 6 per cent greater than the preceding. With samples in which a large amount of inactivation had occurred somewhat larger intervals were used. To each tube was added 1/12 L_f of toxin in 1.0 ml. and 1/10 ml. of the mixture injected intracutaneously into the rabbits. Readings were made after 3 days, and the end-point dilution taken as intermediate between the last negative and first positive reactions. An untreated antitoxin series was included with each group of unknowns, and the per cent of antitoxin remaining was calculated from the ratio of the end-point dilutions of the unknown and standard. The protein concentrations of the unknown samples varied somewhat due to unequal volume changes during the dialysis for removal of the urea; accordingly nitrogen determinations were made on the samples by micro-Kjeldahl analysis (16) and the per cent activity remaining in the sample corrected accordingly.

The quantitative precipitation tests were set up in duplicate in the usual way. Because the flocculation of some of the denatured samples was rather slow, all tests were incubated 20 hours at 42° C. and then left overnight in the refrigerator before analysis. The amount of precipitate was measured by the Folin method as described by Pressman (17); the author is indebted to Mr. Dan Rice for carrying out these analyses.

EXPERIMENTAL

A. Effect of Protein Concentration As Measured by Römer Titration

The effect of protein concentration was studied in 8 molar urea at two pH values. The first experiment was carried out in M/10 sodium borate buffer at pH 9.28. Two protein concentrations were used: 0.4 and 0.04 per cent. Samples were taken at intervals, diluted with an equal volume of saline, and dialyzed against large volumes of saline in the cold to remove the urea; Römer neutralization titrations and protein determinations were then made on the samples. The second experiment was carried out at pH 5.38 in M/10 sodium acetate buffer. The protein concentrations used were 0.4 and 0.08 per cent. The results are recorded in Figs. 1 and 2. It will be observed that in both experiments the results at the two concentrations are identical within the probable experimental error, indicating that the denaturation is first order in

protein. The deviation of the curves from linearity and the effect of pH in changing the course of the denaturation will be considered in a subsequent communication.

B. Effect of Protein Concentration As Measured by Precipitation Titration

Although at pH 7.8 in 8 molar urea the flocculating power of the antitoxin is rapidly lost (1), it was observed that antitoxin denatured at pH 6.20 in 8 molar urea for 25 hours still flocculated with toxin, although the flocculation time was greatly increased. Experiments at different protein concentrations were therefore carried out in acetate buffer under these conditions. The pro-

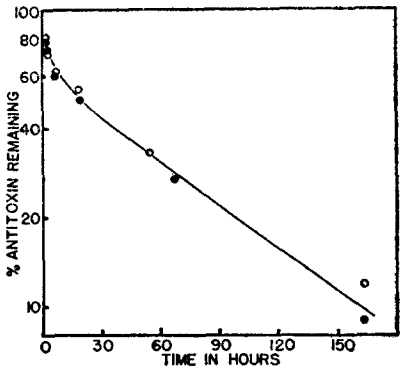

FIG. 1

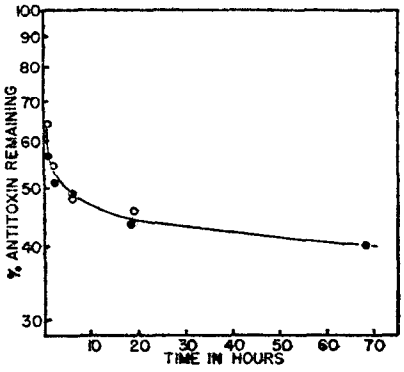

FIG. 2

FIG. 1. Antibody activity as a function of time of denaturation in eight molar urea at pH 9.28, studied at two protein concentrations. Open circles, 0.04 per cent protein; filled circles, 0.4 per cent protein.

FIG. 2. Antibody activity as a function of time of denaturation in eight molar urea at pH 5.38, studied at two protein concentrations. Open circles, 0.08 per cent protein; filled circles, 0.4 per cent protein.

tein concentrations used were 1.6, 0.4, and 0.08 per cent, and the samples were removed and dialyzed against saline, without preliminary dilution, after 1 hour and after 25 hours. In no instance did precipitation occur when the urea was removed; the 1.6 per cent protein sample became somewhat opalescent, however. After ample dialysis the samples were adjusted to approximately 0.4 per cent protein, the concentrated samples by dilution with saline, the dilute samples by fanning in a cellophane bag. The protein concentrations were estimated by micro-Kjeldahl analysis, and in setting up the precipitation tests the volumes of the solutions taken were adjusted to correct for the small variations in protein concentration, so that all tubes in the precipitation tests received the same amount of globulin. The results of the test are plotted in Fig. 3. The units for the amount of precipitate are the colorimeter readings;

they are approximately proportional to the amount of precipitate except at very low readings, where the values appear somewhat too high. The curves have been sketched through the points for convenience and do not represent any theoretical treatment of the data.

