THE APPLICATION OF THE THEORY OF ABSOLUTE REACTION RATES TO PROTEINS[1]

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INTRODUCTION

In discussing protein kinetics we shall make use of quantities related in the following equation (14)

$$k' = \kappa \frac{kT}{h} \exp(-\Delta F^{\ddagger}/RT) = \kappa \frac{kT}{h} \exp(-\Delta H^{\ddagger}/RT) \exp(\Delta S^{\ddagger}/R) \quad (1)$$

$$= \kappa \frac{kT}{h} K^{\ddagger} \frac{\gamma_n}{\gamma^{\ddagger}}$$

Here k' is the specific reaction velocity constant in reciprocal seconds; κ is a transmission coefficient (taken as unity in this paper); k is the Boltzmann constant; h is Planck's constant; T is the absolute temperature; $\Delta F^{\ddagger}$ is the free energy increase when the activated complex is formed from the reactant; $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the analogous increases in heat and entropy; $K^{\ddagger}$ is the equilibrium constant between activated complex and reactant (both taken as ideal solutes); and γ_n and $\gamma^{\ddagger}$ are the activity coefficients of reactants and activated complex, respectively. If there is more than one reactant, then γ_n is the product of the activity coefficients. The same generalized interpretation applies to $\gamma^{\ddagger}$.

From equation 1 we have

$$\Delta H^{\ddagger} = RT^2 \frac{\mathrm{d} \ln k'}{\mathrm{d}T} - RT \quad (2)$$

and

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta F^{\ddagger}}{T}$$

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If the activated complex D is formed by the reaction

$$A + B + C + \cdots = D + E + F + \cdots$$

the concentration of D is

$$(D) = \frac{K^{\ddagger}(A)(B)(C)\gamma_A\gamma_B\gamma_C\cdots}{(E)(F)\gamma_D\gamma_E\gamma_F\cdots}$$

The rate of reaction is then obtained by multiplying this concentration of D by the frequency of flying apart, kT/h, and the transmission coefficient, κ, i.e., the chance that flying apart will successfully end in reaction. This gives:

$$(D)\kappa\frac{kT}{h} = K^{\ddagger}\frac{(A)(B)(C)\cdots\gamma_A\gamma_B\gamma_C\cdots}{(E)(F)\cdots\gamma_D\gamma_E\gamma_F\cdots}\kappa\frac{kT}{h}$$

Dividing out the concentrations on the right of this expression, we obtain the specific reaction rate constant as given in equation 1.

Whether the solvent should be thought of as taking part in the equilibrium leading to D depends on how the standard state is defined, just as in ordinary equilibria. Whenever activity coefficients vary rapidly with concentration, it is an indication that a strong interaction between solvent and reactants has been ignored. An alternative procedure is to redefine the standard state in such a way as to include the solvent as a reactant in the equilibrium. Thus the activity of hydrochloric acid at 1 molal concentration is 0.676, while at 16 molal it has become 478,000 (20). This of course is because of a hydration in the dilute solution which is no longer possible in the concentrated solution. It, however, illustrates the impossibility of ignoring the solvent in choosing a single standard state with reasonably constant activity coefficients in such reactions. The same difficulty is sometimes encountered in rate problems.

I

Part of the difficulty in the productive use of the theory of absolute reaction rates applied to proteins lies in the paucity of reactions characteristic of proteins as such. Reactions of the nature of acetylation, etc., are characteristic of fortuitous groups in particular proteins, but not of a protein. One can imagine a substance which would not undergo any specified reaction of this type but which would yet be a good protein.

Characteristic of proteins as a class are the reactions of hydrolysis and, fairly generally, denaturation. Hydrolysis of a protein is in large measure the hydrolytic splitting of the peptide bond. The theory of absolute reaction rates shows that the quantity that governs rates is the free energy of activation rather than the heat of activation alone, so that it is quite

conceivable that, even though the latter quantity be equal in several cases, the rates may be quite different because of a difference in the entropy change.

In this connection the most unequivocal data are those of Escolme and Lewis (12), who measured the rates of hydrolysis of acetylglycine and benzoylglycine. The values of $\Delta H^{\ddagger}$, the heat of activation, are almost identical in the two cases (21,184 and 21,084 cal. at unit hydrogen-ion activity), but the values of $\Delta S^{\ddagger}$, the entropy of activation (-24.8 and -31.8 cal. per degree, respectively), give reaction rates differing by over thirty-fold. This difference of 7 units in the entropy of activation has been accounted for by the difference in the loss of rotational entropy of internal degrees of freedom on the part of the respective substances when the activated complex is formed (14d).

In general, however, the kinetics of protein hydrolysis are fairly regular, and it is in the case of protein denaturation that the theory of absolute reaction rates exhibits its power as a tool. Denaturation of proteins is characterized by quite abnormal values of activation heats and yet is regularly of the first order with respect to protein.

