STUDIES ON THE DENATURATION OF ANTIBODY

I. The Action of Urea on Diphtheria Antitoxin

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Although a great deal of information has been accumulated regarding the stability of antibody under various conditions and treatments (1), the action of urea and similar substances seems to have been largely neglected. Since the action of urea in denaturing proteins involves weakening the secondary bonds about the peptide linkages (2, 3) it is of interest to know the effect of urea on antibody. Pappenheimer, Lundgren, and Williams (4) report that diphtheria antitoxin is unaffected by "strong" urea solutions. Very recently Erickson and Neurath (5) have reported some experiments on the action of guanidine hydrochloride on horse antipneumococcus antibody.

The present investigation of the stability of antibody in urea was undertaken because of the presumable importance of the results to the theory of antibody structure. Diphtheria antitoxin has been chosen as the antibody for study because the accurate neutralization methods available for its determination permit direct measurement of the fundamental property of antibody, its ability to combine with antigen.

Materials and Methods

The antitoxic globulin used was a commercial preparation obtained from horse plasma by ammonium sulfate fractionation between 32 and 50 per cent saturation. It contained 20 per cent protein, and had a potency of 3000 units of antitoxin per ml.; phenol was present as a preservative. Quantitative precipitation tests (6) showed that 18 per cent of the protein was precipitable with toxin. Electrophoretic analysis in sodium phosphate buffer of pH 7.8 and ionic strength 0.1 indicated that the globulin consisted mainly of the T component (7), plus a small amount of gamma globulin and traces of faster components. The toxin contained 42.5 L_f per ml. Both toxin and antitoxin were obtained from Sharp and Dohme, Glenolden, Pennsylvania, through the courtesy of Dr. Arnold Welch. Most of the antitoxin titrations were carried out by the Römer intracutaneous method in rabbits (8). The accuracy of these titrations, as determined by the dilution intervals used, was better than 5 per cent in most of the determinations. However, in part A and in instances in which a large amount of inactivation occurred the error approached 10 per cent.

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The Ramon flocculation titrations were carried out with toxin in the usual way. The pH measurements were made with a Beckman glass electrode meter and are recorded as determined, no attempt being made to correct for the effect of the urea (9–11). Nitrogen determinations were carried out by the micro-Kjeldahl method of Koch and McMeekin (12). Urea was twice recrystallized from 70 per cent alcohol according to the method of Steinhardt (3).

EXPERIMENTAL

A. Variation of Antibody Inactivation with Urea Concentration.—

The antitoxin was diluted 1–20 in a series of urea solutions of varying concentrations containing neutral sodium phosphate at a final concentration of 0.05 molar. The apparent pH of each sample was measured and is recorded in Table I. The solutions were left at room temperature for 48 hours and were then dialyzed in cellophane against

TABLE I

Stability of Antitoxin for 48 Hours at Various Urea Concentrations

Molar concentration of urea	Apparent pH	Antitoxin remaining		Flocculation time at 42°C.
		Ramon test	Römer test	
		per cent	*per cent*	*min.*
0	7.32	100	100	80
1.9	7.40	100	100	90
3.8	7.48	94	100	105
5.7	7.62	75	80	600
7.6	7.78	*	60	>4000

* No flocculation within 3 days.

0.9 per cent sodium chloride in the cold to remove the urea; no precipitation occurred during dialysis. The samples were then assayed for antitoxin by the Römer intracutaneous method and the Ramon flocculation test. The protein content of the samples was estimated by nitrogen determinations, and the results of the antibody determinations were corrected to uniform protein concentration.

The per cent antibody remaining at each urea concentration as measured by the Römer titration is indicated in Fig. 1. The flocculation time in the Ramon test was greatly increased with the samples which had been exposed to the higher urea concentrations. The flocculation time and the per cent antibody remaining as indicated by Ramon and Römer titrations are recorded in Table I.

Neurath (13) has shown that after removal of urea from pseudoglobulin solutions part of the protein is insoluble in water at pH 6.0. We have obtained similar results with the antitoxin. It seemed of interest to determine whether this water-insoluble material had retained antibody activity. The untreated antitoxin was first dialyzed against distilled water and adjusted to pH 6.0, and the small amount of euglobulin was removed by centrifugation. The sample

was then denatured by 7.5 molar urea as before, and the protein insoluble in water was separated. Römer titration indicated that the water-insoluble fraction contained about half as much antitoxin activity per milligram of nitrogen as the water-soluble material.

