THE KINETICS AND THERMODYNAMICS OF REVERSIBLE DENATURATION OF CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

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(Received for publication, June 10, 1948)

Crystalline soybean trypsin inhibitor which was isolated by the writer in 1945 (1) has the following properties (2).

1. It is a protein of a molecular weight of about 25,000. Its isoelectric point is near pH 4.5.
2. It is a globulin; it is practically insoluble in water near the isoelectric point but it is readily soluble in dilute acid or alkali, or in dilute salt solution even at the isoelectric point.
3. It inhibits trypsin by combining with it irreversibly to form a double protein compound. The compound has been isolated in crystalline form (3) and named "Trypsin-soy inhibitor compound."
4. A solution of the soy inhibitor protein[1] is stable over a wide range of pH at temperatures below 30°C. The protein becomes gradually denatured, however, if heated to higher temperatures. The denaturation is accompanied by a loss in the trypsin-inhibiting power as well as by a change in a number of other properties of the protein, such as, for example, solubility and crystallizing ability. The denatured protein, unlike the unheated protein, is readily digestible by pepsin.
5. The denaturation of the soy inhibitor protein when heated in dilute acid or alkali is not accompanied by precipitation of coagulated protein. A precipitate of denatured protein is, however, formed immediately on addition of buffer solution of pH 4.5 to the heated solution.
6. The heat-denatured protein on cooling is gradually reversed to its native soluble form, and it does not precipitate any longer on addition of buffer solution of pH 4.5. Prolonged heating, however, brings about irreversible denaturation. Irreversible denaturation is also caused by brief heating of the protein solution at its isoelectric point or in the presence of salt, in which cases denaturation is accompanied by precipitation of coagulated protein which does not redissolve on cooling.

Crystalline soybean inhibitor protein when denatured by heat thus behaves in a manner similar to that of a number of other proteins such as hemoglobin

[1] For the sake of brevity the terms "soy inhibitor protein," "soy inhibitor," or "soy protein" are frequently used in the text instead of the full expression "crystalline soybean trypsin inhibitor."

(4), serum albumin (5), pepsinogen (6), trypsin (7), etc., which are reversibly denatured by heat.[2]

The denaturation of the soybean protein on heating and also the reversal of denaturation on cooling generally proceed at a relatively slow rate. Crystalline soybean inhibitor hence offers a convenient material for the study of the effect of temperature on both the rate of denaturation and on the reversal of denaturation. The protein is easily prepared in pure, salt-free, crystalline state from soybean meal which is readily available in unlimited amounts.

Preliminary studies of denaturation of the crystalline soybean protein, of the effect of denaturation on the trypsin-inhibiting power of the protein, and also on its digestibility by pepsin have been made and reported in a previous paper (2). The present paper deals with the results of a more extensive study of the effect of temperature on the rate of denaturation and of the reversal of denaturation of an acidified solution of the protein.

An analysis of the results leads to the conclusion that the soybean inhibitor protein in aqueous solution exists normally in either one or the other of two states, native or denatured, or as a mixture of both in equilibrium with each other.[3] The relative proportion of the two forms of protein in solution is determined by the temperature and the pH of the solution. At low temperatures a solution of soy inhibitor consists mainly, if not entirely, of the native form. As the solution is heated the proportion of denatured form increases so that at temperatures of 60°C. or higher the solution contains mainly denatured protein. On cooling, the process is reversed and the proportion of native protein is gradually increased. At any given temperature an equilibrium is finally established between the two forms of the protein. The equilibrium ratio is the same whether a cold solution is heated or a heated solution is cooled to the given temperature.

A study of the kinetics of the denaturation of the soy inhibitor on heating and of the reversal of denaturation on cooling shows that either process consists of the same pair of simultaneous unimolecular reactions acting in opposite directions until an equilibrium is established. At any given temperature the

[2] An extensive list of references covering the whole subject of denaturation of proteins is given in the review article, by Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O., *Chem. Rev.*, 1944, **34**, 157. See also Anson, M. L., in Chemistry of the Amino acids and Proteins (C. L. A. Schmidt, editor), Springfield, Illinois, Charles C. Thomas Co., 1938, chapter IX; Anson, M. L., in Advances in Protein Chemistry, (M. L. Anson and J. T. Edsall, editors), New York, Academic Press, 1945, **2**, 361.

[3] Anson and Mirsky have demonstrated in 1925 and in 1934 the existence of an equilibrium between native and denatured states for hemoglobin and also for trypsin. See Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1925, **9**, 169; 1934, **17**, 393 and 399.

values of the velocity constants and hence also of the equilibrium constant are the same for the heating as well as for the cooling experiments.

The data obtained from the study of the kinetics of the individual reactions were utilized for the calculations of various thermodynamic values in accordance with the theory of classical thermodynamics and with the "theory of absolute reaction rates."

Preliminary Experiments

1. Tests for Denaturation.—Denatured soy inhibitor protein differs from its native form by its low solubulity, its inability to form crystals, its inability to inhibit the proteolytic activity of trypsin, and by its digestibility by proteolytic enzymes. There is probably also an increase in the reactivity of some chemical groups, such as, sulfhydryl, disulfide, and phenol groups in the denatured protein, since this was found to be true in the case of other proteins (8). Any one of the described properties can be used as a basis for a method of determining the concentration of the native or of the denatured protein in a solution containing both components. It was found most convenient to use in these studies the precipitation method, whereby the denatured protein is removed from solution by precipitation at pH 4.5 in the presence of dilute buffer solution. The concentration of native protein left in solution is then easily measured. This method has the additional advantage that it enables one to stop the progress of denaturation or the reversal of denaturation at any desired moment.

2. Tests for Reversibility of Denaturation.—(*a*) *Trypsin-Inhibiting Power.*—Experiments described in a previous paper (2) have shown that heat-denatured soy protein loses its ability to inhibit the action of trypsin. The trypsin-inhibiting power is reestablished on reversal of denaturation.

(*b*) *Digestion by Pepsin.*—Native soybean inhibitor is not digested by pepsin at pH 3.0 (2). The same holds true for the "reversed" protein, while the denatured protein is readily digestible under the same conditions.

(*c*) *Crystallization.*—The "reversed" protein crystallizes under the same conditions as the original native protein, producing crystals of the same form and habit.

(*d*) *Solubility.*—The "reversed" protein possesses the same solubility as the original native protein. A solution saturated towards the original native protein does not dissolve any of the "reversed" protein and *vice versa.* This test provides strong evidence that the two materials are chemically and physically identical.

