the solubility of the salts and the heat of solution calculated from the slope of the line obtained when the logarithm of the solubility is plotted against the reciprocal of the absolute temperature. The method of least squares was used to find the best slope.

Microscopic Examination of Crystals.—All of the solid salts prepared were examined with a microscope using polarized light. They are all anisotropic. This has been previously reported[11,12,13,14] for all of these salts except anhydrous lithium perrhenate and its hydrates.

Freezing Point of Ammonium Perrhenate.—Although it was found that ammonium perrhenate decomposes rapidly at 365° at atmospheric pressure, its freezing point can be estimated from other properties. Plots of solubility, molar volume and radius of the cation against the freezing points of the alkali metal perrhenates indicate freezing points of 550, 605 and 580°, respectively, for ammonium perrhenate. The average value, approximately 580°, is predicted as the freezing point of this salt.

Summary

The perrhenates of the alkali metals and ammonium have been prepared and the formulas verified. The densities, freezing points and solubilities from 0 to 50° have been measured. The heats of solution have been calculated. The freezing point of ammonium perrhenate has been predicted.

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CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 1072]

Studies on the Denaturation of Antibody. III. Kinetic Aspects of the Inactivation of Diphtheria Antitoxin by Urea

BY GEORGE G. WRIGHT AND VERNER SCHOMAKER

Evidence has been presented[1,2] that the inactivation of antitoxin which takes place in urea solutions, as shown by the change in toxin-neutralizing activity, is a first-order reaction in antibody, since the specific rate of inactivation is independent of the initial concentration of antibody. This indicates that a polymolecular complexing reaction of the protein molecules[3-6] is not the mechanism of the inactivation under consideration, even though the occurrence of complexing as a result of the urea treatment could be inferred from the abnormalities of the quantitative flocculation reaction with toxin of the denatured samples.[2] The course of the reaction is not entirely typical of a classical first-order process, however, since the rate decreases more rapidly as the reaction proceeds than is predicted by the first-order law. Similar results have been obtained in the inactivation of antitoxin by heating in the neighborhood of 65°.[7,8] An understanding and a quantitative formulation of these deviations from first-order behavior are essential if the experimental results on antibody inactivation are to yield the maximum information regarding the chemical nature of the reactions and the structure and stabilizing forces of the combining region of the antibody.

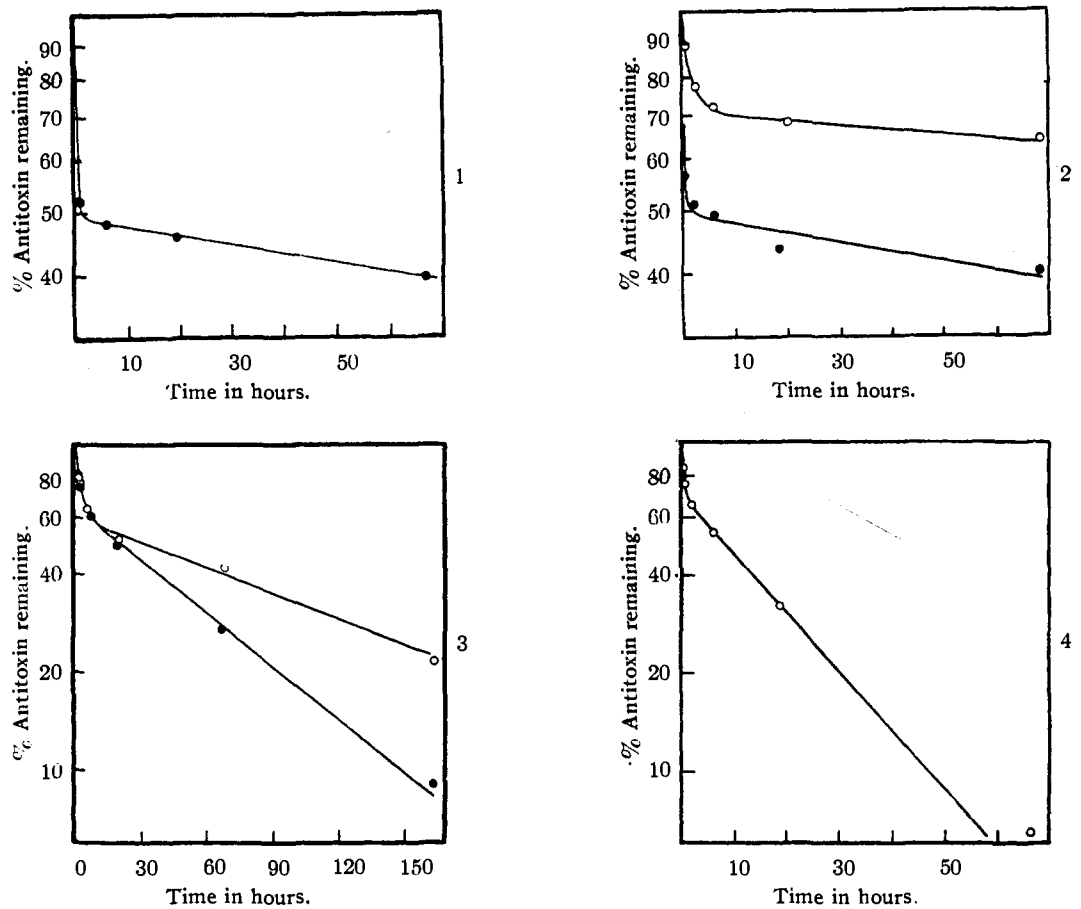
In the present investigation we have studied the effects of *p*H, urea concentration, and temperature on the inactivation reaction, in order to explore the influence of these factors on its abnormalities. The experimental results are shown to be compatible with a simple kinetic mechanism.