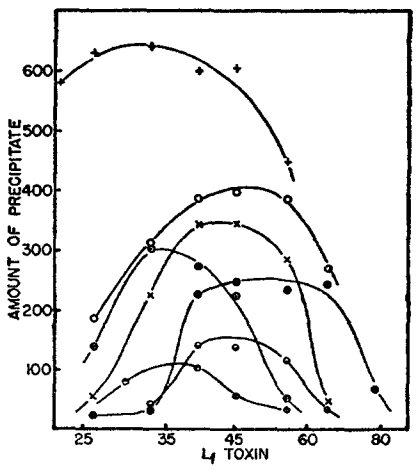

FIG. 3

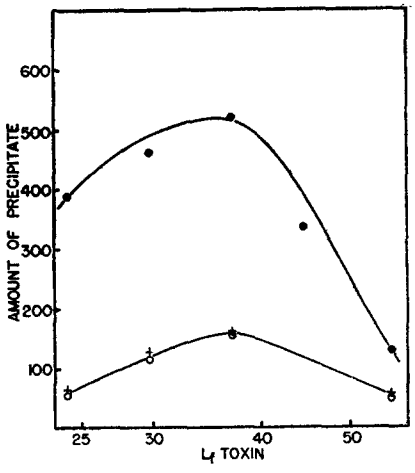

FIG. 4

FIG. 3. The amount of precipitate in arbitrary units as a function of the amount of toxin added, for untreated antitoxin and for antitoxin denatured in eight molar urea at pH 6.20 under various conditions. The amount of globulin was held constant in all of the precipitation tests. The filled circles represent the results with untreated antitoxin; the treatment of the other samples was as follows:—

Symbol	Time of denaturation *hrs.*	Protein concentration *per cent*	Neutralization titer *per cent*
○	1	1.6	75
+	25	1.6	56
×	1	0.4	76
⊙	25	0.4	62.5
◒	1	0.08	78
◓	25	0.08	57

FIG. 4. The amount of precipitate as a function of the amount of toxin added, for samples denatured for 25 hours in eight molar urea at pH 6.20. Filled circles, protein concentration 1.6 per cent during denaturation and during dialysis to remove urea. Open circles, protein concentration 0.32 per cent during denaturation and dialysis. Crosses, protein concentration 1.6 per cent during denaturation, diluted to 0.32 per cent with urea solution before dialysis.

The results, while confirming that the decrease in neutralizing titer is independent of the protein concentration, show that the amount of precipitate obtained in the precipitation test increases greatly with increasing protein concentration during the denaturation. The position of the optimum precipitation region shifts to smaller amounts of toxin, however, indicating decreased combining power for antigen. The shift in optimum is roughly that required by the decreased activity observed in the neutralization test.

In one experiment 0.125 per cent of crystalline carbohydrate-free horse serum albumin (18) was added to 0.4 per cent antitoxic globulin during the denaturation under the same conditions as above. This caused a *reduction* in the amount of precipitate with antigen to less than half the amount obtained when the albumin was not present. Apparently the nature of the protein present as well as its concentration influences the amount of precipitable protein after denaturation by urea, as with denaturation by heat (19).

C. Effect of Protein Concentration during Removal of Urea

Experiments were carried out to determine whether the effect of the protein concentration on the subsequent precipitation reaction occurred while the urea was present, or during the interval in which the urea was dialyzing out of the solution. A solution of 1.6 per cent protein in 8 M urea and M/10 sodium acetate buffer at pH 6.22 was prepared and immediately a portion of the solution was diluted fivefold with more of the urea-buffer solution so arranged that the only variation was in the protein concentration. After 25 hours another portion of the original solution was diluted in the same way, and all three solutions immediately dialyzed against saline to remove the urea. The concentrated solution was diluted fivefold with saline, nitrogen determinations made, and precipitation titrations carried out as in the previous section. The results are recorded in Fig. 4. It is clear that the solution which was denatured at 1.6 per cent protein and dialyzed at 0.32 per cent resembled very closely in its precipitation reactions the solution denatured and dialyzed at 0.32 per cent protein. An experiment set up in the same way but at a lower level of protein concentrations gave similar results; apparently the polymolecular process which leads to increased amounts of precipitate occurs not while the urea is present but while it is being removed. The results of Römer titrations on the samples were again independent of the protein concentration: 1.6 per cent protein sample, 57 per cent; 0.32 per cent protein sample, 62.5 per cent; 1.6 per cent protein sample diluted before dialysis, 62 per cent.

