[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

# The Denaturation of Tobacco Mosaic Virus by Urea. II. Kinetic Aspects[1]

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## I. Introduction

The first study of the kinetics of urea denaturation of a protein, that of Hopkins,[2] is of considerable interest, because an important deviation from the expected behavior was encountered. Hopkins found that the rate of the urea denaturation of egg albumin, contrary to the behavior of most chemical reactions, has a negative differential rate–temperature coefficient. An even more striking departure from the usual was observed in a preliminary study on the urea denaturation of tobacco mosaic virus,[3] TMV. The rate of this reaction was found to have a minimum at room temperature and to increase as the temperature is lowered or as it is raised from that value. In the case of the thermal denaturation of TMV another interesting anomaly was observed. Even though the rate of this reaction was found to follow the first order law quite accurately, its specific reaction velocity was found to increase as the initial virus concentration decreased.[4] In view of the departures from the expected in the behavior of TMV during denaturation under the influence of urea in one case and under the influence of heat in the other, it was thought that it would be of interest to investigate the kinetics of the urea denaturation of TMV in some detail. The results of such a study are presented and discussed in this paper. The biochemical aspects of the urea denaturation of TMV are discussed elsewhere.[5]

## II. Experimental Methods and Materials

Four different samples of TMV were used in these studies. Each was isolated by means of high-speed centrifugation from the juice expressed from frozen and ground diseased Turkish tobacco plants and was purified by three subsequent centrifugation cycles consisting of alternate high and low speed runs. This method of isolation has been demonstrated repeatedly in our laboratory to yield virus preparations high in infectivity and free from all but traces of extraneous matter. No detailed chemical or biological analyses of the individual samples were made. In each experiment designed to test the effect of one variable upon the specific reaction rate, a single virus preparation was used throughout. Most of the experiments were repeated using a different virus sample. In all cases, the general phenomena observed were the same regardless of the sample. Unfortunately, however, no two experiments were carried out under identical conditions except the origin of the virus, and therefore it is impossible to state whether or not minor differences in rate of denaturation can be attributed to the individuality of virus preparations.

Three methods of analysis were used in following the progress of the urea denaturation of TMV. The first of these, which will be referred to as method 1, involved the periodic centrifugation of aliquots of the virus-urea reaction mixture for one and one-half hours at a speed of 30,000 r. p. m. in a Bauer–Pickels type quantity high-speed centrifuge.[6] The undenatured nucleoprotein sediments under these conditions and forms a pellet at the bottom of the tube. This can be separated by decanting the supernatant fluid. The pellets were analyzed for protein by the Kjeldahl method. From the amounts of protein in the pellets after the reaction mixtures had stood for varying periods of time, the specific reaction velocity, $k$, of the denaturation was estimated. This method is not very accurate, due to the difficulty in decanting from the nucleoprotein pellets quantitatively. It was used only in exploratory experiments, but one of which is described in this report.

In method 2, samples of the reaction mixture were periodically diluted very accurately to five times their original volumes and the resulting precipitates were discarded. Triplicate aliquots of the supernatant fluids were placed in centrifuge tubes and immersed in a boiling water-bath for half an hour. This treatment completely denatured and coagulated the virus protein left undenatured by the urea. The coagula in the tubes were then analyzed for protein nitrogen, and quantities proportional to the residual virus concentrations in the reaction mixtures were calculated. This method of analysis is essentially the same as that used by Lauffer and Price[4] in their more critical experiments on the thermal denaturation of TMV. Its advantage lies in the fact that it obviates the potential errors resulting from the separation of nucleic acid from the virus nucleoprotein during denaturation.

The final method, which will be referred to as method 3, involved the use of a photoelectric colorimeter of the Klett–Summerson type.[7] It was found that a Wratten 40A and a Corning didymium filter used in combination provided light of the proper sensitivity for these studies. As was described previously,[5] normal TMV in aqueous and in urea solutions scatters light, while the virus degradation products in urea solutions transmit light almost completely. In a preliminary experiment, carried out at $p$H 5, where TMV is stable in 6 $M$ urea, it was shown that the

(1) Presented at the Protein Symposium of the Division of Biological Chemistry at the 104th meeting of the American Chemical Society, Buffalo, New York, September 7–11, 1942.

(2) F. G. Hopkins, *Nature*, **126**, 328, 383 (1930).

(3) W. M. Stanley and M. A. Lauffer, *Science*, **89**, 345 (1939).

(4) M. A. Lauffer and W. C. Price, *J. Biol. Chem.*, **133**, 1 (1940).

(5) M. A. Lauffer and W. M. Stanley, *Arch. Biochem.*, **2**, 413 (1943).

(6) J. H. Bauer and E. G. Pickels, *J. Expll. Med.*, **64**, 503 (1936).

(7) W. H. Summerson, *J. Biol. Chem.*, **130**, 149 (1939).

colorimeter reading is a linear function of the concentration of native virus in 6 *M* urea when the instrument is adjusted to give a reading of zero with distilled water. It was observed further that the urea degradation products of TMV, when dissolved in 6 *M* urea, gave a small increment to the colorimeter reading. This increment was found to be proportional to the amount of degradation product formed. If there are no stable intermediates capable of scattering light to an intermediate degree, it follows that the difference between the colorimeter reading at any one time and the reading given by the degradation products and urea, that is, the end reading, should be proportional to the concentration of undenatured virus nucleoprotein.