Crystallization and Solubility Tests

Experimental Procedure.—The procedure is similar in principle to that described by Anson and Mirsky for testing the reversibility of denaturation of hemoglobin (9). The steps in the procedure are as follows:—

(*a*) *Denaturation.*—Denaturation of soy inhibitor protein was obtained by heating 25 ml. of 0.4 per cent solution of crystals of soy inhibitor in 0.0025 M HCl for 2 minutes at 80°C. Then 1 ml. sample of the hot solution was added to 5 ml. of 0.06 M acetate buffer pH 4.5 at −5° C.; this resulted in complete precipitation of denatured protein, while unheated native protein is almost completely soluble in 0.05 M acetate buffer pH 4.5.

(*b*) *Reversal of Denaturation.*—The rest of the heated solution was stored for 2 days at about 25°C. A sample of 1 ml. when mixed then with 5 ml. of 0.06 M acetate buffer pH 4.5 produced only a very slight turbidity equal to that of the sample of the unheated "control" solution of 0.4 per cent soy inhibitor, thus proving that on standing for 2 days at 25°C. the heated denatured protein has "reversed" to the soluble native state.

(*c*) *Crystallization of the "Reversed" Protein.*—The "reversed" solution of the soy inhibitor protein and also an equal amount of the non-heated "control" solution of the protein were titrated carefully with 0.1 M NaOH to pH 4.65 (pH tested colorimetrically). This resulted in almost complete precipitation of the protein in each case. The precipitates were centrifuged and then each suspended in 4 ml. of water and dissolved with the aid of several drops of 0.1 M NaOH. The solutions were cooled to about 3°C., 1 ml. of 95 per cent alcohol was added to each solution, and the pH of the solutions was adjusted to about 5.0. A heavy amorphous precipitate was formed in both solutions. The solutions were placed in a constant temperature bath at 30°C. for about 6 hours. The amorphous precipitate in each case gradually changed completely into well formed rhomboid crystals. The crystals in the "control" solution and in the solution of the "reversed" protein appeared to be identical in form and size.

(*d*) *Solubility of Crystals of Native and of "Reversed" Soybean Inhibitor in 0.02* M *Acetate Buffer pH 4.5.*—The crystals were centrifuged and then washed several times in 10 ml. pyrex tubes with the solvent (0.02 M acetate buffer pH 4.5) at 5°C. The tubes, each provided with a glass bead, were filled completely with the solvent, stoppered carefully so as to avoid the formation of air spaces, and then attached by means of rubber bands to the side of a 5 inch pulley driven by a small motor and rotated at about 10 R.P.M. The rotation of the wheel brought about continuous rolling of the glass bead back and forth along the sides of the tubes and thus kept the suspensions stirred slowly and continuously. The suspensions were stirred for 24 to 48 hours at 5°C. The concentration of protein in the supernatant solutions was determined spectrophotometrically at 280 mμ (2). The washings were repeated until nearly constant solubilities were obtained.[4] The final equilibrated supernatant solutions were then crossed, namely the supernatant solution of the control sample was added to the centrifuged crystals of the heated sample, and *vice versa*. The suspensions were stirred again for 48 hours.

[4] The solubility of the crystals, because of the purity of the preparation used in these studies, was apparently not affected by the gradual loss in total protein during the repeated washings. A constant solubility was obtained after three washings and it remained constant during the further successive three washings.

The results are given in Table I.

The tabulated data show: first, that the solubility of the crystals of the "reversed" soy inhibitor protein is 0.02 M acetate buffer pH 4.5 at 5°C., is identical, within the experimental accuracy, with the solubility of crystals of native non-heated protein; and second, that a solution saturated with one of the crystalline materials does not dissolve any of the crystals of the other material. Both tests provide strong evidence that the crystals of "reversed" protein are identical with those of the original native protein.

TABLE I

Comparison of Solubilities of Crystals of Native and of Crystals of "Reversed" Soy Inhibitor Protein

	Solubility in mg. protein per ml. supernatant solution	
	Experiment 1	Experiment 2
Last Washings		
Supernatant solution A: Saturated solution of crystals of native protein	0.220	0.245
Supernatant solution B: Saturated solution of crystals of "reversed" protein	0.230	0.270
Crossed		
Supernatant solution A: Stirred 48 hrs. longer with crystals of the "reversed" protein	0.235	0.265
Supernatant solution B: Stirred 48 hrs. longer with crystals of native protein	0.250	0.275

3. *Effect of pH on the Rate of Reversal of Denaturation of the Soybean Trypsin Inhibitor*

No attempt has been made in the course of these studies to investigate extensively the effect of varying the pH of the solution on the rate of denaturation and of the reversal of denaturation.

The experiment presented here is of a preliminary character and was carried out for orientation purposes.

Experimental Procedure.—A stock of 50 ml. of 0.25 per cent solution of soybean protein in 0.0025 M HCl was adjusted with drops of 0.2 M HCl or 0.2 M NaOH to various pH values. Three series of 1 ml. samples of each pH were pipetted into test tubes and heated for 2 minutes at 90°C.

The samples of one series were mixed immediately after heating with 5 ml. 0.06 M

acetate buffer pH 4.5 at −5°C.; the samples of the second series were allowed to cool for 5 minutes at 10°C., and the samples of the third series were left for 18 hours at 10°C. before they were mixed with acetate buffer pH 4.5. All the tubes were then centrifuged and the optical density of the clear supernatant solutions was measured at 280 mμ. The results are shown graphically in Fig. 1.

The samples that were brought to pH 4.5 immediately after heating for 2 minutes at 90°C. contained very little soluble protein, while those that were allowed to cool and stand at 10°C. gradually gained soluble protein. The reversal of denaturation in the alkaline range of pH is more rapid than in the

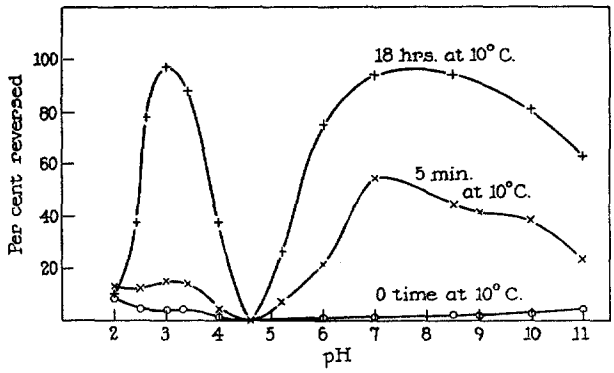

FIG. 1. Effect of pH on reversal of denaturation of crystalline soybean trypsin inhibitor.

acid range. The optimal rates of reversal are at about pH 2.6 to 3.4 on the acid side and at pH 7 to 9 on the alkaline side of the isoelectric point.