Materials and Methods

The diphtheria antitoxin was the same globulin preparation used in previous work,[2] and consisted of the water-soluble portion of the protein precipitated from crude horse antitoxin plasma between 1.38 and 1.65 *M* ammonium sulfate. It contained 16% protein and had an activity of about 4000 units per ml. The urea was twice recrystallized from 70% alcohol. The denaturation reactions were carried out in buffered solutions at ionic strength about 0.1; acetate was used in the acid range and borate in the alkaline. The buffers were prepared by mixing various proportions of 0.2 *N* sodium hydroxide and a solution 0.2 *N* in the acid and 0.2 *M* in sodium chloride, so that the ionic strength remained approximately constant at different *p*H values. The *p*H measurements were made with a glass electrode. The buffers were effective in keeping the *p*H change during the experiment to less than 0.1 *p*H unit in practically every case; the recorded value represents the average of the initial and final *p*H measurements. The reactions were carried out in a thermostat constant to within a few hundredths of a degree, at 25.0° except where otherwise indicated.

The solutions for denaturation were prepared by weighing out the calculated amount of urea (using the density data of Dunstan and Mussel[9]), adding

(1) G. G. Wright, *J. Expll. Med.*, **79**, 455 (1944).
(2) G. G. Wright, *ibid.*, **81**, 647 (1945).
(3) J. van der Scheer, R. W. G. Wyckoff and F. L. Clarke, *J. Immunol.*, **40**, 39 (1941).
(4) A. Kleczkowski, *Biochem. J.*, **37**, 30 (1943).
(5) B. D. Davis, A. Hollander and J. P. Greenstein, *J. Biol. Chem.*, **146**, 663 (1942).
(6) H. Smetana and D. Shemin, *J. Expll. Med.*, **73**, 223 (1941).
(7) T. D. Gerlough and W. White, *J. Immunol.*, **27**, 367 (1934).
(8) F. H. Johnson and G. G. Wright, *Proc. Natl. Acad. Sci.*, **32**, 21 (1946).
(9) A. E. Dunstan and A. G. Mussel, *J. Chem. Soc.*, **97**, 1935 (1910).



Figs. 1–4.—Logarithm of antibody activity as a function of time of denaturation in 8 *M* urea at 25°, studied at various *p*H values. Fig. 1, *p*H 4.91. Fig. 2, filled circles, *p*H 5.38; open circles, *p*H 6.25. Fig. 3, open circles, *p*H 8.85; filled circles, *p*H 9.27. Fig. 4, *p*H 9.73.

an amount of buffer equal to half the final volume of the solution, and the required amount of water. After the urea had dissolved and the solution had come to temperature in the thermostat, the experiment was started by adding the antitoxic globulin. All the denaturation reactions reported in this paper were carried out at a protein concentration of 0.40%. At the desired intervals, 5-ml. samples were removed, diluted with an equal volume of saline to stop the reaction promptly, placed in cellophane tubing, and dialyzed against several large volumes of saline in the cold to removed the urea. The antitoxin in the samples was assayed by the Römer intracutaneous method in rabbits[10] and corrected for volume changes during the dialysis in accordance with the results of micro-Kjeldahl determinations as described previously.[2] Untreated antitoxin was included in each assay in addition to the partially inactivated samples, and the fraction of the antitoxin activity remaining in the samples was taken as the ratio of the largest amounts of untreated antitoxin and partially inactivated antitoxin which permitted a certain minimal skin reaction when added to the constant amount of toxin.

(10) C. Jensen, *Acta Path. Microbiol. Scand., Suppl.*, 14 (1933).

Experimental

A. The Effect of *p*H.—Previous results had shown that the degree of inactivation of antitoxin after urea treatment for a constant time was influenced markedly by the *p*H.[1] Accordingly, the course of the inactivation was investigated in 8 *M* urea at a series of *p*H values. Experiments were carried out in acetate buffer at *p*H 4.91, 5.38 and 6.25, and in borate buffer at *p*H 8.85, 9.29 and 9.73. The results are given in Figs. 1–4, where, as in Figs. 5–8, the curves represent the theoretical formulation advanced in the Discussion.

It is possible that specific salt effects may be involved in the variation of the experimental results with change in *p*H, even though the ionic strength was held approximately constant. Exploratory experiments suggested that such variations of ionic strength as did occur could not be of major importance in influencing the rate and course of the reaction. However, further experi-

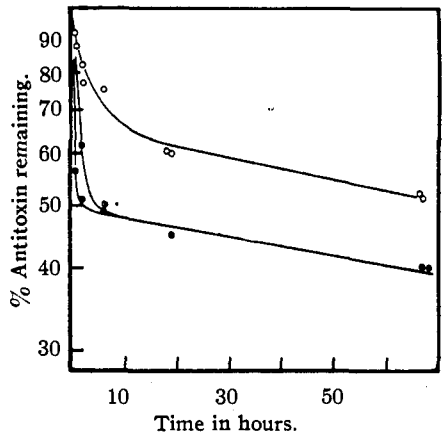


Fig. 5.—Logarithm of antibody activity as a function of time of denaturation in various concentrations of urea at *p*H 5.38 at 25°: open circles, 6 *M* urea (two different experiments); dotted circles, 7 *M* urea; filled circles, 8 *M* urea.

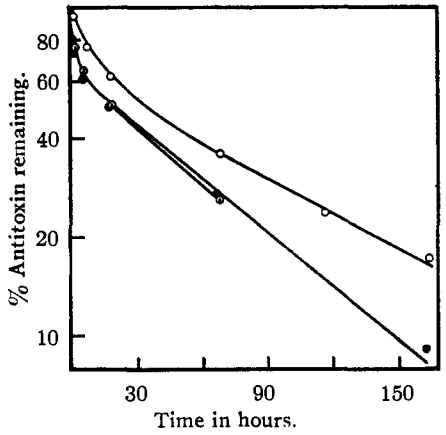


Fig. 6.—Logarithm of antibody activity as a function of time of denaturation in various concentrations of urea at *p*H 9.26 at 25°: open circles, 6 *M* urea; dotted circles, 7 *M* urea; filled circles, 8 *M* urea.

ments on variation of the ionic strength and on the effects of specific buffer salts and buffer acids would be desirable.