A study was made to determine whether or not methods 1, 2 and 3 really measure the same reaction. First of all, the progress of the denaturation of TMV in 6 *M* urea was followed by both methods 2 and 3. The results of a typical experiment are shown in Fig. 1. The triangles represent values for the residual denaturable material as determined by method 2 and the circles represent values determined by method 3. It may be seen that the two methods of analysis gave values for the undenatured protein remaining after various periods in contact with the urea that were the same within experimental error. One irregularity may be observed, however. The concentrations calculated by method 3 are somewhat too high during the first few minutes of the reaction. This situation was rather general. Whether it is due to the rapid formation of a relatively stable undenatured intermediate, to the development of minute air bubbles when the virus and urea solutions are first mixed, or to some other cause is at present not known. The important point, however, is that after the first few minutes the course of the denaturation of TMV in urea, as followed by the disappearance of turbidity measured with the colorimeter according to method 3, parallels the progress as followed by the chemical analysis for material remaining soluble in dilute salt solutions according to method 2. Second, experiments were carried out to determine whether or not method 1 measures the same thing as methods 2 and 3. At the end of one hour in the experiment described in Fig. 1, a small quantity of the virus–urea mixture was acidified to *p*H 6 in order to stop the reaction and was then dialyzed against a great excess of 0.1 *M* phosphate buffer at *p*H 7. The denatured protein which precipitated was removed by low-speed centrifugation. The supernatant fluid was examined in the analytical ultracentrifuge. A single boundary with a sedimentation constant of 186 Svedberg units was observed. No detectable amount of slowly sedimenting material remained in solution. This experiment shows that the virus material remaining soluble in dilute salt solutions after exposure to 6 *M* urea consists entirely of a high molecular weight component comparable to the original virus. Furthermore, ultracentrifuge experiments described in detail elsewhere[5] showed that the dilute salt insoluble fraction, when redissolved in 6 *M* urea or other suitable solvents, consists entirely of material with a sedimentation constant of 8 Svedberg units or less. Hence, when a partially denatured virus solution is spun in the quantity ultracentrifuge as in method 1, all of the undenatured residual virus and none of the material which would be classified as denatured by the criteria of methods 2 and 3 should collect in the pellet. Thus, methods 1, 2 and 3 all measure the same thing, namely, the disintegration of the macromolecular virus nucleoprotein particles. The data published in the preliminary report[3] failed to bring out this fact. This is certainly due to the lack of refinement in the techniques utilized in the exploratory phases of this problem. As is discussed elsewhere,[5] however, the biological inactivation of the virus in strong urea is a different reaction which must precede the disintegration of the virus particles.

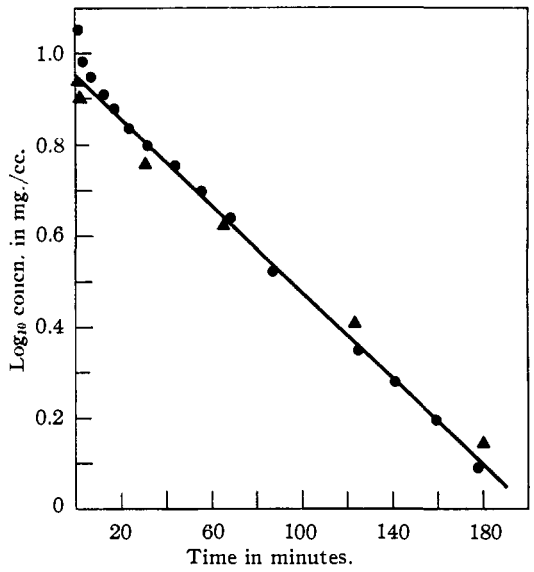


Fig. 1.—The course of the denaturation of tobacco mosaic virus in 6 *M* urea plotted as a first order reaction: ●, data obtained by method 3; ▲, data obtained by method 2.

The author is indebted to Mr. Howard K. Schachman for having solved many of the technical problems associated with the procedure in method 3 and for having carried out most of the analyses reported in this paper.

## III. Presentation and Discussion of Experimental Results

**A. The Order of the Reaction.**—Five cc. of 1 *M* phosphate buffer at *p*H 6.6, 18 cc. of a solution containing 16.7 mg. of TMV per cc., and 18 g. of urea were mixed and immediately diluted to 50 cc. This solution was allowed to stand at 25° and was analyzed periodically according to method 2. The reaction was allowed to proceed to 97% of completion. The results are presented in Fig. 2. It is apparent that the logarithm of the concentration of remaining virus is a linear function of the time of standing in contact with 6 *M* urea. This behavior would seem to classify the urea denaturation of TMV as a reaction of the first order. However, in view of the rather considerable scatter of the experimental points about the best fitting straight line, it was thought

desirable to apply the statistical procedure described by Lauffer and Price[4] to determine the relative goodness of fit of these data to zero, first, and second order reaction equations. The principle of this method is to obtain by least squares the equations of the straight lines which best fit the data when $[V_u]$, $\log_e [V_u]$, and $1/[V_u]$, respectively, are considered as linear functions of time. $[V_u]$ is the concentration of undenatured virus. Then the standard error of estimate, which measures the dispersion of the data about the best fitting straight line, is calculated for each. The slope of each line is divided by the standard error to give a statistic called a $t$ value. The $t$ value of a series of points falling exactly on a straight line would be $\infty$. The $t$ values for the data of Fig. 1 are 11.0, 42.3 and 11.6, respectively, when the reaction is assumed to be zero, first, and second order. Hence, the fit of the data to the equation for a first order process is overwhelmingly the best, and it can be concluded with a reasonable margin of safety that the urea denaturation of TMV really is a process of the first order.

When the denaturation reactions were followed by method 3, it was found that, with the exception of the data obtained during the first few minutes of each reaction, the experimental points followed quite faithfully the graphs of first order reactions. This was found to be true in more than a hundred reactions of this sort carried out at various virus concentrations, urea concentrations, $p$H values, and temperatures. This indicates that the urea denaturation of tobacco mosaic virus is a reaction of the first order with respect to virus concentration under a wide variety of conditions. The thermal denaturation[4] and the high pressure denaturation[8] of TMV are also reactions of the first order.