4. Irreversible vs. Reversible Denaturation

The phenomenon of reversible denaturation is complicated by the fact that the reversible denaturation is accompanied by a secondary "irreversible" denaturation if the heating of the protein solution is prolonged. Thus, when a solution of the soybean protein in dilute acid at pH 2.5 to 3.5 is heated to 90°C. the protein is immediately and completely denatured. The denaturation is of the reversible type since the protein on cooling gradually reverses to the native, soluble state. The reversal is complete if the cooling is initiated within a minute or so after the protein solution has been brought to 90°C. The longer the protein solution is kept at 90°C., the less will be the reversal on cooling. This is shown in the following experiment.

1 ml. samples of 0.5 per cent solution of the soybean protein in 0.0025 M HCl (pH about 3.0) were kept at 90°C. for various lengths of time and then placed at 20°C. for 18 hours. The solutions were afterwards mixed with 5 ml. 0.06 M acetate buffer pH

4.5. The precipitates formed were centrifuged after standing for several hours at room temperature. The concentration of the dissolved native protein in the supernatant solutions was then determined. The results are given in Table II.

Experimental Studies of the Kinetics of Denaturation

1. Effect of Temperature on the Rate of Denaturation of Soybean Inhibitor Protein at pH 3.0

Procedure.—A set of test tubes, each containing 1 ml. of 0.5 per cent solution of crystalline soybean protein in 0.0025 M HCl (final pH about 3.0) was placed in a water bath of the temperature desired. The tubes were removed from the bath after various intervals of time and poured back and forth into test tubes each containing 5 ml. 0.06 M acetate buffer pH 4.5 which had been cooled in an ice-salt bath at about −3°C. This operation brought about the cessation of the progress of denaturation. A precipitate of denatured protein gradually formed in the buffer mixture; the precipitate

TABLE II

Irreversible Denaturation of Crystalline Soybean Trypsin Inhibitor at 90°C.

Time at 90°C., *min*	2	5	10	20	30	45	60
Native protein in supernatant solutions after 18 hrs. at 20°C., *per cent*	95	90	78	65	54	44	36
Irreversibly denatured protein by difference, *per cent*	5	10	22	35	46	56	64

is stable and irreversible. The suspensions were centrifuged after standing for about an hour at 20°C. The concentration of native protein in the supernatant solutions was then determined spectrophotometrically at 280 mμ.

The results of a series of measurements at various temperatures in the range of 40–60°C. are given in Fig. 2. There is a gradual loss in the concentration of native (soluble) protein at all temperatures above 40°C., the rate of denaturation increasing with increase in temperature. At 60°C. or higher the denaturation proceeds nearly to completion, while at lower temperatures the extent of denaturation is gradually decreased, being only 15 per cent at 40°C. At 70°C. the denaturation is completed in less than 2 minutes.

2. Effect of Temperature on the Rate of Reversal of Denaturation of Soy Inhibitor Protein at pH 3.0

Procedure.—A set of test tubes, each containing 1 ml. of 0.5 per cent solution of soy protein in 0.0025 M HCl, was heated for 2 minutes at 90°C. This brought about complete denaturation. All the tubes were then transferred at once into a water bath at the temperature at which the reversal of denaturation was to take place. The samples were removed from the water bath at various times and mixed with 5 ml. 0.06 M

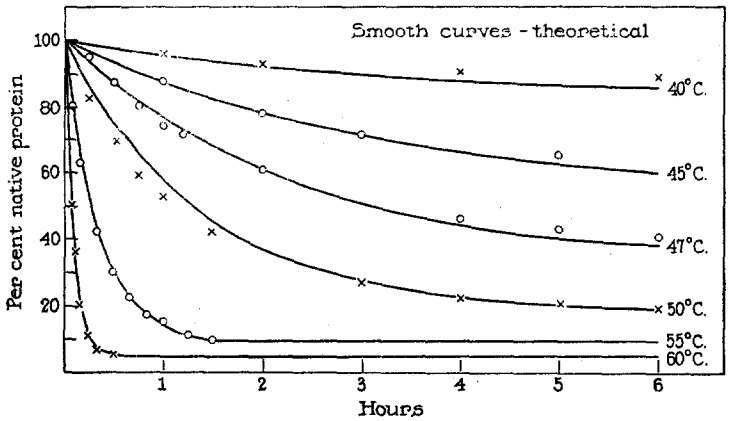

FIG. 2. Rate of denaturation of soy inhibitor protein of pH 3.0 at various temperatures. Smooth curves in Figs. 2 to 5 calculated with the aid of Equation 6, page 252.

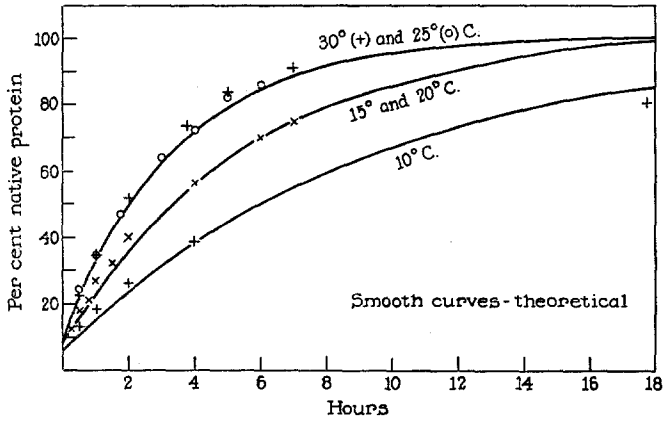

FIG. 3*a*

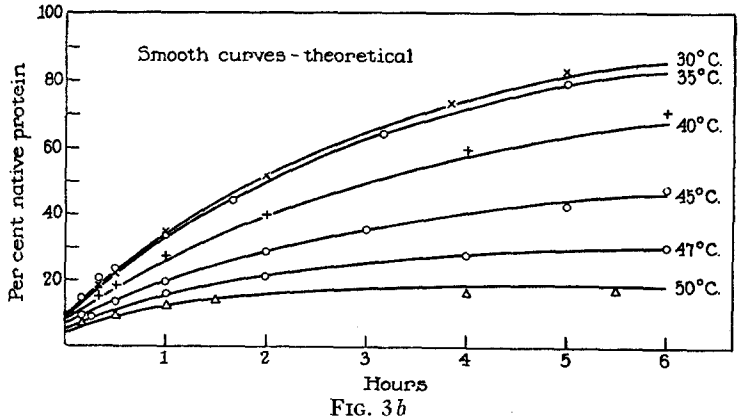

FIG. 3*b*

FIG. 3, *a* and *b*. Effect of temperature on rate of reversal of denaturation. Smooth curves calculated with the aid of Equation 6, page 252.

acetate buffer pH 4.5 at about −3°C. and treated as described in the procedure for denaturation. The amount of precipitate of denatured protein formed on the addition of the acetate buffer decreased progressively with time of cooling, thus indicating the gradual reversal to native (soluble) protein.