B. The Effect of Urea plus Sodium Sulfite.—

Although urea does not set free detectable sulfhydryl groups in serum proteins, it does reveal disulfide linkages which may be reduced to sulfhydryl by suitable reducing agents (14, 15). Experiments were carried out to determine the effect of urea plus sodium sulfite on the antitoxin activity. A series of urea concentrations was set up as in the first experiment except that 0.1 molar

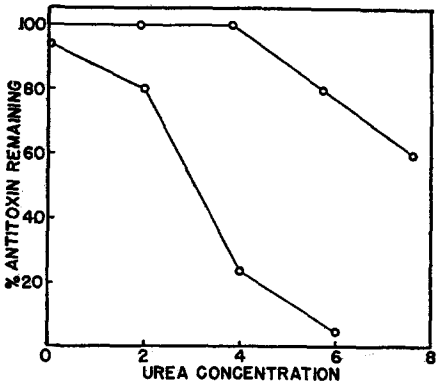

FIG. 1

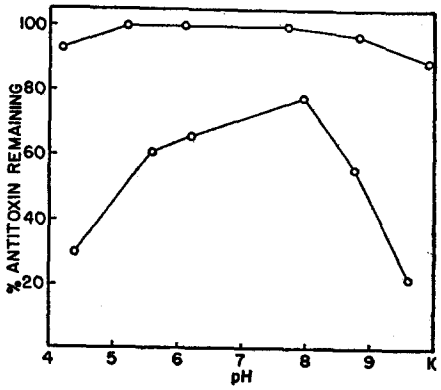

FIG. 2

FIG. 1. Stability of antitoxin for 48 hours at various urea concentrations. Upper curve, urea alone; lower curve, urea plus 0.1 molar sodium sulfite.

FIG. 2. Stability of antitoxin for 48 hours in water and in urea, at various pH values. Upper curve, no urea; lower curve, 6 molar urea. The pH was measured just before the samples were dialyzed.

sodium sulfite was included. The time of action was 48 hours. After dialysis against saline the samples were titrated for antitoxin by the Römer method. The results are plotted in Fig. 1. The 4 and 6 molar urea samples were slightly opalescent after dialysis. Much greater inactivation of antitoxin occurred in urea and sodium sulfite than in urea alone. The sodium sulfite caused slight inactivation of antitoxin in the absence of urea.

C. The Effect of pH.—

The effect of pH on the denaturation of antibody in 6 molar urea was next investigated.

A solution containing urea, neutral sodium phosphate, and antitoxin was divided into portions which were adjusted to pH 4, 5, 6, 8, 9, and 10 with dilute hydrochloric acid or sodium hydroxide and quickly adjusted to a volume such that the antitoxin

dilution was 1–20 and the urea and phosphate concentrations were 6.0 molar and 0.05 molar respectively. A similar set of control samples was prepared without urea. All were left at room temperature for 48 hours, the final pH was determined, the samples were dialyzed in the cold against buffered saline, and the antitoxin was titrated by the Römer method. No precipitation occurred during the dialysis, but the urea sample at pH 4.4 became somewhat opalescent.

The results are recorded in Fig. 2. The antitoxin destruction in the absence of urea is in substantial agreement with the report of Moloney and Taylor (16).

D. The Rate of Antitoxin Inactivation in Urea.—

The inactivation of antitoxin in urea was studied as a function of time. To a solution containing 45 gm. of urea and 0.005 mole of neutral sodium phosphate, with

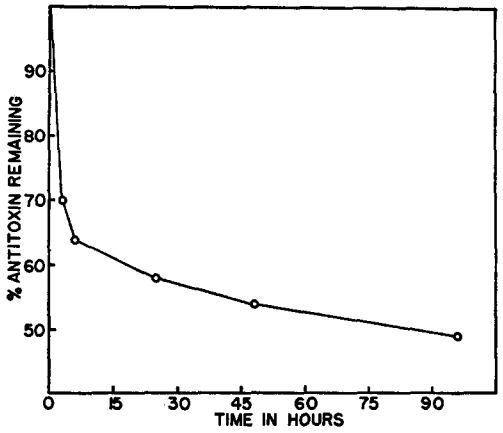

FIG. 3. Stability of antitoxin in 7.5 molar urea at pH 7.82 as a function of time.

a total volume of 95 ml., 5 ml. of antitoxin was added. The final urea concentration was 7.5 molar and the pH was 7.82. The solution was left at room temperature (25–27°), and samples were removed after 3, 6, 25, 48, and 96 hours. The samples were dialyzed with mechanical stirring in thin cellophane tubing for 1 hour at room temperature, and then for several days in the cold.