Recently (3, 26, 14c) the process of denaturation has been pictured as the simultaneous breaking of a number of weak bonds in the molecule with a resulting loss of structure, or, what amounts to the same thing, a large increase in randomness. The fact of finite rates coupled with large heats of activation necessarily implies a large increase of entropy with activation.

Steinhardt (35) and La Mer (18), on the basis of the work of Steinhardt on the inactivation of pepsin, have considered the matter from a somewhat different point of view. Steinhardt's data show that, through a restricted pH range, the rate of pepsin inactivation near the neutral point increases almost exactly as the fifth power of the hydroxide-ion activity. Enzyme inactivation proves that some particular group or groups have disappeared but leaves us in the dark as to what further changes accompany it. This pH dependence indicates, as these authors point out, that the reactive form in this reaction is an ion quintuply more ionized than the stable form. However, to get what they term the true heat of activation, the usually accepted value, i.e.,

$$RT^2 \frac{\mathrm{d} \ln k'}{\mathrm{d}T} - RT$$

is corrected by subtracting the heat necessary to bring about this quintuple ionization.

An analysis of Steinhardt's results is given in table 1. Here the symbol P_0 refers to the protein before the five protons have been lost to transform it into P_5. By a further activation of P_5 the activated complex $P_5^{\ddagger}$ is

formed. The values in the first row are calculated directly from the measured reaction rates at a pH where most of the protein is in the form P_0. We assume, following Steinhardt, five equal dissociation constants, K_0, whose values are 1.74×10^{-7} at 25°C. and 1.024×10^{-7} at 15°C. In row 3, K' is the value of $(P_5)/(P_0)$ at pH 5.7 and is equal to $(K_0/a_H)^5$. From the values in rows 1 and 3 those in row 2 follow at once.

It is apparent that at pH 5.7 almost all of the activation entropy of P_0 going to $P_5^\ddagger$ is associated with the preliminary ionization of protons into solution to give P_5. This large entropy increase is due to the fact that the protons are ionizing into a solution of very low hydrogen-ion activity. When ionization takes place into a solution of unit hydrogen-ion activity, we note that there is little or no entropy of ionization (row 4). This last way of calculating a specific rate constant by including explicit account of

TABLE 1

The kinetics of pepsin inactivation

pH	PROCESS	$\log_{10} k'$ sec.$^{-1}$		$\Delta H^\ddagger$	$\Delta F^\ddagger$	$\Delta S^\ddagger$
		25°C.	15°C.			
				calories	*calories*	
5.7	$P_0 \rightarrow P_5^\ddagger$	−6.996	−8.716	67,470	26,955	135.9
5.7	$P_5 \rightarrow P_5^\ddagger$	−1.696	−2.266	22,360	19,737	8.8
		$\log_{10} K'$		ΔH	ΔF	ΔS
5.7	$P_0 \rightarrow P_5$	−5.3	−6.45	45,110	7,218	127.1
0	$P_0 + 5H_2O \rightarrow P_5 + 5H_3^+O$	−33.8	−34.95	45,110	46,030	−3.1
0	$5HOAc + 5H_2O \rightarrow 5OAc^- + 5H_3^+O$	−23.75		−485	32,350	−110.2

the hydrogen-ion activity is the accepted procedure of adopting a standard state used for ordinary equilibria.

It is highly desirable, wherever possible, to adopt the same standard states for defining reaction rate constants as for equilibria. Reaction rate constants if properly measured, i.e., with due regard to activity coefficients of reactants and activated complex, are functions only of temperature and pressure. However, with the data at present available this is usually impossible, so that the calculated values of $\Delta F^\ddagger$, $\Delta H^\ddagger$, and $\Delta S^\ddagger$ given refer, in general, to the unspecified standard state under which the measurements happen to be made. Sometimes certain reactants are even omitted in calculating the specific reaction rate constant, k'. For such a k' the standard state of the omitted reactant is the activity at which it happened to be present in the solution. Some, although by no means all, of the abnormally large activation entropies of protein reactions are due to failure

to include the hydrogen-ion concentration properly in calculating the specific reaction rate constants.

Row 2 in table 1 shows an apparent entropy of activation of P_5 of only 8.8 units, so that the amount of "opening up" of the protein molecule seems at first glance insignificant. It is, however, apparent that the protein in the preceding ionization acts as a very peculiar acid. When we compare the entropy involved in ionizing five moles of acetic acid, we find -110 units in place of -3. This difference indicates that the actual increase in the entropy of "opening up" the protein molecule may be in the neighborhood of 100 units, when $P_5^{\ddagger}$ is formed from P_5. The opening up of the protein structure measured by this entropy increase is probably associated with the breaking of salt bridges between groups such as $—NH_3^+$ and $—COO^-$.

Mirsky and Pauling (26) assumed that such acid and basic groups were held together by hydrogen bonds which were broken by denaturation. Sookne and Harris (34) find that salt bonds do not contribute greatly to the strength of wool. This is at least consistent with our view that in the process studied by Steinhardt the chief contribution to the free energy of activation is in the breaking of covalent linkages less influenced by pH than the above electrovalent salt bridges.