B. **The Effect of the Kind and the Quantity of Electrolyte on $k$.**—In an exploratory experiment, sixteen solutions consisting of 10 mg. per cc. of TMV dissolved in 6 $M$ urea containing various amounts of different electrolytes were allowed to stand at room temperature. First order $k$ values were determined by method 1. $p$H values were determined by means of a glass electrode on the supernatant fluids of the high-speed centrifugation at the end of twenty-four hours. The results of the experiment are shown in Table I, from which it may be seen that the addition of small amounts of electrolytes tends to increase the reaction velocity and the addition of large amounts tends to suppress it. These data are not particularly accurate and they suffer from a lack of constancy of $p$H throughout the experiment. Because of that, no detailed discussion of the significance of these results is warranted. Nevertheless, a few points merit consideration. In the case of reactions between simple ions, it has been possible to account for the effect of electrolyte concentration upon the specific reaction velocity in terms of the Brønsted–Bjerrum concepts, which take into account the effect of ionic strength upon the activity coefficients of the reactants and the activated complex. In general, when the activation process involves an increase in either positive or negative charge, the effect of the addition of small amounts of electrolyte is to increase the specific reaction rate. If the activation process involves a decrease in charge, the effect is the opposite, and, if no change in charge is involved, the effect is absent. The result observed in the case of TMV might be regarded as an indication that the activation process involves an increase in charge. There is one further point. In general, as judged for example by the effect upon solubility, dilute electrolytes tend to decrease the activity coefficients of proteins, whereas more concentrated electrolyte solutions often tend to have the opposite effect. This means that the increase in the rate of denaturation of TMV by the addition of a small amount of salt followed by the decrease upon the addition of more salt is consistent with the behavior of proteins in general toward electrolytes.

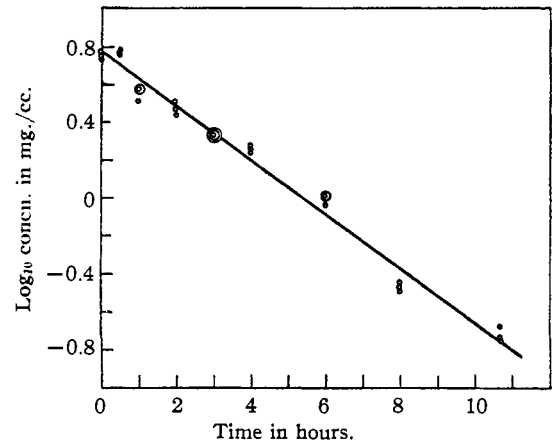


Fig. 2.—The decrease in the amount of undenatured tobacco mosaic virus plotted according to the equation of a first order reaction as a function of the time of standing in 6 $M$ urea.

(8) M. A. Lauffer and R. B. Dow, *J. Biol. Chem.*, **140**, 509 (1941).

TABLE I

THE EFFECT OF VARIOUS ELECTROLYTES ON THE RATE OF UREA DENATURATION OF TMV

| Expt. | Electrolyte | Concentration, $M$ | $p$H | $k$, min.$^{-1}$ |
|---|---|---|---|---|
| 1 | None | ... | 7.6 | 0.000089 |
| 2 | NaCl | 0.024 | 7.4 | .00013 |
| 3 | NaCl | .119 | 7.4 | .00015 |
| 4 | NaCl | .238 | 7.3 | .00016 |
| 5 | NaCl | .714 | 7.35 | .00014 |
| 6 | NaCl | 1.428 | 7.2 | .000072 |
| 7 | $K_xH_yPO_4$ | 0.01 | 7.4 | > .001 |
| 8 | $K_xH_yPO_4$ | .05 | 7.5 | > .001 |
| 9 | $K_xH_yPO_4$ | .10 | 7.4 | > .001 |
| 10 | $K_xH_yPO_4$ | .30 | 7.4 | < .0005 |
| 11 | $K_xH_yPO_4$ | .60 | 7.4 | .000021 |
| 12 | $Na_3$ citrate | .004 | 7.6 | .00036 |
| 13 | $Na_3$ citrate | .02 | 7.7 | .00058 |
| 14 | $Na_3$ citrate | .04 | 7.7 | > .001 |
| 15 | $Na_3$ citrate | .119 | 7.9 | .0016 |
| 16 | $Na_3$ citrate | .238 | 7.9 | .00017 |

**C. The Dependence of $k$ upon the Initial Virus Concentration.**—Lauffer and Price[4] observed that the first order $k$ for the denaturation of TMV by heat was higher for initially dilute virus solutions than for initially more concentrated ones. Price[9] observed that the $k$ of the thermal inactivation of TMV varied in the same manner with the initial concentration. In view of these results, it was thought worth while to investigate the influence of the initial concentration of TMV upon the first order $k$ for the denaturation in urea.

A series of solutions in 6 $M$ urea and 0.1 $M$ phosphate buffer of $p$H 7 of TMV at initial concentrations varying between 9.0 and 1.4 mg. per cc. was allowed to stand at 25°. First order $k$ values were determined by method 3. The results are shown in Fig. 3, where $k$ is plotted as a function of the initial concentration of TMV. It may be seen that $k$ for the urea denaturation of TMV, like that for the thermal denaturation and that for the thermal inactivation of the virus, is higher in initially more dilute systems. The data of Fig. 3 can be described quantitatively by equation 1.

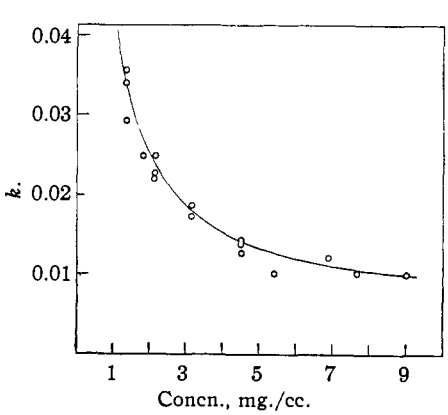


Fig. 3.—The specific reaction velocity in min.$^{-1}$ of the urea denaturation of tobacco mosaic virus plotted as a function of the initial virus concentration.