In all of the Figs. 1–8 the fraction of the original antibody activity is plotted on a logarithmic scale and the time on a linear scale, a treatment which reduces the data for a simple first-order reaction to a straight line. Clearly, the reaction deviates from this behavior, and the degree of deviation is influenced markedly by the *p*H. Each of the curves, however, shows a definite tendency to approach linearity after the initial curved portion.

The possibility that the deviation from linearity might be due to some uncontrolled variable in the experimental method has been considered rather extensively. Although it is difficult to exclude this possibility entirely, considerable evidence now supports the conclusion that the deviation is in fact a property of the reaction under consideration. The solutions were adequately buffered, and no

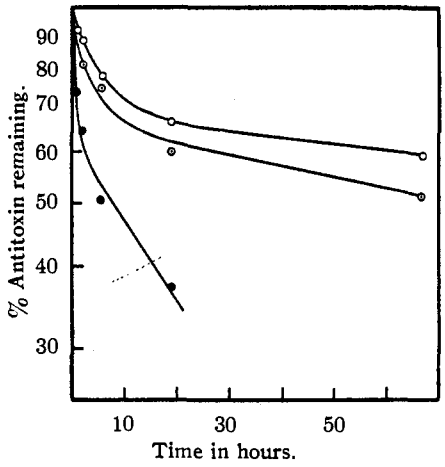


Fig. 7.—Logarithm of antibody activity as a function of time of denaturation in 6 *M* urea at *p*H 5.38 at various temperatures: open circles, 1.6°; dotted circles, 25°; filled circles, 35°.

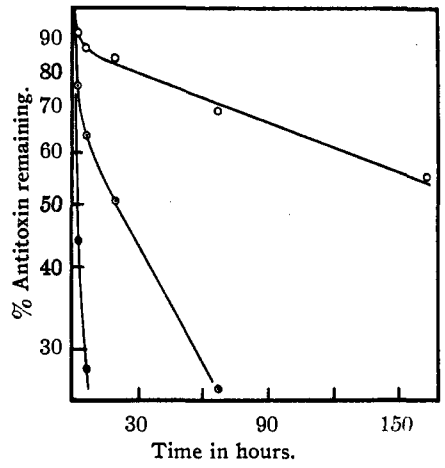


Fig. 8.—Logarithm of antibody activity as a function of time of denaturation in 7 *M* urea at *p*H 9.23 at various temperatures: open circles, 10°; dotted circles, 25°; filled circles, 40°.

significant *p*H change occurred during the reaction. The curves were reproducible and seemed to show an orderly change with *p*H. No change was observed with different urea preparations, and, as noted below, a similar deviation was observed in guanidine hydrochloride inactivation. Perhaps most important, however, was the fact that the shape of the curve was independent of the initial concentration of protein,[2] for if the exhaustion of some contaminant or component of the solution not contributed by the antitoxin solution were responsible for the decrease in specific rate, it should in general occur more rapidly at high protein concentration. The same would be true even if such a substance originated in the antitoxin solution itself.

B. The Effect of Urea Concentration.—The effect of urea concentration was investigated at

two representative *p*H values, one in the acid and the other in the alkaline region. Data were obtained for the reaction at 6 and 7 *M* concentrations of urea, and compared with the data already obtained at 8 *M* urea. Since the urea concentration influences the *p*H of the solution, small changes were made in the composition of the buffer used in the 6 and 7 *M* experiments, in order to keep the *p*H constant, as indicated by measurements with the glass electrode. The results are given in Figs. 5 and 6.

It is evident that the effect of increasing the urea concentration is not a uniform increase in the rate of over-all reaction, since the early part of the reaction is greatly speeded up by increase in urea and the latter part of the reaction is speeded up relatively slightly.

C. The Effect of Temperature.—An interesting aspect of the denaturation of proteins in urea is the frequent occurrence of a negative temperature coefficient.[11] Lauffer[12] found that the denaturation of tobacco mosaic virus shows a negative temperature coefficient below room temperature and a positive coefficient above; he advanced a quantitative formulation of this behavior. In a study of the urea denaturation of ovalbumin by measurement of the turbidity after removal of urea by dialysis, however, no negative coefficient was found from 4–40°.[13] It is reasonable that various phases of the denaturation reactions of different proteins with urea should have somewhat different activation energies, so that rather diverse temperature characteristics would be found depending upon the protein and the assay employed for the estimation of extent of denaturation. The results for our reaction, which are recorded in Figs. 7 and 8, show an over-all positive temperature coefficient through the region 0–40°, with, as in the case of ovalbumin, an indication that the reaction may have a minimum rate in the neighborhood of 0° and a negative temperature coefficient at lower temperatures. Although our data are too few and too scattered to demonstrate the existence of such a minimum in the rate of denaturation of antitoxin, they do show that if it does exist it must lie considerably below 25°, the temperature of minimum rate of denaturation of tobacco mosaic virus.[12]

The experiments were carried out under two conditions of urea concentration and *p*H, urea concentrations of less than 8 *M* being necessary because of the reduced solubility of urea at the lower temperatures. In each experiment a single mixture was divided into three parts which were quickly adjusted to the desired temperatures. Accordingly, the three solutions were identical at 25°, but no doubt there were minor differences in *p*H, etc., at the temperatures at which they were allowed to react.

(11) F. G. Hopkins, *Nature*, **126**, 328, 383 (1930).
(12) M. A. Lauffer, THIS JOURNAL, **65**, 1793 (1945).
(13) J. H. Clark, *J. Gen. Physiol.*, **27**, 101 (1943).

D. Effect of Guanidine Hydrochloride.—The action of guanidine hydrochloride on proteins is generally rather similar to that of urea, but, at equimolar concentration, the denaturing action of the guanidine hydrochloride is considerably greater.[14] A preliminary experiment was carried out to explore the denaturing action of guanidine hydrochloride on antibody activity. Eight *M* guanidine hydrochloride was used, and no buffer or other electrolyte was added; otherwise the methods were the same as in the experiments with urea. The initial *p*H was 6.26, and after forty-eight hours had changed only to 6.28. The results resembled rather closely those for denaturation in 8 *M* urea at *p*H 4.91, but the deviation from linearity was even more extreme. The activity dropped to about 45% of the original in one hour, but after forty-eight hours was still 40%.