The results of a series of measurements of the reversal of denaturation by cooling the heated protein at various temperatures are shown in Figs. 3 *a* and 3 *b*. In the range of temperatures of 10–25°C. the rate of formation of native protein gradually increases with increase in temperature and the reversal proceeds to practical completion (100 per cent). Complete reversal occurs also in the range of temperatures of 25–35°C., although the rate of reversal is practically identical for all the temperatures in that range, so that the curves for 25, 30, and 35°C. almost coincide. At temperatures above 35°C. the rate of formation of native protein decreases with increase in temperature and the reversal does not proceed to completion but reaches definite levels which decrease as the temperatures are raised, so that at 50°C. the reversal reaches a level of only 18 per cent. A comparison of the curves on Figs. 3 *a* and 3 *b* with those on Fig. 2 shows that the final levels reached on reversal are equal to those reached on denaturation at the corresponding temperatures. It thus appears that in a given solution of protein at any temperature in the range of 35–50°C. or even higher, the final quantitative relationship between the concentration of native form and that of the denatured form of the protein is fixed. It is the same whether a freshly made up solution of native protein or whether a solution of protein which had been exposed for 2 minutes at 90°C. is allowed to come to equilibrium at the given temperature. A true equilibrium which may be approached from either direction thus exists between the two forms of protein in solution in the range of temperature of 35–50°C. (and probably also above and below that range).

3. *Effect of Varying the Concentration of Protein on the Rate of Denaturation and on the Rate of Reversal of Denaturation*

At constant pH and temperature the percentage rate of denaturation is independent of the concentration of the protein as shown in Fig. 4. In case of reversal experiments there is a complication because of partial *irreversible* denaturation of the protein during the preliminary heating for 2 minutes at 90°C. The percentage of protein irreversibly denatured at 90°C. apparently increases considerably as the concentration of protein is increased. This anomaly is discussed in the following section, on page 255.

4. *The Order of the Reaction*

The kinetics of heat denaturation of proteins (mostly irreversible) and of inactivation of enzymes has been studied extensively and it has been generally found that the process of denaturation follows the course of a reaction of the

first order, similar to that of a simple unimolecular reaction. It is to be shown here that the reversible denaturation of the soybean protein on heating and also the reversal of denaturation on cooling follow the course of a reversible unimolecular reaction, the main characteristics of which are as follows: (1) The rate of reaction when expressed in percentage change is independent of the concentration of the reactant. (2) In any reversible reaction there is a tendency towards the establishment of a state of equilibrium between the concentrations of the initial and the final products of the reaction. This is expressed by an equilibrium constant which is independent of the direction in which the process is initiated. In the case of a unimolecular reversible reaction the equi-

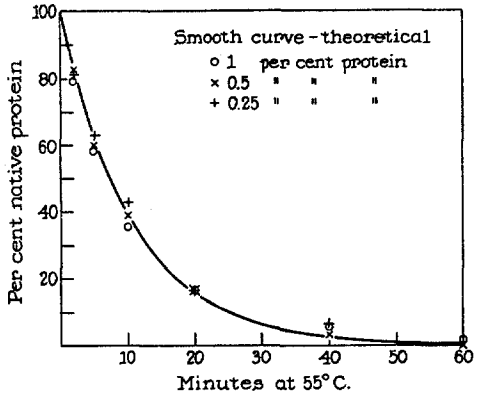

FIG. 4. Effect of concentration of protein on rate of denaturation at 55°C.

librium constant is a pure number and is independent of the original concentrations of the reactants. (3) The rate of change of the concentration in a reversible unimolecular reaction at any given time is proportional to the divergence of the system from the equilibrium state; *i.e.*, to the difference in concentration of the reactant at the given time and its concentration at equilibrium.

The equation for the kinetics of a reversible unimolecular reaction (10) when applied to reversible denaturation of protein in dilute solution at constant pH and temperature is as follows:—

Let A_0 = the total concentration of protein
a = concentration of native protein at any time t
a_0 = concentration at $t = 0$
a_e = concentration at equilibrium
$A_0 - a$ = concentration of denatured protein
K = equilibrium constant $= \dfrac{A_0 - a_e}{a_e}$
k_1 = unimolecular velocity constant for the reaction: native → denatured; *i.e.*,

$$k_1 = -\frac{da}{adt}$$

k_2 = unimolecular velocity constant for the reaction: denatured → native; *i.e.*,

$$k_2 = \frac{da}{(A_0 - a)\,dt}$$

The equation expressing the rate of reversible denaturation of protein is

$$-\frac{da}{dt} = k_1 a - k_2(A_0 - a) \tag{1}$$

At equilibrium

$$k_1 a_e = k_2(A_0 - a_e) \quad \text{or} \quad \frac{k_1}{k_2} = \frac{A_0 - a_e}{a_e} = K \quad \text{(by definition)} \tag{2}$$

Equation 1 may be written as

$$-\frac{da}{dt} = (k_1 + k_2)a - k_2 A_0$$

At equilibrium

$$k_2 A_0 = (k_1 + k_2)a_e$$

Hence, by substitution, we get

$$-\frac{da}{dt} = (k_1 + k_2)(a - a_e) \tag{3}$$

Equation 3 states that the rate of the reaction is proportional to the distance that the reaction has to cover; in other words, the rate is proportional to the divergence of the system from the state of equilibrium. Equation 3 is usually written as

$$-\frac{da}{(a - a_e)\,dt} = k_1 + k_2$$

and is equivalent to

$$-\frac{d \log (a - a_e)}{dt} = \frac{k_1 + k_2}{2.3} \tag{4}$$

In accordance with Equation 4, the values of log $(a - a_e)$ when plotted against time t should fall on a straight line, the slope of which is

$$\frac{k_1 + k_2}{2.3}$$

It is to be recalled that Equation 2 gives the expression for $\frac{k_1}{k_2}$; the values of k_1 and k_2 individually can thus readily be determined.

The complete integral of Equation 4 is

$$\log \frac{a_0 - a_e}{a - a_e} = \frac{k_1 + k_2}{2.3} t \tag{5}$$

or

$$a = a_0 e^{-kt} + a_e(1 - e^{-kt}) \tag{6}$$

where $k = k_1 + k_2$ and $e =$ the base of the Napierian system of logarithms.