(9) W. C. Price, *Arch. ges. Virusforsch.*, **1**, 373 (1940).

Eq. 1 $$k = 38.4 \times 10^{-3} (0.15 + 1/[V_0])$$

If first order $k$ values are calculated for a reaction which is really zero order, the improperly assigned first order $k$ should increase as initial concentration is decreased. Since this is the behavior observed in the case of the urea denaturation of TMV, it might be argued that the explanation of the dependence of $k$ upon $1/[V_0]$ is that the reaction progresses, not according to the first order law, but according to the zero order law. If this situation obtained, however, one should get a better fit when the virus concentrations of an individual experiment are plotted as a linear function of time than when the logarithms of the concentrations are plotted as a linear function of time. The statistical treatment of the data in Fig. 2 shows that by far the best fit is obtained when the log of $[V_u]$ is plotted against time. Hence, it is necessary to conclude that in individual systems of TMV in urea the denaturation proceeds according to the law of a first order process, even though the $k$ of that reaction depends upon the initial virus concentration.

The observed dependence of $k$ upon initial virus concentration can be explained quantitatively on the basis of one major and two minor assumptions. The major assumption is that TMV contains some substance, called an inhibitor, I, which is capable of reacting with native virus, $V_n$, to form a complex $V_nI$, which denatures more slowly in urea than the native virus. Stated more quantitatively, it may be assumed that $V_nI$ denatures with a specific reaction velocity of $k_i$ which is $z$ times that, $k_n$, of the denaturation of $V_n$, where $z$ is a number greater than zero and less than unity. Thus, the denaturation is pictured as consisting of two parallel reactions, in one of which inhibitor–virus complex is denaturing and in the other of which the free virus is denaturing.

The first minor assumption is that the concentration of I is proportional to the initial virus concentration and, molecule for molecule, is great com-

pared to the virus concentration. This can be expressed by the equation $[I] = c[V_0]$, where $c$ is a constant of large magnitude and $[V_0]$ is the initial virus concentration. (To be strictly correct, activities should be substituted for concentrations.) The second minor assumption is that the reaction between I and $V_n$ is molecule for molecule and is reversible, as expressed by the equation $I + V_n \rightleftarrows V_nI$. If the equilibrium constant of this reaction is designated by $K_i$, it is possible to derive equation 2 by simple algebraic processes.

Eq. 2 $$k = k_n\left(\frac{1 + zK_ic[V_0]}{1 + K_ic[V_0]}\right)$$

When $K_ic[V_0] \gg 1$, this expression reduces to equation 3

Eq. 3 $$k = \frac{k_n}{K_ic}\left(zK_ic + \frac{1}{[V_0]}\right)$$

Equation 3 is of exactly the same form as equation 1, which was fitted empirically to the data of Fig. 3. Obviously, equation 2 could also be fitted to the data of Fig. 3. Hence, it may be concluded that the assumptions upon which equations 2 and 3 are derived form a possible basis for explaining the unusual dependence of the $k$ of the urea denaturation of TMV upon the initial virus concentration. The inhibitor postulated in this treatment could be an impurity present at a very low concentration, or it could possibly be a portion of the virus molecule. It is recognized that the final acceptability of an explanation of this sort must rest upon independent evidence, and it is accordingly proposed only as a possibility.

Effects of concentration of the reactants on the specific reaction rate more or less comparable to that here described have been observed for numerous chemical reactions. The $k$ for the reaction between sodium ethoxide and alkyl iodide doubles when the concentrations of the reactant are reduced from normal to 0.01 $N$. Results such as this have been interpreted as being due to the existence of two parallel reactions, one between the ethoxide ion and the iodide and the other between the neutral molecules of both species.[10] In some respects, this mechanism is analogous to the one proposed above. As was already mentioned, the rates of numerous reactions between ions have been found to vary with the over-all dilution. In some cases the rates are depressed and in others they are increased when the dilution is increased. When reasonably dilute solutions are considered, these results can be explained satisfactorily according to the Brønsted–Bjerrum concept in terms of the effect of ionic strength upon the relative activity coefficients of the reactants and the activated complex as given by the Debye–Hückel ionic interaction theory even in its simplest form.[11] According to current theories, reactants form by a reversible process a critical complex or an activated complex which then decomposes into the reaction products. The reaction rate is thus dependent upon the concentration of the activated complex, and this, in turn, is dependent upon the concentration of the reactants and the equilibrium constant for the system, complex–reactants. Thus, the effect of a variable upon the specific reaction rate may be regarded as an effect upon the equilibrium constant (on a concentration basis) for the formation of the activated complex. The equilibrium constant for the keto–enol isomerism of ethyl acetoacetate in various solvents has been found to vary with the concentration of the ester in a manner more or less comparable to the variation of the rate of denaturation of tobacco mosaic with initial concentration. The effect in the case of the ester has been explained as the influence of concentration upon the relative activity coefficients of the keto and the enol forms arising from non-electrostatic interaction.[12]

The effect of initial concentration upon the specific reaction velocity in the case of the urea denaturation of TMV may be thought of as being an effect of virus concentration upon the relative activity coefficients of the activated complex and the original virus. However, the virus degradation products must be regarded as having the same effect as the original virus. Viewed in this light, the explanation of the influence of initial concentration based upon the presence of an inhibitor is merely a possible mechanism to account for the change in activity coefficient ratios. If the influence of initial concentration is to be ascribed to the effect of interaction upon the relative activity coefficients, the requirement that the virus and the degradation products behave the same presents some difficulty, for there is every indication that the over-all interaction in the virus solution is much greater per unit concentration expressed in grams per cc. than that of the degradation products. TMV particles in solution are

(10) H. C. Robertson, Jr., and S. F. Acree, THIS JOURNAL, **37**, 1902 (1915).