Discussion

Although the specific rate of inactivation of antitoxin by urea is independent of the initial concentration of antitoxin,[2] it is clear that the course of the reaction deviates from the typical behavior of a first-order reaction, the specific rate of inactivation decreasing as the reaction proceeds so as to produce a strong deviation from linearity of the log activity *vs.* time plots. The present work shows that this deviation varies greatly with the *p*H and to a lesser extent with the temperature and urea concentration even though it is independent of the protein concentration.[2] The kinetic situation appears in some of these respects to be the opposite of that for the denaturation of tobacco mosaic virus in urea as studied by the disintegration of the virus or by its insolubility on dilution.[12] Nearly straight lines were obtained when the log of the concentration of remaining virus was plotted against the time, but the specific rate varied considerably with the initial concentration of the virus. Preliminary results also suggest that the rate of inactivation of the antibody is less dependent on the ionic strength of the solution than is the rate of denaturation of the virus. It is not clear whether these differences in behavior are due to the very considerable differences in the proteins, or to the different properties of the proteins which were under consideration in the two cases.

It was suggested[1] that the deviation of the inactivation reaction from typical first-order behavior may be due to a heterogeneity of the antibody combining groups in their susceptibility to denaturation by urea. This view was strengthened when it was found to be possible to account quantitatively for the action of simple haptens in inhibiting the precipitin reaction by assuming a normal-error-curve distribution of the free energies of interaction of the antibody molecules with simple hapten.[15] For, although a heterogeneity of

(14) J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938); **128**, 233 (1939); **130**, 519 (1939); **136**, 795 (1940).
(15) L. Pauling, D. Pressman and A. L. Grossberg, THIS JOURNAL, **66**, 784 (1944).

antibody molecules with respect to their interactions with hapten does not require that the molecules be correspondingly heterogeneous in their susceptibility to the denaturing action of urea, such a situation is definitely possible. Attempts were accordingly made to fit the data for antibody denaturation on the assumption, analogous to that adopted for the discussion of the hapten inhibition experiments, that the free energy of activation for the first-order denaturation process is distributed according to a normal-error-curve

$$N(E^*)\mathrm{d}E = \frac{1}{\sigma\sqrt{2\pi}} e^{-(E^*-E_0^*)^2/2\sigma^2}\mathrm{d}E^*$$

Tables and plots of the resulting expression

$$\text{Remaining fraction of antibody activity} = \frac{1}{\sqrt{\pi}}\int_{-\infty}^{\infty} e^{-k_0 t e^{-bx} - x^2}\mathrm{d}x$$

were prepared[16] for comparison with tl. observed curves. Here t is the time, k_0 is the specific rate of denaturation for antibody with activation energy E_0^* and b is $2^{1/2}\sigma/RT$. The fits were often unsatisfactory, since the tails of the plots of this function do not tend to approach straight lines, as seems to be required by the data, and the rapid decline in activity is predicted to amount always to about 50%, in very pronounced disagreement with some of the experimental curves (for example, the upper curve of Fig. 2). Although the shapes of the curves could, of course, be made to fit the data by the assumption of special, non-normal distribution functions for the free energy of activation—as will be seen below, they would indeed need to be simple bimodal distributions—the notion that heterogeneity of the antibody is the main reason for the peculiar kinetics of the reaction was discarded because the values of b required are sometimes greater by an order of magnitude than for the case of hapten inhibitions,[15] and they vary with change in experimental conditions, particularly the pH, by what seems to be an unreasonably great amount.

It is, of course, entirely possible that the reaction would proceed by more than one route or mechanism. If each of the mechanisms led independently to inactivation according to the simple first-order law, however, their sum would also follow the first-order law, as has been mentioned by Lauffer[17]; evidently the observed deviation is not to be accounted for in this manner.

A significantly different approach is suggested by the notions that an antibody globulin molecule may undergo denaturation in a number of different ways[18]; that some of these may involve an unfolding of the antibody region, with consequent inactivation, while others may not[18]; and that reactions of this second type can lead reversibly to a product which retains its activity (or regains it when the urea is dialyzed out) but is not susceptible to the inactivation by the first type of reaction. It turns out that the particular, comparatively very simple formulation of these notions

$$I \xleftarrow{k_1} N \underset{k_3}{\overset{k_2}{\rightleftarrows}} P$$

is consistent with all our data. Here N represents the original, native antibody (or altered antibody not distinguishable from the native antibody in our experiments), I represents the inactivated antibody, and P stands for the postulated active, protected antibody. Transformations among these three molecular species by first-order reactions proceed according to the specific rate constants, k_1, k_2 and k_3, which, although constant in any one experiment, are dependent on the experimental conditions. It is easy to see how this mechanism leads to the correct general results including the straight-line tails of the log activity *vs.* time plots. At the beginning of the reaction the competing transformations of N into I and P predominate, and, although N disappears according to the first-order law, the total activity decidedly does not. Instead, it decays with a rapidly decreasing specific rate, corresponding to an incomplete limiting degree of inactivation. Gradually, however, the reverse action $P \xrightarrow{k_3} N$ becomes important (if $k_3 \neq 0$) and, by providing a continuing source of N such that ultimately a constant fraction of the active material is present as N in steady-state equilibrium with P, leads to ultimate complete inactivation of the antibody according to the first-order law.