Cohn, McMeekin, Edsall, and Blanchard (10) have considered the possibility of salt-bond formation in proteins. They were, however, concerned with the additional bonds which can form in the denatured protein as a result of the greater flexibility consequent upon loss of structure. The salt bonds here considered are those which stabilize the native protein with respect to the activated complex. Dr. Anson[2] points out that stabilization by salt bonds involving free amino groups cannot be of primary importance, since substances such as formaldehyde, although they react with the amino group reducing the basicity, do not greatly reduce the stability of the native protein.

The apparent entropy of activation for denaturation passes through a maximum at a pH of maximum stability. On the alkaline side the decrease in $\Delta S^{\ddagger}$ below the maximum value is due to a change in process. This is brought out in table 1 where, as the pH increases, the activation process will be seen to change from $P_0 \rightarrow P_5^{\ddagger}$ with $\Delta S^{\ddagger} = 135.9$ units to the process $P_5 \rightarrow P_5^{\ddagger}$, with $\Delta S^{\ddagger} = 8.8$ units. On the other side of the pH of maximum stability, as the pH decreases, the same process can take place with values of $\Delta S^{\ddagger}$ decreasing from 127.1 at pH 5.7 to -3.1 at pH 0.

The final step in activation lies in the breaking of one or more bridges which are not destroyed by ionization, such as a covalent bonded cystine bridge. Such a process cannot "pay its way" with an increase in entropy

[2] Private communication.

compensating for the higher energy, since it probably involves hydrolysis of the S—S bond, at least as a first step.

Salt bridges and covalent cross linkages particularly due to cystine have been frequently spoken of (37, 5, 8, 10).

The behavior of cystine itself fits this picture well. Lavine (19) finds an easy hydrolysis of cystine into cysteine and sulfinic acid when catalyzed by mercuric ion. The ease of reversibility of this dismutation is suggestive in connection with the reversibility of denaturation. Lavine found a 90 to 92 per cent cystine recovery on removing the mercury. Shinohara and Kilpatrick (33) noted a spontaneous dismutation of cystine to cysteine and oxy acids under proper conditions. From their semi-quantitative data we obtain a value for $\Delta H^{\ddagger}$ of the order of 20,000 cal. By analogy with hydrolysis of peptide linkages (12) we expect about this value for $\Delta H^{\ddagger}$ and also a negative $\Delta S^{\ddagger}$ of the order of -20 to -30 units. The fact that in the case of pepsin there is an actual increase in entropy of 8 E.U. may well arise from the consequent loosening of the protein structure.

It is interesting to inquire why the activated complex should be more highly ionized than the normal pepsin. The explanation follows immediately from the concept that the activated complex contains broken salt bridges and as a result approximates the isoelectric state over a much shorter pH range. The reason is that, since the positive and negative groups which in the normal state formed the salt bridges are no longer adjacent, it becomes easier in alkaline solution for the amino or for the imino group to lose its proton and likewise, in the more acid range, easier for the negative carboxyl group to acquire a proton.

If the breaking of a salt bridge is, as we believe, an important step in the activation process leading to denaturation, there is a critical pH on either side of which salt bridges are automatically broken. The pH of maximum stability will not in general coincide with the isoelectric point. Thus if a protein contains a large excess of acid groups and a low isoelectric point, the pH of maximum stability will be higher than the isoelectric point. This is well exemplified in the cases of pepsin (isoelectric point, 2.85; pH of maximum stability, 5.28) and of egg albumin (isoelectric point, 4.55; pH of maximum stability, 6.76) (28). By the same token, if basic groups are largely predominant, the relation between these two points should be reversed. With a protein like hemoglobin, the isoelectric point of which is very close to neutrality and which probably has nearly the same number of acid and basic bridge-forming groups, the pH of maximum stability should practically coincide with the isoelectric point. Table 3 shows that this is the case. Proteins with very acid isoelectric regions will, according to this rule, be expected to have maximum stabilities at more alkaline pH values, since their comparatively acid isoelectric points arise from an excess of acid over basic groups.

An instructive example of the fact that the important quantity in activation is the free energy rather than the heat is given by the case of trypsin. From Pace's data (29) we get a value for $\Delta H^{\ddagger}$ of activation of 40,000 cal. at pH 6.5, while Anson and Mirsky (2), from thermal equilibrium data at pH 2 to 3, find ΔH for the equilibrium to be 67,600 cal. Unless there is a change of nearly 30,000 cal. in these values in the pH range between the two sets of measurements, one is led to a negative value for the heat of activation for recovery of enzymatic activity. At 50°C., however, this latter rate is seven times smaller than the rate for the loss of enzymatic activity. Any cutting down of Pace's value for the heat of activation because of the difference in pH for the two experiments would lead to a still more negative heat of activation for the recovery of enzymatic activity. Such a reaction, interpreted in terms of collisions between water and enzyme molecules, would involve an exceedingly small steric factor.