(11) V. K. LaMer, *Chem. Rev.*, **10**, 179 (1932).

(12) G. Scatchard, *ibid.*, **8**, 321 (1931).

rod-shaped bodies about 300 × 15 mμ with a molecular weight of around 4 × $10^7$. The dependence of the rates of sedimentation and of diffusion upon concentration and the tendency to form gel-like systems at moderate concentrations show that TMV departs from ideality to a somewhat greater extent than most proteins of lower molecular weight.[13] The degradation products, on the other hand, are known to consist of much smaller particles.[5] Viscosity data indicate that they are much less asymmetrical than the original virus particles, and diffusion data seem to indicate the lower departure from ideality that would be anticipated on the basis of the greater particle symmetry.[14] Nevertheless, in spite of the fact that the over-all interactions encountered in the virus and the degradation products are probably different, the possibility that the effect upon relative activity coefficients is due to some particular component of the over-all interaction which happens to be about the same for both the degradation products and the initial virus cannot be excluded on the basis of the data at present available.

**D. The Variation of $k$ with Temperature.**—Hopkins[2] reported that the urea denaturation of egg albumin has a negative differential coefficient of rate with respect to temperatures between 0 and 37°. Diebold and Juhling[15] showed that the urea denaturation of fibrinogen has a positive coefficient over a narrow range, and Drabkin[16] found that the urea denaturation of carboxyhemoglobulin also has a positive coefficient between 20 and 38°. Stanley and Lauffer[3] reported that the differential rate–temperature coefficient of the denaturation of TMV in urea was negative below room temperature and positive above. Bawden and Pirie[17] have since confirmed this observation and have reported in addition that comparable anomalies may be observed in the urea denaturation of potato virus X, tomato bushy stunt virus, and tobacco necrosis virus. It is entirely possible that the unusual effect of temperature upon the $k$ of the urea denaturation of viruses is general for the urea denaturation of proteins.

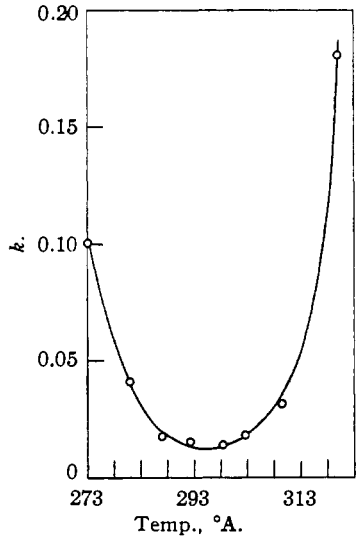


Fig. 4.—The specific reaction velocity in min.$^{-1}$ of the urea denaturation of tobacco mosaic virus plotted as a function of the absolute temperature.

In order to investigate more thoroughly the dependence of the $k$ of the urea denaturation of TMV upon temperature, a series of identical solutions of the virus at a concentration of 4.5 mg. per cc. in 6 $M$ urea buffered to about $p$H 7 with 0.1 $M$ phosphate was studied at various temperatures between 0 and 45°. First order $k$ values were determined for each by method 3. The results are presented in Fig. 4, where $k$ is plotted against the absolute temperature. Quite in accordance with the results of the preliminary experiments,[3] the rate is at a minimum near room temperature and increases both as the temperature is lowered and as it is raised from that value.

It has been possible to account quantitatively for this curious temperature–rate effect on the basis of a few simple assumptions. The principal assumption is that undenatured TMV is transformed into the denatured material by two or more parallel simultaneous reactions, one or more of which have negative differential rate–temperature coefficients and one or more of which have positive coefficients.

If TMV reacts reversibly with urea to form a complex which then denatures, the rate of appearance of denatured protein will depend at every instant upon the number of molecules of undenatured virus in the combined state and upon the specific reaction rate of the denaturation of the complex. The over-all differential coefficient of such a mechanism could be either positive or nega-

(13) M. A. Lauffer and W. M. Stanley, *Chem. Rev.*, **24**, 303 (1939); W. M. Stanley and T. F. Anderson, *J. Biol. Chem.*, **130**, 325 (1941); M. A. Lauffer, *J. Phys. Chem.*, **44**, 1137 (1940); H. Neurath and A. M. Saum, *J. Biol. Chem.*, **126**, 435 (1938).

(14) V. L. Frampton and A. M. Saum, *Science*, **89**, 84 (1939).

(15) W. Diebold and L. Juhling, *Biochem. Z.*, **296**, 389 (1938).

(16) D. L. Drabkin, *Proc. Soc. Expll. Biol. Med.*, **41**, 225 (1939).

(17) F. C. Bawden and N. W. Pirie, *Biochem. J.*, **34**, 1258 (1940).

tive, depending solely upon the relative effects of temperature increases upon the extent of dissociation and upon the velocity of denaturation of the urea-virus complex. Reasoning similar to this was used by Hopkins[2] to account for the negative coefficient of the urea denaturation of egg albumin.

It is convenient to utilize the Eyring[18] concept of absolute reaction rates in attempting to account for the effect of temperature on the rate of denaturation of TMV in urea. This concept may be represented by equation 4

$$\text{Eq. 4} \quad k = \kappa \frac{k'T}{h} e^{-\Delta F^{\ddagger}/RT} = \kappa \frac{k'T}{h} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} = \kappa \frac{k'T}{h} K^{\ddagger}$$

$R$ is the gas constant, $T$ is the absolute temperature, $k$ is the specific reaction rate, $\kappa$ is a transmission coefficient usually with a value of about unity, $k'$ is the Boltzmann constant, $h$ is Planck's constant, $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the free energy, the heat, and the entropy of the activation process, and $K^{\ddagger}$ is the equilibrium constant between the normal and the activated states. In unimolecular activation processes $K^{\ddagger}$ is approximately the probability that a particular molecule of reactant will be in the activated state.