It is not difficult to solve the differential equations

$$\mathrm{d}[N]/\mathrm{d}t = -(k_1 + k_2)[N] + k_3[P]$$
$$\mathrm{d}[P]/\mathrm{d}t = +k_2[N] - k_3[P]$$

for this mechanism. The result is

$$[N] = N_1 e^{-\lambda_1 t} + N_2 e^{-\lambda_2 t}$$
$$[P] = P_1 e^{-\lambda_1 t} + P_2 e^{-\lambda_2 t}$$

provided that λ_1 and λ_2, the roots (always positive, real numbers) of the determinantal equation

$$\begin{vmatrix} \lambda - k_1 - k_2 & +k_3 \\ +k_2 & \lambda - k_3 \end{vmatrix} = 0; \quad \begin{matrix} \lambda_1 + \lambda_2 = k_1 + k_2 + k_3 \\ \lambda_1\lambda_2 = k_1k_3 \end{matrix}$$

are not equal. The constants N_1, N_2, P_1 and P_2 are determined by the general equations

$$N_1(\lambda_1 - k_1 - k_2) + P_1k_3 = 0$$
$$N_2(\lambda_2 - k_1 - k_2) + P_2k_3 = 0$$

and the conditions for the initial amounts of N and P

$$N_{t=0} = N_0 = N_1 + N_2 \text{ and } P_{t=0} = P_0 = P_1 + P_2$$

In this way the constants $T_1 = N_1 + P_1$ and $T_2 = N_2 + P_2$ of the expression

$$[T] = [N] + [P] = T_1e^{-\lambda_1 t} + T_2e^{-\lambda_2 t} \quad (1)$$

for the total activity are also determined

$$N_1 = \frac{(\lambda_1 - k_3)N_0 - k_3P_0}{\lambda_1 - \lambda_2} \qquad N_2 = \frac{(\lambda_2 - k_3)N_0 - k_3P_0}{\lambda_2 - \lambda_1}$$

(16) L. Pauling, V. Schomaker and E. E. Hammond, Jr., to be published.

(17) M. A. Lauffer, *Arch. Biochem.*, **8**, 272 (1945).

(18) G. G. Wrigbt and L. Pauling, *Science*, **99**, 198 (1944).

TABLE I

CONSTANTS OF THE EQUATION $T = T_1e^{-\lambda_1 t} + T_2e^{-\lambda_2 t}$ AS FITTED TO THE DATA OF FIGURES 1–8 ON THE INACTIVATION OF DIPHTHERIA ANTITOXIN BY UREA

T, °C.	Molar concn. of urea	pH	n[a]	T_1	T_2	λ_1[b]	$100\lambda_2$[b]
25	8	4.91	5	0.513 ± 0.042	0.487 ± 0.027	4.0 ± 1.6	0.31 ± 0.16
		5.38	6	.505 ± .038	.495 ± .023	2.92 ± 0.66	.34 ± .14
		6.25	6	.281 ± .043	.713 ± .037	0.59 ± .18	.17 ± .12
		7.82	6[c]	.381 ± .048	.618 ± .038	.45 ± .11	.25 ± .11
		8.85	7	.357 ± .044	.598 ± .039	.402 ± .080	.60 ± .10
		9.27	7	.328 ± .045	.649 ± .039	.67 ± .15	1.26 ± .18
		9.73	7	.282 ± .051	.701 ± .048	1.82 ± .48	4.11 ± .65
25	7	5.38	5	.504 ± .044	.490 ± .033	0.68 ± .18	0.32 ± .17
	6		10	.306 ± .038	.671 ± .033	.365 ± .086	.41 ± .11
25	7	9.26	5	.328 ± .057	.670 ± .051	.50 ± .14	1.42 ± .25
	6		7	.38 ± .13	.63 ± .13	.089 ± .032	0.82 ± .21
1.6	6	5.38	6	.291 ± .059	.692 ± .058	.194 ± .091	.24 ± .16
35			5	.375 ± .057	.618 ± .051	1.28 ± .34	2.79 ± .74
10	7	9.23	6	.130 ± .043	.870 ± .034	0.40 ± .26	0.292 ± .048
40			4			(>2.3)	(11.3)

[a] Number of experimental points. Altogether, fifty-six constants were determined from the eighty-eight experimental points of the fourteen curves which were fitted by least squares, leaving thirty-two degrees of freedom for the estimation of error. [b] In reciprocal hours. [c] Data previously reported,[2] not shown in the figures.

$$P_1 = \frac{k_2N_0 + (\lambda_2 - k_3)P_0}{\lambda_2 - \lambda_1} \quad P_2 = \frac{k_2N_0 + (\lambda_1 - k_3)P_0}{\lambda_1 - \lambda_2}$$

$$T_1 = \frac{(k_1 - \lambda_2)N_0 - \lambda_2P_0}{\lambda_2 - \lambda_2} \quad T_2 = \frac{(k_1 - \lambda_1)N_0 - \lambda_1P_0}{\lambda_2 - \lambda_1}$$

For our experiments the values of k_1, k_2 and k_3 were obtained by fitting equation (1) to the data, thereby fixing the values of λ_1, λ_2, T_1 and T_2, and applying the formulas

$$k_1 = \frac{\lambda_1T_1 + \lambda_2T_2}{T_1 + T_2} \quad k_3 = \lambda_1\lambda_2/k_1$$
$$k_2 = \lambda_1 + \lambda_2 - k_1 - k_3 \qquad (2)$$

which may be derived from the above expressions on substituting our initial conditions, $P_0 = 0$ and $N_0 = T_0 = T_1 + T_2$. If the conditions $T_1 + T_2 \backsimeq 1$ (T_1 and T_2 are both positive) and $\lambda_1T_1 >> \lambda_2$ are satisfied, as they are for many of our experiments, k_1, k_2 and k_3 are approximated by the much simpler relations $k_1 \backsimeq T_1\lambda_1$, $k_2 \backsimeq T_2\lambda_1$, $(k_1/k_2 \backsimeq T_1/T_2)$ and $k_3 \backsimeq \lambda_2/T_1$.