DISCUSSION

The experimental results show that the rate of denaturation of the antitoxin, as measured by its neutralizing power for toxin, is independent of the initial concentration, and that by this criterion the inactivation is a reaction of the first order The amount of precipitate formed by the denatured material with toxin, on the other hand, depends greatly upon the protein concentration. After denaturation at 1.6 per cent protein, for example, the amount of precipitate was more than twice that obtained from an equal amount of the untreated antitoxin. It seems probable that the process responsible for this effect is a complexing of the protein molecules so that non-antibody globulin is combined with the antibody globulin and carried down with it when it is precipitated with antigen. This sort of complexing has been observed following other types of

denaturation (3–9). The experimental results appear to eliminate complexing as the principal mechanism for the inactivation of antibody by urea, because the rate of the inactivation is independent of the protein concentration, and the amount of complexing, as estimated by the increased amount of precipitate with antigen, increases greatly with increasing protein concentration.

The effect of the protein concentration on the degree of complexing, and therefore presumably the complexing reaction itself, occurs during the removal of the urea from the solution. This is in accord with physical measurements, which indicate that the molecular weights of serum proteins are unchanged in urea solution (20, 21). Although the actual complexing occurs during the removal of urea, it is evident that it is dependent upon a relatively slow process occurring in the urea solution, since, at 1.6 per cent protein, the amount of precipitate with toxin is greater at 25 hours than at 1 hour, despite the reduction in the activity as shown by the neutralizing titer and the shift in the position of the optimum in the precipitation test. At 0.08 per cent protein the effect of the inactivation predominates, and the amount of precipitate is smaller at 25 hours than at 1 hour. At 0.4 per cent protein the two effects approximately balance each other.

With these facts it is not difficult to propose a reasonable picture of the nature of the complexing reaction. The urea causes a partial unfolding of polypeptide chains of the protein, presumably largely because of its neutralization of the hydrogen bonding affinity of the peptide linkage and other regions. In the presence of urea, then, the partially extended chain configuration is stable, and there is little tendency for the formation of either intra- or intermolecular bonds, since the bond-forming affinities are largely neutralized. As the urea is removed this bond-forming affinity returns; it may be satisfied either by the formation of intramolecular bonds approximating more or less those of the native protein or by the formation of similar bonds which happen to involve parts of two protein molecules. It is clear that the number of intermolecular bonds formed would vary with the protein concentration during the removal of the urea. It seems probable that the "irreversibly denatured" fraction of Neurath *et al.* (21) may be formed by a process of this sort, especially since the amount formed is dependent upon the concentration of protein (22).

It is probable that even if no complexing were to occur, the amount of precipitate with antigen would be a complex function of the number of intact antibody groups remaining. If it be postulated that the action of urea leads to a random inactivation of the combining sites of bivalent antibody, then the first portion of the reaction would lead preponderantly to the formation of univalent antibody, and this material could, up to a certain point, be built into the precipitate framework, so that the amount of precipitate would remain unchanged. At some point in the destruction of antibody, however, the univalent material would reach a concentration where it would interfere appreciably

with the precipitation of the unaltered antibody, and would eventually inhibit precipitation completely, long before the antibody activity was largely destroyed. With the possibility of complexing added to these considerations it is evident that the interpretation of the amount of precipitate in terms of antibody activity becomes very difficult.

SUMMARY

The specific rate of inactivation of antitoxin in urea solutions, as measured by the Römer neutralization test with toxin, has been shown to be independent of the concentration of protein under the conditions studied. The amount of precipitate obtained in the quantitative precipitation test with toxin, however, increases greatly with increasing protein concentration during denaturation. The time during which the protein concentration is important in this respect has been shown to be the interval in which the urea is being dialyzed from the solutions. The meaning of the results is discussed.

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