In order to explain the effect of temperature on the particular reaction under consideration, it is necessary to assume only that urea can react reversibly with TMV and that in so doing it increases the entropy of activation and/or decreases the energy of activation for denaturation. The treatment can be simplified greatly by supposing that these changes are proportional to the number of urea molecules, $a$, which happen to be combined with a particular virus molecule. If $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ represent the entropy and energy of activation in the absence of urea and if $+\Delta S^*$ and $-\Delta H^*$ represent the changes per molecule of urea, then the entropy and energy of activation of a virus molecule with $a$ urea molecules combined would be $(\Delta S^{\ddagger} + a\Delta S^*)$ and $(\Delta H^{\ddagger} - a\Delta H^*)$.

The number of groups on a virus molecule capable of reacting with urea may be taken as $n$. The reaction between the $i^{\text{th}}$ group of a virus molecule and one urea molecule can be represented by equation 5

$$\text{Eq. 5} \quad 1V_i + 1U \rightleftarrows V_iU$$

If $K$, the equilibrium constant, is not too large, the probability that one urea molecule will really be combined with the $i^{\text{th}}$ group at any instant will be $K[U]$. If all $n$ of the groups which can react with urea have roughly the same reactivity, the probability that one urea molecule will be combined with a particular virus molecule will be approximately $nK[U]$.

The probability that there will be $a$ urea molecules combined with a particular virus molecule at a given time is $(nK[U])^a$ for all values of $a$ which are much less than $n$. This expression is a good approximation if the values of $K$ for all groups are similar and are not more than about $0.003/n$ and if $n \gg a$. On the basis of the above reasoning, the probability, $P_a$, that a given virus molecule will be combined with $a$ urea molecules is given by equation 6.

$$\text{Eq. 6} \quad P_a = (n[U])^a K^a = (n[U])^a (e^{\Delta S/R} e^{-\Delta H/RT})^a = (n[U])^a e^{a\Delta S/R} e^{-a\Delta H/RT}$$

$\Delta S$ and $\Delta H$ are the entropy change and the heat of the reaction of one molecule of urea with one molecule of virus. According to equation 4 and the reasoning in the paragraph following the one in which equation 4 is presented, the probability that one of these virus molecules combined with $a$ urea molecules will be in the activated state is $e^{(\Delta S^{\ddagger} + a\Delta S^*)/R} e^{-(\Delta H^{\ddagger} - a\Delta H^*)/RT}$. Therefore, the probability, $P_a^{\ddagger}$, that a virus particle can be simultaneously combined with $a$ urea molecules and in the activated state is given by equation 7.

$$\text{Eq. 7} \quad P_a^{\ddagger} = (n[U])^a e^{a\Delta S/R} e^{-a\Delta H/RT} e^{(\Delta S^{\ddagger} + a\Delta S^*)/R} e^{-(\Delta H^{\ddagger} - a\Delta H^*)/RT} = (n[U])^a e^{(S^{\ddagger} + a(\Delta S + \Delta S^*))/R} e^{-(\Delta H^{\ddagger} + a(\Delta H - \Delta H^*))/RT}$$

The probability that a virus particle will be in the activated state no matter how many urea molecules it has on it will be $\sum_{a=0}^{a=n} P_a^{\ddagger}$, and, according to equation 4

$$k = \kappa \frac{k'T}{h} \sum_{a=0}^{a=n} P_a^{\ddagger}$$

Hence, equation 8 may be written

$$\text{Eq. 8} \quad k = \kappa \frac{k'T}{h} \sum_{a=0}^{a=n} (n[U])^a e^{(\Delta S^{\ddagger} + a(\Delta S + \Delta S^*))/R} e^{-(\Delta H^{\ddagger} + a(\Delta H - \Delta H^*))/RT}$$

Equation 8 is a reasonable approximation only if the effective values of $a$ are fairly small compared to $n$. As will be seen later, the effective values of $a$ encountered in this study are fairly small numbers, and $n$ may be assumed with reasonableness to be very large. Equation 8 was derived on the basis of the laws of ideal solutions. Although it is reasonable to expect that this equation

(18) H. Eyring, *Chem. Rev.*, **17**, 65 (1935).

is capable of elucidating the fundamental nature of the unusual dependence of the rate of denaturation of TMV in urea upon temperature, it cannot be expected to describe the behavior of any real solution with absolute fidelity. Even if 8 is assumed to be approximately correct, it is obviously only a partial equation in the sense that it describes only the effect of temperature and urea concentration upon the reaction rate. A complete equation for the reaction rate must obviously be a product of terms taking care of the effects of initial virus concentration, *p*H, ionic strength, etc., as well as those considered here. The $\Delta H^{\ddagger}$ and $\Delta H^{*}$ of equation 8 are energies of activation and should therefore be positive in sign. $\Delta H$ is the molar heat of the equilibrium reaction between virus and urea. For the present purpose, it must be negative in sign. When $a > -\Delta H^{\ddagger}/(\Delta H - \Delta H^{*})$, the second exponential in equation 8 will be positive, but when $a < -\Delta H^{\ddagger}/(\Delta H - \Delta H^{*})$, that exponential will be negative. Since $a$ can have many values in the range from 0 to $n$, equation 8, when written in its expanded form, will consist of a sum of terms which are positive exponential functions of $1/T$ plus a sum of terms which are negative exponential functions of $1/T$. A clearer physical picture of this theory can be obtained by supposing that it is a fair approximation to say that urea can react with TMV in only two ways. When $x$ molecules of urea react reversibly with 1 molecule of virus to form a complex which then denatures, the heat of the exothermic equilibrium reaction will be $x\Delta H$ calories per mole and the energy of activation (endothermic) for the denaturation step will be $\Delta H^{\ddagger} - x\Delta H^{*}$. If $x$ is small enough, the algebraic sum of these two energy terms will be positive and the second exponential of equation 8 will be negative. When $y$ molecules of urea react with 1 virus molecule, the corresponding terms will be $y\Delta H$ for the heat of the exothermic equilibrium reaction and $\Delta H^{\ddagger} - y\Delta H^{*}$ for the energy of activation for the denaturation of this complex. When $y$ is a sufficiently large number, the algebraic sum of these terms will be negative and the second exponential of equation 8 will be positive for this reaction. Thus, the over-all denaturation would be the sum of two parallel reactions, the rate of one of which would be a negative exponential function of $1/T$ and the rate of the other of which would be a positive exponential function of $1/T$. The smooth curve fitting the data of Fig. 4 is a graph of equation 9 which is of exactly this form. It is possible, of course, that a more complex expansion of equation 8 could also be fitted to the data of Fig. 4.