Equation (1) was fitted to the data (except for the 40°, 7 *M* urea, *p*H 9.23 experiment) by the method of least squares,[19] starting with approximate graphical fits. Equal weights were assigned to all the points of the curves including the initial points, which were not assumed to be necessarily correct inasmuch as the assays of undenatured antitoxin samples were conducted with no greater precision than were the assays of the partially denatured samples. It was assumed that the nominal times of reaction were not in error. The resulting theoretical curves (Figs. 1–8), corresponding to the constants given in Table I and Figs. 9–11, represent the data very well, the root-mean-square of the deviations ($T_{obs.} - T_{calcd.}$) for the 88 experimental points being only 0.019, or 1.9% of the initial activity. The corresponding error of the experimental points ($\epsilon = ([v^2]/s-m)^{1/2}$, with $(s-m)$ equal to 32; see ref. 19, p. 245), estimated by the theory of least squares on the assumption that equation (1) is indeed the correct equation for the representation of the data, is 0.031, in good agreement with the accuracy that might be expected from the assay method. This agreement and the generally satisfactory random nature of the deviations provide some additional evidence that equation (1) is an adequate representation of the data. It is true that the average value of $T_1 + T_2$, 0.990, represents a tendency for the curves to pass below the initial assay points, but the discrepancy is not great enough to be regarded as significant.

Although the agreement between the curves and the data is good, the uncertainty in the derived constants is often great because the reactions were not followed intimately enough, particularly at very short and very long times of denaturation, to avoid ambiguity arising from the possibility of approximately fitting data for the intermediate region with different curves having rather different constants. The estimates of standard error of these constants depend on the shape of the particular curves and on the number and distribution of the measured points as well as on the estimated standard error (0.031) of the observations. For T_1, T_2, λ_1 and λ_2 the estimated standard errors are given in the table; for k_1, k_2 and k_3 representative values are perhaps the following for 25° and 8 *M* urea: at *p*H 4.91—0.78, 0.87 and 0.0033, respectively; at *p*H 5.38—0.37, 0.30 and 0.0026; and at *p*H 9.73—0.09, 0.47 and 0.034. These values, which are so large that only the major trends exhibited by Figs. 9–11 need be regarded as significant, do not tell the whole story, however,

(19) E. T. Whittaker and G. Robinson, "The Calculus of Observations," Blackie and Son Limited, London, 1944, pp. 209–250.

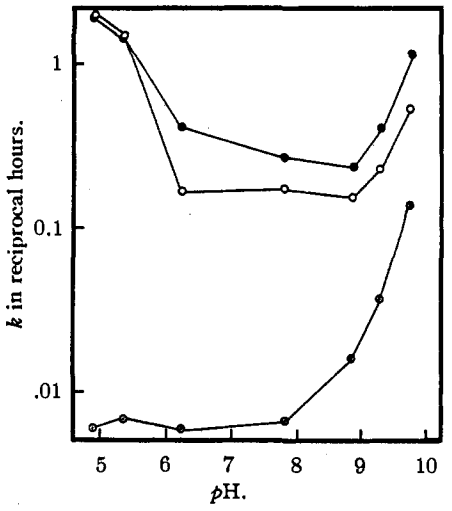


Fig. 9.—The variation of the rate constants discussed in the text with changes in pH; plots of log k *vs.* pH: open circles, k_1; filled circles, k_2; dotted circles, k_3.

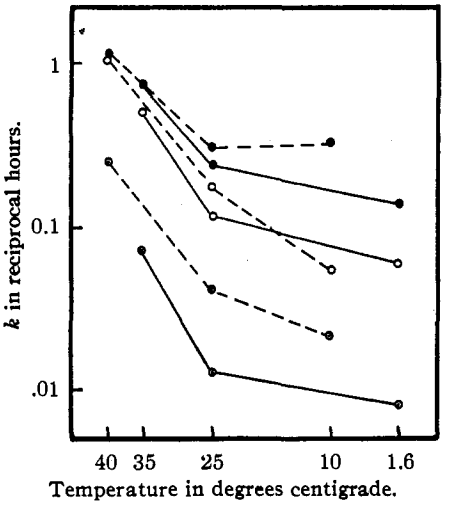


Fig. 10.—The variation of the rate constants with change in temperature; plots of log k against the reciprocal of the absolute temperature: open circles, k_1; filled circles, k_2: dotted circles, k_3. Solid lines connect points at pH 5.38 and 6 M urea; dotted lines, pH 9.23 and 7 M urea.

because various combinations of the constants may be more or less subject to error, particularly in accordance with their special dependence on the ambiguity just mentioned. This ambiguity is likely to lead to a set of errors such as, $\delta T_1 < 0$, $\delta T_2 > 0$, $(\delta(T_1 + T_2) \backsimeq 0)$, $\delta\lambda_1 > 0$ and $\delta k_1 > 0$ (increase in initial slope), and $\delta\lambda_2 > 0$, corresponding to $\delta k_2 > 0$, and $\delta k_3 > 0$ as well as $\delta(k_1/k_2) < 0$.

The notable features of the dependence of the k's on pH (Fig. 9) are the almost parallel behavior of k_1 and k_2, which decrease with increasing pH to about pH 6.25 and then increase again beyond pH 9.0, and the strikingly different behavior of k_3—hence the great effect of pH on the shapes of

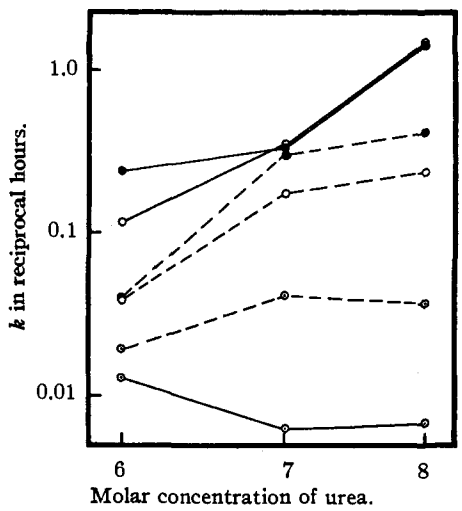


Fig. 11.—The variation of the rate constants with change in urea concentration at 25°; plots of log k *vs.* log urea concentration: open circles, k_1; filled circles, k_2; dotted circles, k_3. Solid lines connect points at pH 5.38; dotted lines, pH 9.26.