Anson and Mirsky find a total entropy increase of 213.1 units for the equilibrium, whereas Pace's data on activation show that the activated complex has only 44.7 entropy units more than active trypsin. This means that the formation of the activated complex from the inactive trypsin, for the reaction in the reverse direction, must take place with the loss of 213.1 − 44.7 or 168.4 E.U. Thus the free energy of this reverse reaction is not negative but has at 50°C. the value 26,800 cal. ($\Delta H^{\ddagger} - T\Delta S^{\ddagger} = 40{,}000 - 67{,}600 + 323 \times 168.4$), giving a normal reaction rate. It will be seen below (table 3) that a negative temperature coefficient is obtained for the denaturation of egg albumin by urea. In such a case the ordinary collision viewpoint is less illuminating than thinking in terms of an equilibrium between the activated complex and normal molecules. In the latter case we draw on whatever knowledge there is of analogous equilibria. More accurate trypsin rate measurements will probably alter somewhat the above provisional calculations.

It is unfortunate that there are no other data analogous to those for trypsin discussed above. The results of Anson and Mirsky (1) on chemical denaturation involve the heat of combination of the protein with the denaturing catalyst, the value of which is not known. The unusual fact that ΔH for equilibrium exceeds $\Delta H^{\ddagger}$ for activation means, of course, that after activation there are many bonds that open spontaneously, because for them the net value of $T\Delta S$ exceeds ΔH.

II

A general view of the kinetics of protein denaturation (and enzyme or hormone inactivation) is presented in tables 2 and 3. Table 2 includes for the most part data for ordinary water denaturation (or inactivation).

These data bring out in a very striking manner the fact that the apparent heat of activation, obtained from the temperature coefficient, while important, is not in itself the dominating factor. Specifically one may point out two reactions at 50°C. (those of pancreatic proteinase and goat hemolysin), with heats varying between 38,000 and 198,000 cal. but with rates which are equal to within a factor of less than 2. The ratio of these rates, if the entropy were the same in the two cases, should be 10^{107} at 50°C.

TABLE 2

Kinetics of denaturation and enzyme inactivation

SUBSTANCE	t	k' SEC.$^{-1}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$	$T\Delta S^{\ddagger}$	pH
	°C.		*calories*	*calories*	*calories*		
Insulin (16)*	80.	$1. \times 10^{-4}$	35,600	23.8	27,200	8,400	1.5
Pepsin (36)	25.	5.47×10^{-4}	55,600	113.3	21,900	33,750	6.44
Leucosin (23)	55.	1.2×10^{-3}	84,300	185.	23,620	60,680	6.1
Egg albumin (21)	65.	2.54×10^{-4}	132,100	315.7	25,400	106,700	5.0
Hemoglobin (21)	60.5	4.3×10^{-4}	75,600	152.7	24,700	50,900	5.7
Trypsin (29)	50.	2.83×10^{-5}	40,160	44.7	25,700	14,440	6.5
Enterokinase (33)	50.	7.37×10^{-5}	42,160	52.8	25,100	17,050	
Trypsin kinase (34)	50.	3.17×10^{-5}	44,260	57.6	25,700	18,600	
Proteinase (pancreatic) (35)	50.	1.27×10^{-4}	37,860	40.6	24,750	13,100	
Lipase (pancreatic) (25)	50.	1.18×10^{-3}	45,360	68.2	23,330	22,030	
Hemolysin (goat) (15)	50.	2.09×10^{-4}	198,000	537.	24,550	173,500	
Vibriolysin (15)	50.	$3. \times 10^{-3}$	127,950	326.	22,650	105,300	
Tetanolysin (15)	50.	2.1×10^{-4}	172,650	459.	24,350	148,400	
Peroxidase (milk) (42)	70.1	5.1×10^{-4}	185,300	466.4	25,300	160,000	
Rennen (4)	50.	6.7×10^{-3}	89,350	208.1	22,130	67,200	
Amylase (malt) (24)	60.	$8. \times 10^{-4}$	41,630	52.3	24,230	17,400	
Invertase (yeast) (13)	50.	7.7×10^{-5}	52,350	84.7	25,000	27,350	5.7
Invertase (yeast) (13)	55.	$5. \times 10^{-5}$	86,350	185.	25,700	60,680	5.2
Invertase (yeast) (13)	50.2	3.5×10^{-5}	110,350	262.5	25,500	84,840	4.0
Invertase (yeast) (13)	55.	3.75×10^{-4}	74,350	152.4	24,400	50,000	3.0
Emulsin (wet) (39)	60.	$4. \times 10^{-3}$	44,930	65.3	23,200	21,750	
Emulsin (dry) (39)	100.	1.48×10^{-4}	25,550	−7.86	28,500	−2,930	
Lipase (dry) (27)	120.	$4. \times 10^{-4}$	(24,200)	(−13.)	(29,300)	(−5,100)	

* References to the literature are given in parentheses.