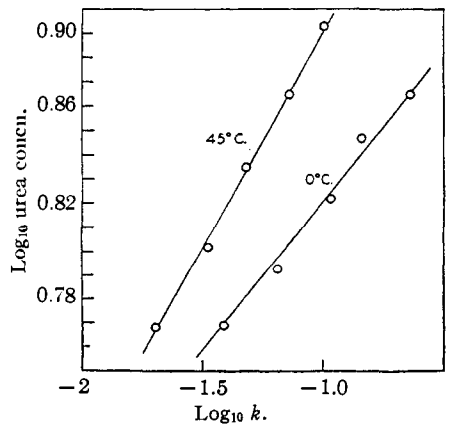


Fig. 5.—The relationship between the specific reaction velocity in min.$^{-1}$ at 0° and 45° of the urea denaturation of tobacco mosaic virus and the molar concentration of urea.

Eq. 9 $\quad k = 10^{-16.6}\, e^{+19,400/RT} + 10^{17.7}\, e^{-27,000/RT}$

The important point is that the fundamental assumptions upon which equation 8 is based form a possible explanation of the nature of the variation of the rate of denaturation of TMV in urea with temperature.

**E. The Dependence of $k$ upon Urea Concentration.**—At constant temperatures, equation 8 may be written, $k = \Sigma C_a[\mathrm{U}]^a = C_0[\mathrm{U}]^0 + C_1[\mathrm{U}]^1 + C_2[\mathrm{U}]^2 + \ldots + C_n[\mathrm{U}]^n$, where $C_0, \ldots, C_n$ are constants. According to equation 8, values of $C_a$ for which $a > -\Delta H^{\ddagger}/(\Delta H - \Delta H^{*})$ will decrease with increasing temperature and values of $C_a$ corresponding to smaller values of $a$ will increase with increasing temperature. Thus, at a high temperature, the reaction velocity should be approximately proportional to some fairly low power of the urea concentration, while at a lower temperature it should vary roughly with some higher power of the urea concentration. An experiment was designed to test this prediction.

A series of solutions containing 6 mg. of TMV per cc. dissolved in 0.1 $M$ phosphate buffer and urea at concentrations varying from 6 to 8 $M$ was allowed to stand at 45°. First order $k$ values were measured by method 3. A comparable test was carried out at 0°. The results are presented in Fig. 5, where the logarithm of the urea concentration is plotted against the logarithm of $k$ at 0 and 45°. Both graphs are approximately linear.

It is obvious that the reciprocals of the slopes of such graphs should be equal to the exponents discussed above. The reciprocal of the slope for the reaction at 0° is 8.1 and that for the reaction at 45° is 5.7, showing that the denaturation rate depends upon a higher power of the urea concentration at a low temperature than at a higher temperature. This result is completely in accord with the theory and affords at least a partial confirmation of the theory.

A complication is introduced by virtue of the fact observed by Burk and Greenberg[19] that the *p*H of a buffer measured in an urea solution is higher than that of the same buffer in water. The higher the urea concentration, the greater is the *p*H shift. Whether this shift is due to an actual change in hydrogen ion activity or to a liquid junction potential effect is still an open question. Therefore, it is not possible to decide whether or not to correct the slopes of the graphs shown in Fig. 5 for the *p*H difference between systems of low and high urea concentration. Since the difference in slope between the 0 and the 45° graphs is increased somewhat when the correction is applied, this *p*H complication does not affect the conclusions drawn in the preceding paragraph.

**F. Dependence of *k* upon *p*H.**—Steinhardt[20] has shown that the rate of thermal inactivation of pepsin is directly proportional to the reciprocal of the 5th power of the hydrogen ion activity. Steinhardt concluded from this result that the pepsin molecule must dissociate 5 protons before it can be inactivated. Lauffer and Price[4] found that in solutions more alkaline than *p*H 5 the thermal denaturation of TMV proceeded faster the higher the *p*H. Stanley and Lauffer[3] reported substantially similar results in the case of the urea denaturation of the virus, and Bawden and Pirie[17] confirmed this result.

In order to investigate more fully the effect of *p*H upon the rate of urea denaturation of TMV, solutions containing 5 mg. per cc. of virus in 6 *M* urea and potassium phosphate buffers of various *p*H values and at ionic strengths of 0.2 were allowed to denature at 0° and at 45°. First order *k* values were measured by method 3. *p*H values were measured on each virus–urea–buffer system using a glass electrode. The results are shown in Fig. 6, where $\log_{10} k$ is plotted against

(19) M. F. Burk and D. M. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

(20) J. Steinhardt, *Kgl. Danske Videnskab Selskab. Math.-fys. Med.*, **14**, No. 11 (1937).