the log activity *vs.* time curves—which is independent of pH below 8 but above pH 8 increases rapidly. If it is assumed that the effect of variation of pH on the rate is simply one of changing the equilibrium concentration of the critical reaction intermediate (the specific rates of transformation of the intermediate being unaffected by its ionization), these facts may be interpreted in terms of acidic (or basic) ionization constants which are different for the ordinary molecule and the intermediate. If there are n such groups, taken as acidic groups, with the same ionization constants K and K^* in the ordinary molecule and the intermediate, respectively, the specific rate constant turns out to be given by the expression

$$\log k = \log k_0 + \log ([H^+]^n + K^*) - \log ([H^+]^n + K)$$

where k_0 is the specific rate constant at hydrogen ion concentrations so high that the groups are not ionized in either the ordinary molecule or the intermediate. A plot of this expression as a function of pH will be seen to consist essentially of two horizontal straight lines and, joining these with short intermediate curves, another straight line of slope $\doteq n$. Also, the pH values at which the extended straight lines intersect are the values pK^* (*i. e.*, $-\log K^*$) and pK, pK being the greater of the two values if the intermediate line has sloped upward, and *vice versa*. Since the slopes of the steep sections of the curves of Fig. 9 are all essentially unity, one pH unit corresponding to a ten-fold change in the rate constant, it may be inferred that in effect only one group of a given kind is coupled with the denaturation reactions; the ionizations are one-step ionizations. It would appear also that only two such groups are strongly coupled with the reactions; regarded as acidic groups, one has $pK \backsimeq 6.2$ for P and the critical

intermediates of the reactions $N \rightarrow I$ and $N \rightleftarrows P$ and $pK \geqslant 5$ for N, and the other has $pK \backsimeq 8.8$ for the critical intermediates and $pK > 10$ for P and N. The reaction intermediates and P are accordingly all alike in regard to their reaction-coupled acidic properties in the low pH range (excepting the relatively slight difference that results in the change in the ratio of k_1 to k_2 on going from acidic to basic solutions), but are different at high pH, where P appears to resemble N. With change in temperature the over-all rate of inactivation varies greatly, although the shapes of the log activity *vs.* time curves (Figs. 7 and 8) are not much changed; the same observations are brought out by Fig. 10, which also emphasizes a rather sharp decrease (perhaps not significant) of the ratio k_1/k_2 at the lowest temperature at pH 9.23 and the suggestion, mentioned above, of a minimal over-all rate of reaction in the neighborhood of 0°. The most important point brought out by Fig. 11 is the relative independence of k_3 on urea concentration in contrast to the average eighth-order dependence of k_1 and k_2. In the light of the relatively great probability of errors of the same sign for the k's, particularly for k_2 and k_3, it seems likely that the deviations from these generalizations by the separate results for pH 5.23 and pH 9.26 may not be real.

The substance P is at present hypothetical, and as yet there is no independent evidence of its existence. In addition to the work presented in this paper we have evidence from viscosity studies of the antitoxin system during denaturation that seems to be compatible with the mechanism involving a substance P. Moreover, preliminary experiments in which the denaturation was interrupted by dialysis showed a tendency for the subsequent reaction (when urea was again added) to simulate the original course of reaction with its characteristic plot of log activity *vs.* time. This behavior to us seemed entirely incompatible with the heterogeneity hypothesis. It seems now to be also the sole experimental reason for choosing the scheme $N \underset{k_3}{\overset{k_2}{\rightleftarrows}} P$, $N \xrightarrow{k_1} I$ as the basis for our considerations of mechanism rather than the slightly more elaborate scheme $N \underset{k_3}{\overset{k_2}{\rightleftarrows}} P$, $N \xrightarrow{k_1} I \xleftarrow{k_4} P$ or perhaps its other simplified form with $k_3 = 0$: if the sum of the concentrations of N and P are assumed always to be measured in the assays, the latter scheme gives the same results as the former for the dependence of remaining activity on time of denaturation provided that the rate constants are appropriately adjusted to fit the data. In fact, with $k_3 = 0$, and $\lambda_1 T_1 >> \lambda_2$, the same results are obtained for k_1 and k_2 as before, while for k_4 is found $k_4 = \lambda_2$, in close analogy to the corresponding approximate expression $k_3 = \lambda_2/T_1$ for the first scheme. It should be possible to measure k_3 independently of k_4 by carrying out the crucial interrupted experiment on a quantitative basis with the aid of more data on the denaturation reaction at lower urea concentrations to provide a sounder basis for estimating the rate of reconversion of P to N on dialysis. It should also be possible to gain information about the nature of P by performing interrupted experiments of other sorts, especially with abrupt changes of pH. If these experiments could be performed with enough precision, they, like the crucial dialysis experiment, would also serve to distinguish between our steps in k_3 and k_4, as considerations with the help of the constants shown in Table I and the equations for $k_4 = 0$ with general initial conditions will show.

It is interesting to speculate that from a general chemical point of view P is very similar to I, and that the reactions which form the two products are essentially identical except for the failure in the formation of P of the unfolding of the polypeptide chain to include those regions of the chain which constitute the combining sites[20] of the antibody. The similarity of dependence of k_1 and k_2 on pH, urea concentration, and temperature is in agreement with this view. The partially unfolded globulin molecule (it is known that the unfolding of serum globulin in urea stops far short of the point at which the polypeptide chain is completely extended[21]) may then be stabilized with respect to further unfolding or to refolding. Further processes of refolding and unfolding will no doubt occur, however, and we are particularly interested in the molecule P, which may need to refold into something like its native configuration in order that the antibody combining region may be exposed and thereby again be subjected to the possibility of unfolding and undergoing the detectable change of inactivation. The dependence of the rate constants on urea concentration suggests that the unfolding processes, being highly dependent on urea concentration, may be regarded tentatively as involving actual combination of the protein molecule with several urea molecules—the same number (about eight) for $N \rightarrow P$ as for $N \rightarrow I$—whereas the refolding processes, revealed in the case of $P \rightarrow N$ as independent of the urea concentration, involve dissociation of the protein molecule–urea complexes. According to this interpretation, which certainly cannot be regarded as strictly valid, P may be regarded as a complex formed between a globulin molecule and about eight urea molecules, as may I also if the thesis of general similarity of I and P is correct.