Naturally $\Delta F^{\ddagger}/T$ does not vary greatly for these reactions. For a conveniently measurable reaction rate between, say, 25°C. and 70°C. the free energy cannot vary much. (For example, for specific rates between 10^{-3} and 10^{-5} at temperatures between 25°C. and 70°C., $\Delta F^{\ddagger}$ can vary through a range of 6500 cal.) In table 2, except for the two cases of dry heating, the values of $\Delta F^{\ddagger}$ vary from 21,900 to 27,200, or through a range

of 5300 cal. These $\Delta F^{\ddagger}$ values are to be compared with the corresponding values of $T\Delta S^{\ddagger}$, where an entirely different situation obtains. $T\Delta S^{\ddagger}$ values vary over a range of more than 150,000 cal.

In many studies the pH has not been reported. It will be considered in discussing table 3, but in table 2 the data for invertase are striking. Here any variation of $\Delta F^{\ddagger}$ is small enough so that the slightly different temperatures at which the measurements were made obscure any noticeable trend, whereas $T\Delta S^{\ddagger}$ varies by over 57,000 cal. in the pH range 3 to 5.5, and passes through a distinct maximum at about pH 4.

The apparent abnormalities met with in reactions of the type summarized in table 2 are, at least in some cases, due to the method of calculating k', a method which does not take into full account all substances involved in the equilibrium between the normal and the activated states of the reacting system. As we have previously stated, in effect it amounts to choosing a variable standard state for this equilibrium. To obtain a value of k' which is really constant (i.e., depending only on temperature and pressure), it would be necessary to include in this constant the activities of all substances which enter the equilibrium between the normal and the activated states. Suppressing any of these amounts to treating as unit activity the actual activity of the suppressed substance at which k' is measured, and thus may involve a different standard state for each measurement. It is analogous to ignoring the activities of certain of the substances entering into any equilibrium. The value of K would change with changes in the activities of these ignored substances. For example, if the constant for the ionization of phosphoric acid were formulated as $(PO_4^{---})/(H_3PO_4)$, the value of this ratio would certainly change enormously when measured at widely differing hydrogen-ion activities.

However, even if we define our standard state in solution by taking the activity equal to the molality at infinite dilution, as Lewis and Randall do, there will still be reactions with abnormal entropies of activation just as one finds for the hydrogen chloride liquid–vapor equilibrium. If an activation process involves an increase or decrease in the number of ions present, the consequent "freezing" or "melting" of water around the ion will insure abnormal changes in entropy which render hopeless any attempt to treat the process by simple kinetic theory. There is, of course, no such limitation on the general statistical-mechanical treatment.

The proteins whose behavior on denaturation has been most widely studied are egg albumin and hemoglobin. In table 3 data on the kinetics of denaturation of these proteins under varying catalytic conditions are given. Specific numerical data are available, in one or the other case, for denaturation by urea, acid, alcohol, and water; data are available on denaturation by water both in the presence and in the absence of high salt

TABLE 3

Kinetics of denaturation with various catalysts

PROTEIN AND REFERENCE	t	pH	CATALYST	K' SEC.$^{-1}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$	$T\Delta S^{\ddagger}$
	°C.							
Egg albumin (21)	65.	5.0	Water	2.54×10^{-4}	132,100	315.7	25,400	106,700
	65.	6.76	Water	2.8×10^{-6}	128,200	295.2	28,400	99,800
	65.	7.7	Water	1.8×10^{-5}	134,300	317.1	27,100	107,200
	70.2	5.0	Water	5.1×10^{-3}	132,100	315.7	23,750	108,350
	70.2	6.76	Water	5.15×10^{-5}	128,200	295.2	26,900	101,300
	70.2	7.7	Water	3.8×10^{-4}	134,300	317.1	25,500	108,800
Hemoglobin (21)	60.5	5.7	Water	4.3×10^{-4}	75,600	152.7	24,700	50,900
	60.5	6.8	Water	1.05×10^{-4}	76,300	152.0	25,600	50,700
	60.5	8.0	Water	3.35×10^{-4}	77,300	157.3	24,840	52,460
	68.	5.7	Water	5.4×10^{-3}	75,600	152.7	23,530	52,070
	68.	6.8	Water	1.35×10^{-3}	76,300	152.0	24,470	51,830
	68.	8.0	Water	4.45×10^{-3}	77,300	157.3	23,660	53,640
Egg albumin (9)	51.1	3.4	Water	2.76×10^{-4}	96,750	223.7	24,300	72,500
	56.2	3.4	Water	2.88×10^{-3}	96,750	223.7	23,100	73,640
	64.8	9.8	Water	1.15×10^{-5}			27,500	
Egg albumin (9)	70.9		2 N $(NH_4)_2SO_4$	1.94×10^{-5}	87,500	174.2	27,600	59,900
	75.4		2 N $(NH_4)_2SO_4$	1.03×10^{-4}	87,500	174.2	26,800	60,700
Hemoglobin (22)	64.	6.76	0.15 N $(NH_4)_2SO_4$	3.41×10^{-4}	76,500	152.5	25,100	51,400
	70.	6.76	0.15 N $(NH_4)_2SO_4$	2.57×10^{-3}	76,500	152.5	24,200	52,300
	64.	6.76	1.14 N $(NH_4)_2SO_4$	5.45×10^{-4}	87,000	184.6	24,800	62,200
	70.	6.76	1.14 N $(NH_4)_2SO_4$	5.4×10^{-3}	87,000	184.6	23,700	63,300
	64.	6.76	2.27 N $(NH_4)_2SO_4$	9.1×10^{-5}	103,800	230.9	26,000	77,800
	70.	6.76	2.27 N $(NH_4)_2SO_4$	1.4×10^{-3}	103,800	230.9	24,600	79,200
	64.	6.76	3.03 N $(NH_4)_2SO_4$	2.44×10^{-5}	119,800	275.8	26,900	92,900
	70.	6.76	3.03 N $(NH_4)_2SO_4$	5.7×10^{-4}	119,800	275.8	25,200	94,600