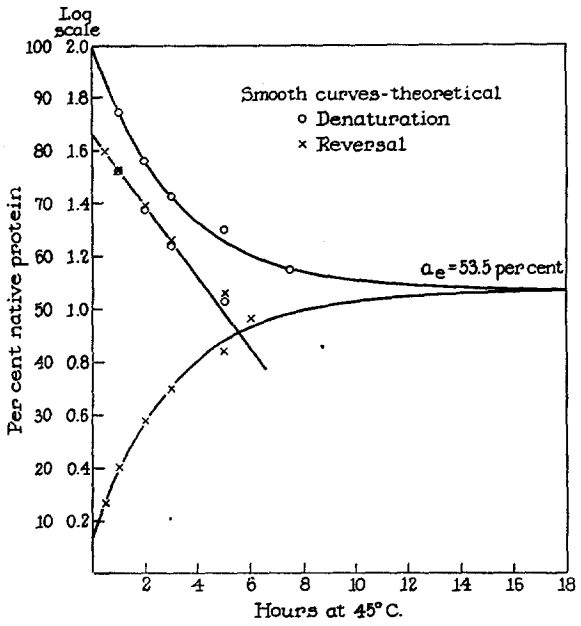

FIG. 5*a*

FIG. 5, *a*, *b*, and *c*. Rate of denaturation and of reversal at various temperatures. Logarithmic plot in accordance with Equation 4, page 251.

At temperatures above 60°C. where a_e approaches zero and k_2 is negligibly small as compared with k_1 all the relationships described above assume the form of an ordinary unimolecular reaction. Thus Equation 5 becomes

$$\log \frac{a_0}{a} = \frac{k_1 t}{2.3}$$

or

$$a = a_0 e^{-k_1 t}$$

The equations for the kinetics of the reversal of denaturation are identical with those given for denaturation, except for changes in plus and minus signs.

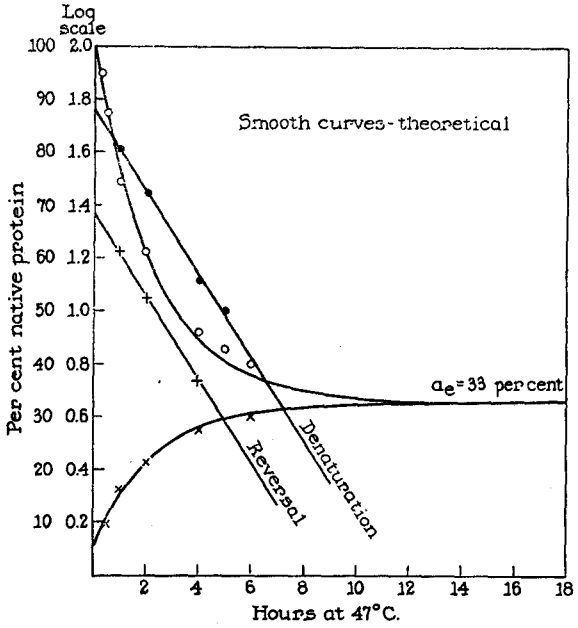
Log scale
100 2.0
90 1.8
80 1.6
70 1.4
60 1.2
50 1.0
40 0.8
30 0.6
20 0.4
10 0.2
Per cent native protein
Smooth curves - theoretical
a_e = 33 per cent
Denaturation
Reversal
2 4 6 8 10 12 14 16 18
Hours at 47°C.

FIG. 5*b*

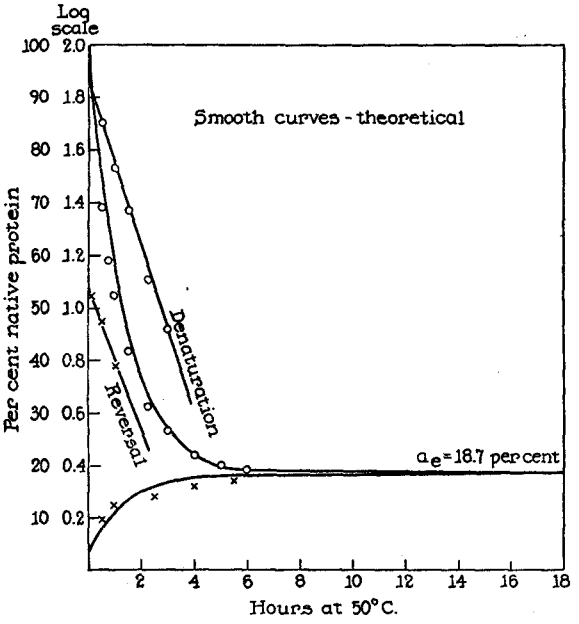
Log scale
100 2.0
90 1.8
80 1.6
70 1.4
60 1.2
50 1.0
40 0.8
30 0.6
20 0.4
10 0.2
Per cent native protein
Smooth curves - theoretical
Denaturation
Reversal
a_e = 18.7 per cent
2 4 6 8 10 12 14 16 18
Hours at 50°C.

FIG. 5*c*

At temperatures below 35°C. where a_e approaches A_0 and k_1 is small as compared with k_2, all equations are reduced to those of an ordinary unimolecular reaction, namely,

$$\frac{da}{dt} = k_2(A_0 - a)$$

and

$$\log \frac{A_0 - a_0}{A_0 - a} = \frac{k_2 t}{2.3}$$

It can be easily shown that, as in the case of any unimolecular equation, the values of a, a_e, a_0 in all the given equations can be expressed in terms of percentages of A_0 (*i.e.*, they can all be divided by 0.01 A_0) without affecting either the character of the equations, or the values of the various constants k_1, k_2, and K.

The experimental results check closely with the described theoretical consideration, as follows:—

1. The value of a_e (and hence of $\frac{k_1}{k_2}$ or K) at a given temperature is the same for the denaturation experiment as in the experiment on reversal of denaturation. The value of a_e when expressed in per cent is independent of the total concentration of protein.

2. The plotted values of log $(a - a_e)$ *vs.* t for denaturation and of log $(a_e - a)$ *vs.* t for reversal fall on straight lines. At any given temperature the straight lines for both processes are parallel as shown in Figs. 5 *a*, 5 *b*, 5 *c* thus proving that the values of $(k_1 + k_2)$ are identical for both processes. Since the values of $\frac{k_1}{k_2}$ were also found to be identical for the two processes at any given temperature, it follows that k_1 and also k_2 are identical and can thus be determined independently either from the data obtained in the denaturation experiment or from those obtainable in the reversal experiment.