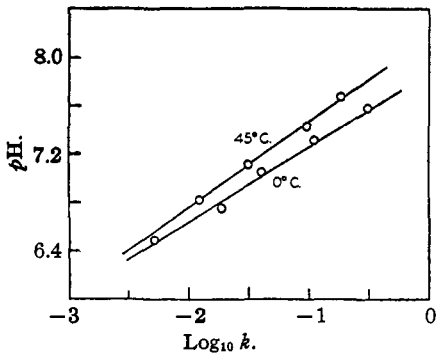


Fig. 6.—The relationship between the specific reaction velocity in min.$^{-1}$ at 0° and 45° of the urea denaturation of tobacco mosaic virus and the *p*H.

*p*H for the reactions at zero and 45°. The slopes of the two graphs are 1/1.57 and 1/1.38, respectively. This means that the rate of denaturation of TMV in 6 *M* urea varies with about 1.5th power of the reciprocal of the hydrogen ion activity. Following Steinhardt's reasoning, it is possible to conclude that, on the average, about 1.5 protons are dissociated by a virus molecule before it denatures in urea. Since the isoelectric point of TMV is at about *p*H 3.5, the particles are negatively charged at *p*H 7. If protons must be dissociated in the formation of the activated complex, the negative charge would be increased. The conclusion is the same as the one reached on the basis of the discussion of the effect of electrolytes upon the specific reaction rate. The dependence of the virus denaturation rate upon the 1.5th power of the *p*H applies only to a limited *p*H range, for preliminary experiments have shown that the rate of urea denaturation reaches a minimum somewhere in the region of the isoelectric point of the virus and increases again at lower *p*H values.

The author wishes to express his gratitude to Professor Henry Eyring and Mr. R. W. Williams of Princeton University for aid in formulating the theoretical aspects of this paper.

## IV. Summary and Conclusions

The denaturation of tobacco mosaic virus in strong solutions of urea proceeds as a reaction of the first order.

The specific reaction velocity is increased by the addition of small quantities of salt and is depressed by the addition of larger quantities of electrolyte.

In spite of the fact that an individual reaction proceeds according to the first order law, the

specific reaction velocity is a linear function of the reciprocal of the initial virus concentration.

The specific reaction velocity varies in a complex way with changes in temperature. Between 0 and 23° the rate decreases as temperature is increased, but above 23° the rate increases as temperature is increased. This behavior was explained by assuming that the denaturation of tobacco mosaic virus in concentrated urea can proceed by several parallel reactions, some with negative differential rate–temperature coefficients and some with positive coefficients. Both types of reaction were visualized as taking place in two stages: first, a reversible reaction between urea and virus to form a readily denaturable complex and, second, the denaturation of the complex. The important difference between the two types of postulated reactions is that in the one with negative coefficients the $\Delta H$ values of the equilibrium reactions are greater than the energies of activation of the denaturation steps, while in the reactions with the positive coefficients the opposite is true.

It was found that the specific reaction velocity varied approximately with the 8.1th power of the urea concentration at 0° and with the 5.7th power at 45°. This observation is evidence in favor of the validity of the assumptions made to explain the complex rate–temperature dependence.

It was observed that the specific reaction velocity varied with about the 1.5th power of the reciprocal of the hydrogen ion activity. This can be explained as meaning that on the average about 1.5 protons must be dissociated by a virus particle before it denatures in urea.

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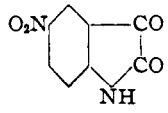
[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WESTERN KENTUCKY STATE TEACHERS COLLEGE]

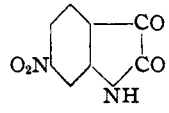
# The Nitration of Isatin

BY WARD C. SUMPTER AND WILLIS F. JONES

The results of the several studies which have been made of the nitration of isatin are somewhat conflicting and confusing. The first nitration of isatin was carried out by Baeyer[1] through the action of potassium nitrate on a solution of isatin in concentrated sulfuric acid. Baeyer reported the melting point of his product as 226–230° and assumed that it was 5-nitroisatin (I). Liebermann



I



II

and Krauss[2] prepared nitroisatin in essentially the same manner but reported the melting point as 245° (dec.).

Procedures for the nitration of isatin are given in German Patent 221,529 and the melting point reported as 253–255° when the nitration is effected by nitric acid in sulfuric acid solution and 248–250° when the nitration is accomplished by the action of potassium nitrate on a solution of isatin in sulfuric acid.

In 1924 Rupe and Stocklin[3] published a paper in which they claimed that 6-nitroisatin (II) was obtained when isatin was nitrated by treating its solution in concentrated sulfuric acid with the calculated quantity of fuming nitric acid (sp. gr. 1.5). They reported the melting point of their product as 244° and stated that it was not identical with the product obtained by following the procedure of Baeyer.

In 1925 Calvery, Noller and Adams[4] employing a procedure essentially the same as that used by Rupe and Stocklin, obtained a product melting at 254–255° which they assumed to be 5-nitroisatin. They cited Baeyer's paper and German Patent 221,529 but seemingly were not aware of the fact that Rupe had claimed the preparation of 6-nitroisatin by a procedure practically identical with their own.

Subsequently Rupe and Kersten[5] presented what appeared on its face to be definite proof of the structure of Baeyer's 5-nitroisatin and of Rupe's 6-nitroisatin.[6] They did not offer any explanation for the difference in their own results and those of Calvery, Noller and Adams but

(1) Baeyer, *Ber.*, **12**, 1312 (1879).
(2) Liebermann and Krauss, *ibid.*, **40**, 2492 (1907).
(3) Rupe and Stocklin, *Helv. Chim. Acta*, **7**, 557–566 (1924).
(4) Calvery, Noller and Adams, THIS JOURNAL, **47**, 3059 (1925).
(5) Rupe and Kersten, *Helv. Chim. Acta*, **9**, 578 (1926).
(6) In his second paper Rupe used a different system of numbering and designated the compound as 4-nitroisatin. Structural formulas in the paper show that he meant 6-nitroisatin.