Egg albumin (17)	0.	5.9	Urea, 0.6 g. per cc.	1.7×10^{-3}	−6,750	−95.6	19,350	−26,100
	23.	5.9	Urea, 0.6 g. per cc.	$7. \times 10^{-4}$	−6,790	−95.8	21,560	−28,350
Hemoglobin (7, 21)	60.5	6.0	Alcohol (none)	2.3×10^{-4}	76,250	153.4	25,100	51,150
	45.	6.0	Alcohol (20 volume %)	4.56×10^{-4}	107,400	263.9	23,500	83,900
	33.	6.0	Alcohol (30 volume %)	5.3×10^{-4}	117,000	308.9	22,500	94,500
	60.5	6.5	Alcohol (none)	1.22×10^{-4}	75,050	148.5	25,550	49,500
	45.	6.5	Alcohol (20 volume %)	4.5×10^{-4}	102,600	248.8	23,500	79,100
	33.	6.5	Alcohol (30 volume %)	6.8×10^{-4}	120,100	319.5	22,300	97,800
	60.5	7.0	Alcohol (none)	1.22×10^{-4}	76,050	151.5	25,550	50,500
	45.	7.0	Alcohol (20 volume %)	8.0×10^{-4}	98,200	236.1	23,100	75,100
	33.	7.0	Alcohol (30 volume %)	(1.1×10^{-3})	(128,500)	(348.)	(22,000)	(106,500)
Hemoglobin (16)	45.	4.85	Acid	1.7×10^{-4}	17,760	−19.9	24,100	−6,330
	45.	4.23	Acid	2.53×10^{-3}	10,660	−36.9	22,400	−11,730
	37.	4.64	Acid	2.78×10^{-4}	11,300	−38.3	23,200	−11,870
	37.	4.11	Acid	3.06×10^{-3}	10,700	−35.5	21,700	−11,000
	25.	4.52	Acid	2.29×10^{-4}	11,100	−37.8	22,400	−11,260
	25.	4.08	Acid	1.76×10^{-3}	11,100	−33.7	21,150	−10,040
Egg albumin (16)	37.	2.00	Acid	2.15×10^{-5}	48,400	76.3	24,750	23,650
	37.	1.62	Acid	5.68×10^{-5}	36,900	41.2	24,100	12,770
	37.	1.32	Acid	8.83×10^{-5}	36,700	41.4	23,900	12,830
	25.	1.72	Acid	4.8×10^{-6}	34,400	32.6	24,700	9,700
	25.	1.52	Acid	6.4×10^{-6}	35,400	36.5	24,500	10,900
	25.	1.35	Acid	8.2×10^{-6}	35,200	36.3	24,400	10,800
	25.	1.02	Acid	1.59×10^{-5}	(35,400)	(38.5)	24,000	(11,400)

TABLE 3—*Concluded*

PROTEIN AND REFERENCE	t	pH	CATALYST	K' SEC.$^{-1}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$	$T\Delta S^{\ddagger}$
	°C.							
Hemoglobin (16)	45.	5.5		9.6×10^{-6}			25,920	
	45.	4.9		1.21×10^{-4}			24,320	
	45.	4.52		7.26×10^{-4}			23,190	
	45.	4.23		2.53×10^{-3}			22,400	
Egg albumin (16)	45.	3.52		1.2×10^{-5}			25,780	
	45.	2.68		3.5×10^{-5}			25,100	
	45.	1.98		1.73×10^{-4}			24,100	
	45.	1.34		3.88×10^{-4}			23,590	
Hemoglobin (7)	45.	6.57	Alcohol (20 volume %)	4.52×10^{-4}	100,450	242.1	23,500	77,000
	40.	6.57	Alcohol (20 volume %)	3.5×10^{-5}	100,450	242.1	24,700	75,800
	60.5	6.0	Alcohol (2 volume %)	5.06×10^{-4}	83,500	176.7	24,600	58,950
	55.	6.0	Alcohol (2 volume %)	6.0×10^{-5}	83,500	176.7	25,600	57,950
Egg albumin (11)	25.	1.35	Acid	8.2×10^{-6}	35,100	36.1	24,350	10,750
	37.	1.35	Acid	8.5×10^{-5}	35,100	36.1	23,900	11,200
	45.	1.35	Acid	3.83×10^{-4}	36,200	39.7	23,600	12,600

concentration. The process which takes place at pH values fairly close to the pH of maximum stability we call denaturation by water. Qualitatively the experimental behavior may be summarized in the following statements:

(*1*) For a given reaction $\Delta F^{\ddagger}$ at constant pH decreases slowly but steadily with temperature except for urea denaturation.

(*2*) On either side of a pH of maximum stability $\Delta F^{\ddagger}$ decreases respectively with increasing or decreasing pH.

(*3*) Again excepting the case of urea denaturation, $T\Delta S^{\ddagger}$ increases with increasing temperature. This may be entirely due to the temperature factor, but there are indications that $\Delta S^{\ddagger}$ increases slightly with temperature.

(*4*) Owing to the possibility of confusing side reactions, such as partial digestion, data in alkaline solutions are scarce. In sufficiently acid solutions, however, $\Delta S^{\ddagger}$ decreases with decreasing pH. In the cases of egg

TABLE 4

Relative effectiveness of denaturing catalysts

CATALYST	ORDER OF INCREASING VALUES OF		
	$\Delta F^{\ddagger}$	$\Delta H^{\ddagger}$	$T\Delta S^{\ddagger}$
Urea	1	1	1
Acid	2	2	2
Alcohol	3	5	5
Water	4	4	4
Salt solution	5	6	6
Dry heat	6	3	3

albumin and pepsin, and ostensibly in general, $\Delta S^{\ddagger}$ also decreases with increasing pH in sufficiently alkaline solutions.

(*5*) It is instructive to arrange the various denaturing catalysts in the order of increasing values for the three important quantities $\Delta F^{\ddagger}$, $\Delta H^{\ddagger}$, and $T\Delta S^{\ddagger}$. This is done in table 4.

(*6*) The effect of alcohol and of ammonium sulfate should be noted. In the concentration ranges covered, the values of $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ are almost parallel. At the maximum respective concentrations, however, $\Delta F^{\ddagger}$ is rapidly decreasing with increase in alcohol concentration, whereas it is increasing with increasing salt concentration. This signifies a more rapid increase in $\Delta S^{\ddagger}$ with alcohol concentration, where $T\Delta S^{\ddagger}$ is gaining on $\Delta H^{\ddagger}$, than with salt concentration, where $T\Delta S^{\ddagger}$ is falling behind $\Delta H^{\ddagger}$.

(*7*) Finally a certain consistency in behavior between egg albumin and hemoglobin appears. The value of $\Delta S^{\ddagger}$ for egg albumin decreases by some 250 units between pH 5 measured at 65°C. and pH 1.5 measured at 37°C.,

while $\Delta S^{\ddagger}$ for hemoglobin decreases by about 190 units between pH 5.7 measured at 60°C. and pH 4.6 measured at 37°C. The change in $\Delta S^{\ddagger}$ for the two substances would no doubt agree even more closely for more nearly the same change in pH.

III

From equation 1 we see that any change in environment which renders the activated complex more soluble (comparatively) than native protein, i.e., which preferentially decreases $\gamma^{\ddagger}$, will increase the rate of denaturation. It is at once apparent: (*1*) that the temperature coefficient of solubility of the activated complex in water and most aqueous solutions is greater than that of native protein (concentrated urea solution is an exception); (*2*) that a shift in pH in either direction from a critical value characteristic of the particular protein increases the solubility of the activated complex preferentially; (*3*) that its solubility is also increased preferentially by alcohol–water mixtures, at least up to 30 per cent alcohol, and by other chemical agents such as urea, salicylate, etc.; (*4*) that, with increasing salt concentration, the activated complex is first preferentially "salted in" and finally preferentially "salted out".

In each of the above-mentioned environments the general mechanism of activation is the same. The protein chain is held in a specific configuration by side bridges, among which salt bridges and the cystine type of bridge are essential. In this latter type we include any bridge not automatically broken by an equilibrium shift due to change in pH and not included in so-called hydrogen bridges.

Salt bridges may be broken either by neutralization due to a pH shift, in which case the heat involved will be written off as heat of neutralization and will not appear in the measured $\Delta H^{\ddagger}$, or they may be broken "thermally", in which case the heat involved will be the difference between the strength of the bridge bond and the strength of any bonds subsequently formed with solvent. This net heat may at times be negative, as in the case of concentrated urea solutions, or it may reach values higher than for water, as in alcohol or in concentrated salt solutions where the water is already tied up by salt ions. So long as we treat denaturation as a monomolecular reaction, it is perfectly legitimate to speak of the $\Delta H^{\ddagger}$ given by equation 2 as the heat of activation for the particular experimental conditions under which it is measured.