Results of Römer titrations on the samples are recorded in Fig. 3. The urea seemed to dialyze out of the samples very rapidly and the error due to continuation of the reaction during dialysis is presumably not great. This conclusion is supported by an experiment in which the sample after 3 hours' denaturation under the same conditions was diluted 100-fold in saline before dialysis; the activity in this case was essentially identical with that of the undiluted dialyzed sample. It was also determined that at 3 hours the proportion of antitoxin remaining undenatured was the same if the initial antitoxin concentration was one-quarter of that used above. A preliminary study has indicated that the

viscosity of the antitoxin in urea also fails to reach a final value immediately, but increases appreciably over a considerable period.

DISCUSSION

In a recent theory of the nature of antibody, Pauling (17) has suggested that the antibody activity of globulin is due to a configuration of the surface of the molecule complementary to the antigen. This configuration is visualized as being the result of a particular folding of the polypeptide chain, which is stabilized by hydrogen bonding and other forces. Once these bonds are broken, the molecules would be free to assume any of a great number of configurations, and the specificity of the globulin for the antigen would disappear.

By means of viscosity and diffusion measurements, Neurath and coworkers (13) have studied the dimensions of serum protein molecules and have concluded that considerable unfolding of the molecules occurs in urea solution, as indicated by the shape of the molecules. The molecular weights of serum albumin and globulin are unchanged in urea solution, even if the disulfide linkages are reduced (15, 18). The action of urea (2, 3) involves a weakening of the association of adjacent polypeptide chains by competition of the urea for the hydrogen bonding affinity of the peptide linkage and the breaking of other hydrogen bonds, between side chains, and possibly also of other linkages in the protein molecule. On the basis of ultraviolet absorption studies at different pH values, Crammer and Neuberger (19) have recently concluded that in native ovalbumin the phenolic hydroxyl groups in the tyrosine residues are involved in hydrogen bonds of considerable strength. The importance of the bonds formed by these and other residues in maintaining protein structure and the susceptibility of these bonds to the various denaturing agents await further investigation.

The present investigation demonstrates that diphtheria antitoxin may be inactivated by urea; this result is readily explained by the theory of antibody structure outlined above. Many points remain to be clarified, however, before a comprehensive theory of the process can be formulated. It is not clear whether the observed inactivation represents a general weakening of all the antitoxin combining groups or a complete destruction of a portion of them. We are inclined to favor the second possibility. On this basis, the loss of flocculating power with toxin after urea treatment may be accounted for by assuming that a considerable proportion of the antibody becomes univalent, and therefore inhibits the precipitation of the unaltered bivalent antibody.

The appreciable antibody activity of the protein which is water-insoluble after urea treatment suggests that the inactivation of antibody may be due to a different process or involve a different part of the molecule than that which causes part of the protein to become insoluble in water. There would seem to be no *a priori* reason why one process should be considered a more funda-

mental criterion of denaturation than the other. The marked effect of pH suggests that destruction of the antibody configuration may be correlated with the ionization of acidic or basic groups. A similar effect of alkali in greatly increasing the rate of denaturation of hemoglobin has been observed by Drabkin (20). In a recent comprehensive study of the denaturation of tobacco mosaic virus in urea, Lauffer (11) has demonstrated that pH, ionic strength, and temperature are among the factors determining the rate of denaturation. In view of these conclusions, no quantitative interpretation of the observed pH effect is possible, since it may be due in part to the variation of the ionic strength of the phosphate. It is probable that the effect of sulfite is due to its power of splitting the disulfide links, with production of sulfhydryl groups, and this suggests that the disulfide links play a part in holding the molecule in its original configuration.

The observations on the rate of denaturation do not seem to be explicable on any simple kinetic basis. The fact that the rate is independent of the initial antitoxin concentration indicates that the denaturation is first order with respect to antitoxin, as would be expected. It is probable that the decreasing rate of the reaction with time is due to a heterogeneity of the antibody groups or molecules. A similar decrease in reaction rate is observed in the denaturation of tetanus antitoxin by heat (21). Further studies on the reaction rate and other problems suggested by the present investigation are in progress in these Laboratories.

SUMMARY

Diphtheria antitoxin has been shown to be inactivated in the presence of urea. In 7.5 molar urea at pH 7.82 nearly half the activity is destroyed in 24 hours, as measured by the neutralizing power for toxin. The reaction appears to be first order with respect to antitoxin, since the initial rate is independent of the antitoxin concentration, but the rate decreases rapidly with time. Higher or lower pH or the presence of M/10 sodium sulfite increases the antitoxin inactivation.

The meaning of the results is discussed.

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