3. Theoretical values for the two processes were calculated with the aid of Equation 6 by substituting the experimental values of a_e and also the values of k; *i.e.*, of $k_1 + k_2$ as obtained from the slopes of the lines plotted in accordance with Equation 4. The calculated theoretical values for the percentage of native protein found at any time t for both processes are represented by the smooth curves in Figs. 2 to 5. The experimental points generally lie close to the theoretical curves.[5]

The values of the unimolecular velocity constants k_1 and k_2, as calculated with the aid of Equations 2 and 4 and also the value of the equilibrium constant K for various temperatures are given in Table III.

[5] It is to be observed that at temperatures of 10°C. or lower there is a gradual falling off in the rate of reversal and the experimental points do not coincide with the theoretical curves.

Is Irreversible Denaturation a Reaction of the Second Order?

Preliminary experiments indicate that irreversible heat denaturation of the bean protein follows the course of a reaction of the second order. It was found, for example, that the percentage of protein irreversibly denatured in a given time increases considerably as the concentration of protein is increased. Also, the data on the rate of irreversible denaturation given in Table II fit closer the theoretical curve of a reaction of the second order than that of the first order. The preliminary character of the experiments, however, do not warrant a definite conclusion on this question.

TABLE III

The Evaluation of the Unimolecular Velocity Constants of Heat Denaturation (k_1) and of the Reversal of Denaturation (k_2) of Soybean Trypsin Inhibitor at pH 3.0

Temperature, °C.	10	15	20	25	30	35	40	45	47	50	55	60	65
$\frac{k_1}{k_2} = \frac{100 - a_e}{a_e} = K$					0.010	0.042	0.22	0.87	2.03	4.35	10.1	19.0	(31)
$k_1 + k_2 = -2.3 \frac{d \log (a_e - a)}{dt}$ per sec. ($\times 10^5$)	2.95	4.67	5.30	8.32	9.10	8.80	7.66	8.90	12.1	20.8	80.7	300	1000
$k_2 = \frac{k_1 + k_2}{K + 1}$ ($\times 10^5$)	2.95	4.67	5.30	8.32	9.00	8.44	6.28	4.75	4.00	3.89	7.30	(15)	(31)
k_1 by difference ($\times 10^5$)	—	—	—	—	0.10	0.36	1.38	4.15	8.1	16.9	73.4	285	(970)

The Thermodynamics of Reversible Denaturation of Soybean Trypsin Inhibitor

The first attempt to determine the heat of reaction of reversible denaturation of proteins was made by Anson and Mirsky (11) in connection with their experimental studies on the effect of temperature on the equilibrium between native and denatured trypsin in acid solution. Applying the classical van't Hoff equation they found that the heat of the reaction "native → denatured" equals 67,600 calories per mole.

The studies of the thermodynamics of reversible denaturation of proteins have been further extended by Stearn (12) and by Eyring and Stearn (13). Using the data of Anson and Mirsky they calculated the change in free energy and the entropy of the reaction of reversible denaturation of trypsin. The same authors initiated the application of the "theory of absolute reaction rates" (14) to the kinetics of protein denaturation. Because of scarcity of material on the kinetics of reversible denaturation, the authors were forced to use the data of Pace (15) on the kinetics of irreversible inactivation of crude trypsin in neutral solution in conjunction with Anson and Mirsky's data on the equilibrium between native and denatured trypsin (crystalline) in acid solution.

The present studies of the kinetics of denaturation and of the reversal of denaturation of the soybean trypsin inhibitor protein offer the necessary data on the effect of variation of temperature on the equilibrium constant and on the velocity constants so as to be able to evaluate both, the energies of reaction and the energies of activation, on the same experimental material.

1. The Energies of Reaction, Based on Equations of Classical Thermodynamics. —The following equations were used here in evaluating the energies of the reaction:

Native protein → denatured protein

(*a*)
$$\Delta F = \Delta H - T\Delta S \tag{1}$$

where ΔF = change in the free energy of the system when one mole of native protein is transformed into a mole of denatured protein both at the standard state; *e.g.*, at infinite dilution. At the low concentration of the protein solution used (about 0.0002 M), the conditions are nearly those of the standard chosen state. The same holds true for the other terms of Equation 1 where

ΔH = change in total heat (energy) of the reaction per mole
ΔS = change in the entropy of the reaction per mole
T = absolute temperature

All energy values are expressed in small calories. Equation 1, on partial differentiation with respect to T, gives[6]

$$\frac{d\Delta F}{dT} = -\Delta S \tag{2}$$

(*b*)
$$\Delta F = -RT \ln K \quad \text{or} \quad K = e^{-\Delta F/RT} \tag{3}$$

where K = equilibrium constant and R = gas constant = 1.98 calories. Substituting for ΔF its equivalent $\Delta H - T\Delta S$ we get

$$-\ln K = \frac{\Delta H}{R} \cdot \frac{1}{T} - \frac{\Delta S}{R} \tag{4}$$

Differentiating with respect to $\frac{1}{T}$ we get

$$-\frac{d \ln K}{d\left(\frac{1}{T}\right)} = \frac{\Delta H}{R} \quad \text{(van't Hoff's equation)}$$

[6] It is to be noted that

$$\frac{d\Delta H}{dT} = T\frac{d\Delta S}{dT} \text{ at constant pressure.}$$

See Lewis, G. N., and Randall, M., Thermodynamics, New York, McGraw-Hill Book Company, 1923, 162.

or

$$\frac{4.58\, d \log K}{d\left(\frac{1}{T}\right)} = -\Delta H \tag{5}$$

Equation 5 (van't Hoff's equation) states that if we should plot log K against $\frac{1}{T}$ the slope of the curve at each point when multiplied by -4.58 gives the values of ΔH at that point. A constant value of ΔH is obtained if the plot of log K *vs.* $\frac{1}{T}$ results in a straight line.

TABLE IV

Temperature, °C.	30	35	40	45	47	50	55	60	65
a_e, *per cent*	99	96	82	53.5	33.0	18.7	9	(5)	(3)
$K = \frac{100 - a_e}{a_e}$	0.010	0.042	0.220	0.870	2.03	4.35	10.1	(19)	(31)
$\Delta F = -4.58\, T \log K$	2780	1920	950	87.5	−450	−950	−1515	(−1950)	(−2310)
$\Delta H = -4.58 \frac{d \log K}{d\left(\frac{1}{T}\right)}$ (See Fig. 6)	←———			57300			———→		
$\Delta S = \frac{\Delta H - \Delta F}{T}$	180	180	180	180	180	180	179	(178)	(177)
$\Delta S = -\frac{d\Delta F}{dt}$ (See Fig. 7)	about 180 calories per degree								

The values of ΔF for various temperatures are calculated with the aid of Equation 3 while ΔS is obtained by difference or by plotting ΔF against temperature in accordance with Equation 2.