Further studies on the nature of the reactions are in progress. Preliminary experiments with a horse antitoxin against *Staphylococcus* α-hemolysin have indicated that the course of this reaction is closely similar to that of diphtheria antitoxin, and

(20) L. Pauling, THIS JOURNAL, **62**, 2643 (1940).

(21) H. Neurath, G. R. Cooper and J. O. Erickson, *J. Phys. Chem.*, **46**, 203 (1942).

the simpler technique of the *in vitro* assay for the antitoxin should make the system a favorable one for further kinetic investigations. It is possible that the mechanism proposed for the antitoxin inactivation may prove to be applicable to other denaturations which deviate in a similar way from first-order behavior.

This investigation was carried out with the aid of a grant from The Rockefeller Foundation. The authors are indebted to Professor Linus Pauling for suggestions and discussion, to J. Y. Chien for carrying out preliminary experiments on the effect of temperature, and to Sharp and Dohme, Glenolden, Pennsylvania, for supplying the antitoxin and toxin.

Summary

A study has been made of the influence of *p*H, urea concentration, temperature and certain other factors on the course of the inactivation of diphtheria antitoxin in urea solutions. The reaction is first-order with respect to initial antibody concentration but departs from typical first-order behavior in that the specific rate decreases as the reaction proceeds. The degree of this effect was found to vary greatly with the *p*H of the solution, and somewhat with the urea concentration. No negative temperature coefficient was observed in the temperature studies, although it was clear that the effect of change in temperature is not uniform over the region studied and possibly tends toward a minimum rate of inactivation at or below 0°. The interpretation of the experimental results has been discussed, and a simple kinetic mechanism which accounts for the observed behavior within the error of the measurements has been proposed. The proposed mechanism involves two competing reactions of the protein, one of which leads irreversibly to inactivation of the antibody and the other of which leads reversibly to an active product, more stable in urea solution than the original antibody and subject to inactivation only by way of slow reconversion to the original antibody or its kinetic equivalent.

PASADENA, CALIFORNIA RECEIVED JULY 2, 1947

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF MARYLAND]

Tetratriacontanoic Acid and Related Compounds[1]

BY NATHAN L. DRAKE AND SIDNEY MELAMED[1a]

The present research was undertaken with the object of preparing certain aliphatic carboxylic acids and ethers in the C_{20}–C_{40} range. Francis, King and Willis[2] have prepared tetratriacontanoic acid with a yield of 14% from docosanoyl chloride and ethyl sodio-α-acetylbrassylate. More recently dotriacontanoic acid was obtained with a yield of 26% by the reaction of docosanyl zinc iodide and 9-carbethoxynonanoyl chloride.[3] The procedure employed in the present work gave greatly improved yields and is applicable to the preparation of relatively large amounts of material.

The reaction of a dialkyl cadmium with an ω-ester-acid chloride has been extensively studied by Cason.[4] The method has been applied in this work to the preparation of 10-ketohexacosanoic acid from 9-carbethoxynonanoyl chloride and dihexadecyl cadmium and of 18-ketotetratriacontanoic acid from 17-carbethoxyheptadecanoyl chloride and dihexadecyl cadmium; the yields obtained were of 32 and 79%, respectively. The reaction has been further applied to the condensation of an acid chloride and an ω-alkoxyalkyl cadmium compound. In this manner 1-cyclohexoxy-11-ketodotriacontane was obtained from di-(10-cyclohexoxydecyl)-cadmium and docosanoyl chloride and 1-cyclohexoxy-11-ketohexatriacontane was similarly prepared from hexacosanoyl chloride and the same dialkyl cadmium compound. The requisite 1-bromo-10-cyclohexoxydecane was obtained from 1,10-dibromodecane and sodium cyclohexoxide.[5]

The conversion of the keto-acids and keto-ethers to the desoxy acids and ethers, respectively, was accomplished in excellent yield by the application of a recently described modification of the Wolff–Kishner reaction.[6] The only adaptation required was the use of larger amounts of solvent. It is of interest that the method of Clemmensen proved less satisfactory.[7]

The use of the cadmium alkyl reaction for the preparation of these keto-acids is dependent upon the availability of long-chain acid esters. Ethyl hydrogen sebacate[8] is readily obtained by the partial esterification of sebacic acid and can be isolated by fractional distillation. In the preparation of ethyl hydrogen hexadecanedicarboxylate, distillation is not a practicable method of isolation. The partial saponification of the diester[9] leads to

(1) The work described in this paper was done under a contract between the University of Maryland and the Bureau of Aeronautics, Navy Department.

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(2) Francis, King and Willis, *J. Chem. Soc.*, 999 (1937).

(3) Schuette, *Oil & Soap*, **22**, 107 (1945).

(4) Cason, THIS JOURNAL, **68**, 2078 (1946), and previous papers.

(5) Drake, Anspon, Draper, Haywood, VanHook, Melamed, Peck, Sterling, Walton and Whiton, THIS JOURNAL, **68**, 1540 (1946).

(6) Huang-Minlon, *ibid.*, **68**, 2487 (1946).

(7) "Organic Reactions," Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1942, Chap. 7.

(8) "Organic Syntheses," Coll. Vol. II, 276 (1943).

(9) The diethyl hexadecanedicarboxylate was prepared electrolytically from etbyl potassium sebacate by the procedure of Swann, Oehler and Pinkney, "Organic Syntbeses," **21**, 48 (1941).