The evaluated values of ΔF, ΔH, and ΔS for the reversible denaturation of the soybean protein are given in Table IV and in Figs. 6 and 7.

2. Energies of Activation. Equations Based on the "Theory of Absolute Reaction Rates."—The equations used for evaluating energies of activation are similar to those of classical thermodynamics and are as follows:—

$$\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{1 a}$$

$$\frac{d\Delta F^{\ddagger}}{dT} = -\Delta S^{\ddagger} \tag{2 a}$$

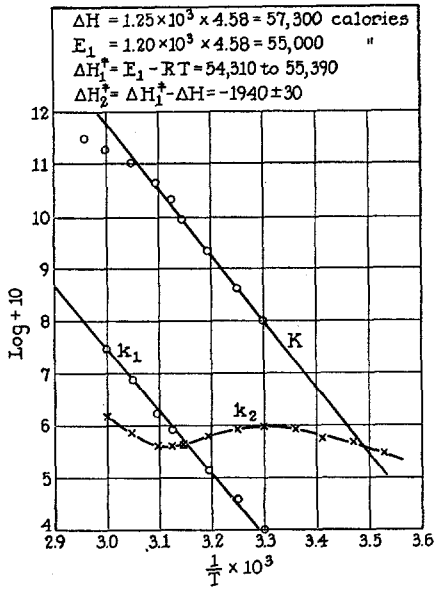

FIG. 6. Heat of reaction *vs.* heat of activation

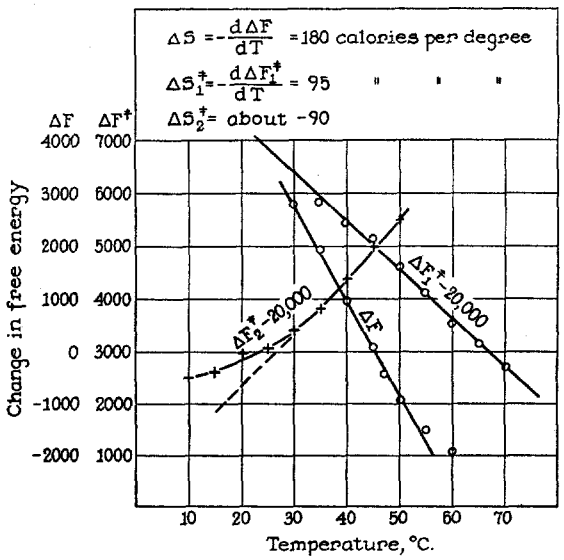

FIG. 7. Entropy of reaction *vs.* entropies of activation

$$k = \frac{\kappa}{h} T e^{-\Delta F^{\ddagger}/RT} \tag{3 a}$$

where $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ = free energy, heat, and entropy of activation, respectively.

k = the velocity constant per second
κ = Boltzman constant = 1.38×10^{-16} erg degree^{-1}
h = Plank's constant = 6.62×10^{-27} erg second
$\frac{\kappa}{h} = 2.08 \times 10^{10}$ degree^{-1} second^{-1}; $\log \frac{\kappa}{h} = 10.318$

The logarithmic expression of equation (3 *a*) is

$$\Delta F^{\ddagger} = RT\left(\ln \frac{\kappa}{h} + \ln T - \ln k\right)$$

or

$$\Delta F^{\ddagger} = 4.58T(10.318 + \log T - \log k) \tag{4 a}$$

Thus, $\Delta F_1^{\ddagger}$, *i.e.* the free energy of activation for the denaturation reaction, and $\Delta F_2^{\ddagger}$ for the reversal reaction at various temperatures are evaluated from their respective velocity constants, k_1 or k_2, with the aid of Equation 4 *a*; the values of $\Delta S_1^{\ddagger}$ and $\Delta S_2^{\ddagger}$, the entropies of activation for the corresponding reactions, are obtained from the slopes of the plotted curves of $\Delta F_1^{\ddagger}$ *vs.* T and of $\Delta F_2^{\ddagger}$ *vs.* T, respectively in accordance with Equation 2 *a*. $\Delta H_1^{\ddagger}$ and $\Delta H_2^{\ddagger}$ are then found by addition of the corresponding $\Delta F^{\ddagger}$ and $T\Delta S^{\ddagger}$.

3. The Arrhenius Equation.—The heat of activation, $\Delta H^{\ddagger}$, can be evaluated directly from the velocity constants by means of the classical Arrhenius equation

$$k = Ae^{-E/RT}$$

or

$$\ln k = -\frac{E}{R} \cdot \frac{1}{T} + c$$

whence

$$\frac{d \ln k}{d\left(\frac{1}{T}\right)} = -\frac{E}{R} \tag{5 a}$$

or

$$4.58 \frac{d \log k}{d\left(\frac{1}{T}\right)} = -E$$

where E = Arrhenius' constant. The relationship between $\Delta H^{\ddagger}$ and E is[7]

$$\Delta H^{\ddagger} = E - RT \qquad (6\,a)$$

Thus, by plotting the values of log k *vs.* $\frac{1}{T}$ the value of E and hence $\Delta H^{\ddagger}$ is readily determined. The evaluated values of activation energies for denaturation are given in Table V and those for the reversal of denaturation of the soybean protein are given in Table VI.

4. Reaction Energies vs. Activation Energies.—There is a simple relationship between the energies of activation as evaluated from the values of the velocity constants, k_1 and k_2, and of the reaction energies as obtained from the values

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[7] Proof:

Substituting in equation (3 *a*) for $\Delta F^{\ddagger}$ its equivalent $\Delta H^{\ddagger} - T\Delta S^{\ddagger}$ we get

$$k = \frac{\kappa}{h} T e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \qquad (7\,a)$$

or

$$\ln k = \frac{-\Delta H^{\ddagger}}{R} \cdot \frac{1}{T} + \ln T + \frac{\Delta S^{\ddagger}}{R} + \ln \frac{\kappa}{h} \qquad (8\,a)$$

Differentiating equation (8 *a*) with respect to $\frac{1}{T}$ and observing that

$$d \ln T = \frac{dT}{T}; \qquad d\left(\frac{1}{T}\right) = -\frac{dT}{T^2};$$

or

$$\frac{d \ln T}{d\left(\frac{1}{T}\right)} = -T$$

and

$$\frac{dH^{\ddagger}}{dT} = T \frac{dS^{\ddagger}}{dT} \quad \text{(See footnote 6)}$$

we get

$$\frac{d \ln k}{d\left(\frac{1}{T}\right)} = \frac{-\Delta H^{\ddagger}}{R} - T = -\frac{\Delta H^{\ddagger} + RT}{R} \qquad (9\,a)$$

Hence, by comparing Equation 9 *a* with Equation 5 *a*, we get

$$\Delta H^{\ddagger} + RT = E \quad \text{(Arrhenius' constant)} \qquad (6\,a)$$

of the equilibrium constant K for any given temperature. Thus from Equations 3 or 3 a and from the definition of $K = \frac{k_1}{k_2}$ we have

$$\frac{k_1}{k_2} = e^{(-\Delta F_1^{\ddagger} + \Delta F_2^{\ddagger})/RT} = e^{-\Delta F/RT}$$

TABLE V

Temperature, °C.	30	35	40	45	47	50	55	60	65	70
k_1 per sec. ($\times 10^5$)	0.10	0.36	1.38	4.15	8.1	16.9	73.4	285	970	2700
$\Delta F^{\ddagger}_1$ calories per mole	26200	25800	25400	25100	24800	24600	24100	23500	23100	22700
$\Delta H^{\ddagger}_1 = E_1 - RT$ (See Fig. 6)	55394	55384	55374	55364	55360	55354	55344	55334	55324	55314
$\Delta S^{\ddagger}_1 = \frac{\Delta H^{\ddagger}_1 - \Delta F^{\ddagger}_1}{T}$	96.5	96.2	95.8	95.6	95.6	95.4	95.4	95.8	95.5	95.3
$\Delta S^{\ddagger}_1 = -\frac{d\Delta F^{\ddagger}_1}{dt}$ = about 95 (See Fig. 7)										

TABLE VI

Temperature, °C.	10	15	20	25	30	35	40	45	47	50	55	60	65
k_2 per sec. ($\times 10^5$)	2.95	4.67	5.30	8.32	9.00	8.44	6.28	4.75	4.00	3.39	7.30	(15)	(31)
$\Delta F^{\ddagger}_2$ calories per mole	22500	22600	22950	23050	23400	23820	24400	25000	25300	25500	25600	(25400)	(25450)
$\Delta H^{\ddagger}_2 = \Delta H - \Delta H^{\ddagger}_1$	−1866	−1876	−1886	−1896	−1906	−1916	−1926	−1936	−1940	−1946	−1956	(−1966)	(−1976)
$\Delta S^{\ddagger}_2 = \frac{\Delta H^{\ddagger}_2 - \Delta F^{\ddagger}_2}{T}$	−86	−85	−85	−84	−84	−84	−84	−85	−85	−85	−84	(−82)	(−80)

Hence

$$\Delta F = \Delta F_1^{\ddagger} - \Delta F_2^{\ddagger}$$

Also from equations (2) we have

$$\frac{d\Delta F}{dt} = -\Delta S$$

or

$$\frac{d\Delta F_1^{\ddagger}}{dT} - \frac{d\Delta F_2^{\ddagger}}{dT} = -\Delta S$$

but

$$\frac{d\Delta F_1^{\ddagger}}{dT} = -\Delta S_1^{\ddagger} \quad \text{and} \quad \frac{d\Delta F_2^{\ddagger}}{dT} = -\Delta S_2^{\ddagger} \tag{2 a}$$

Hence $\Delta S = \Delta S_1^{\ddagger} - \Delta S_2^{\ddagger}$. It follows also that $\Delta H = \Delta H_1^{\ddagger} - \Delta H_2^{\ddagger}$.

The values of the reaction energies as compared with the activation energies for the reversible denaturation of the soybean protein are shown in Figs. 6 and 7 and summarized in Table VII.

DISCUSSION

The present studies were undertaken mainly for methodological purposes with regard to the experimental methods of studying the kinetics of reversible denaturation of proteins as well as to the methods of evaluating the thermodynamics of the reaction. The experiments described here are more or less

TABLE VII

ΔH	= 57300 calories per mole
$\Delta H^{\ddagger}_1$	= 55350 ± 50 calories per mole
$\Delta H^{\ddagger}_2$	= −1900 " " "
ΔS	= 180 calories per degree per mole
$\Delta S^{\ddagger}_1$	= 95 " " " " "
$\Delta S^{\ddagger}_2$	= −84 " " " " "

introductory and limited in character; a more complete study would embrace a detailed study of the effect of variation of pH on the various thermodynamic data evaluated here for one pH only.

Is Reversibility Complete?—It has been pointed out by Neurath *et al.* that this question cannot be decided categorically as it would be necessary to prove that every possible chemical, physical, and biological property of the native protein molecule is reestablished completely on reversal of denaturation. With this limitation in mind it is possible, however, to state that according to the tests performed no distinct difference could be detected between the originally native soybean inhibitor protein molecules and the protein formed on reversal of denaturation. It is of course to be expected that neither denaturation nor the reversal of denaturation can proceed to completion with regard to all molecules in solution, since the denaturation discussed here is a reversible reaction tending towards an equilibrium between native and denatured protein molecules. It is possible, however, to separate the denatured protein from the native fraction by precipitation and to test the properties of the reversed protein molecules.

SUMMARY

Crystalline soybean trypsin inhibitor protein undergoes denaturation on heating which is reversed on cooling. In the range of temperature of 35 to 50°C. a solution of the protein consists of a mixture of native and denatured forms in equilibrium with each other. The equilibrium is only slowly established and its final value at any temperature is the same whether a heated, denatured solution of the protein is cooled to the given temperature or whether a fresh solution is raised to that temperature. The kinetics of reversible denaturation of the soybean protein as well as the reversal of denaturation is that of a reversible unimolecular reaction, each process consisting at a given temperature of the same two simultaneous reactions acting in opposite directions.

The experimental data on the effect of temperature on the velocity and the equilibrium constants of the opposing reaction were utilized in evaluating the reaction energies and activation energies.

The reaction energies for denaturation were found to be as follows:—

Change in total heat of reaction $\Delta H = 57{,}000$ calories per mole

Change in entropy of reaction $\Delta S = 180$ calories per degree per mole

The heat of activation $\Delta H_1^{\ddagger}$ for denaturation $= 55{,}000$

The heat of activation $\Delta H_2^{\ddagger}$ for the reversal of denaturation $= -1900$

The entropy $\Delta S_1^{\ddagger}$ for denaturation $= 95$

The entropy $\Delta S_2^{\ddagger}$ for reversal of denaturation $= -84$

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