We speak of the process of breaking a bridge, or a set of bridges, as paying its way if the total $T\Delta S^{\ddagger}$ for the process equals or exceeds $\Delta H^{\ddagger}$, giving $\Delta F^{\ddagger} \leqslant 0$. In general we do not expect the breaking of a bridge of the cystine type to pay its way unless the process is accompanied by a large opening of the protein structure, since hydrolysis is probably involved,

where $\Delta S^{\ddagger}$ will be negative unless the remainder of the protein molecule can more than make it up.

Certain of the salt bridges cannot pay their way in breaking; others can. In general the last few bridges broken more than pay their way, since their breaking will permit complete opening up of the protein structure. The entropy change for breaking a particular bridge will be the sum of three contributing terms: (*1*) that involved in freeing the side chains which form the bridge, (*2*) that involved in freezing solvent molecules to the free side chains, and (*3*) that due to the effect of the breaking of the bridge on the opening up of the protein molecule. It will be realized that two samples of the bridge in a protein molecule may make entirely different contributions to the net $\Delta S^{\ddagger}$. If one is among the first broken, its contribution may even be negative, whereas if the other is among the last broken, the contribution may be very large, owing to the third term listed above.

If, when breaking, all bridges paid their way under all conditions, denaturation would proceed spontaneously and rapidly to completion. Indeed, native protein would be unknown. The process of activation, then, consists of the breaking of those bridges which will not pay their way, the number of such varying under different conditions. As a specific example, egg albumin has many salt bridges at pH 6.76 and a highly restricted configuration. When it activates $\Delta H^{\ddagger}$ is over 130,000 cal. and $\Delta S^{\ddagger}$ about 300 units. When the pH is shifted to 1, all the salt bridges are broken by ionization. There are, however, two cystine residues found per molecule of egg albumin (8). It was shown above (33) that we expect a $\Delta H^{\ddagger}$ of about 20,000 cal. and a $\Delta S^{\ddagger}$ of -20 to -30 units for the hydrolysis of the S—S bond. For two cystine bridges these values agree satisfactorily with the measured value of $\Delta H^{\ddagger}$ for denaturing egg albumin at pH 1, i.e., about 35,000 cal. With $\Delta S^{\ddagger}$ we do not, of course, expect such agreement, since the opening of the protein molecule brings the measured $\Delta S^{\ddagger}$ of denaturation up to about 36 units. With hemoglobin the data are equally interesting, though the case is more complicated. Hemoglobin has one cystine residue per molecule of weight 68,000 (8). Its pH stability range, however, unlike that of egg albumin, is only from about 6 to 8 (38). In urea solution it goes to a molecular weight of 34,000 (8). At pH 4.1, therefore, we assume hemoglobin with molecular weight 34,000 and only a few salt bridges. (Unless the two dissociated varieties differ, there would be only the equivalent of one-half of a cystine residue per molecule of weight 34,000.) Under these conditions the heat of activation is so small (about 11,000 cal.) that the molecule can activate by breaking a very few bridges which do not pay their way ($\Delta S^{\ddagger}$ about -35 units). Indeed, if the pH were lowered much farther, $\Delta F^{\ddagger}$ would decrease so that the rate of denaturation would be immeasurably rapid. In table 3 it is seen that in the

pH range 5.5 to 4.2 $\Delta F^{\ddagger}$ for hemoglobin is dropping by almost 3000 cal. per pH unit, whereas, even in the lower pH range 3.5 to 1.3, not only is $\Delta F^{\ddagger}$ for egg albumin about 700 cal. higher than for hemoglobin, but it is much more nearly constant, dropping by only 1000 cal. per pH unit. Apparently, if we were to speak, in terms of Steinhardt's interpretation, of a "real" $\Delta H^{\ddagger}$ for hemoglobin denaturation independent of pH, its value would not be greater than zero and might conceivably be negative.

As a résumé of the general denaturation behavior a few remarks may be made. The pH of maximum stability is on the same side of the isoelectric point as that for the maximum in the arithmetic sum of charges in the protein. In the case of the proteins this will be the pH of minimum entropy. Where the isoelectric point and the pH of maximum stability coincide, as in the case of hemoglobin, we expect that the basic and acidic bridge-forming groups are essentially equal in number. This need not necessarily mean exact equivalence in acid- and base-binding capacity, since at high pH the phenolic group of tyrosine, for example, may affect the titration curve but will not form a salt bridge.

When salt bridges are broken, we expect a certain binding or "freezing out" of polar solvent molecules on the freed charges (14c). Change of solvent from dilute aqueous solution to more concentrated solution will in every case affect this "freezing out" process. If the molecules frozen out are water, then addition of non-aqueous solvent molecules will lower the freezing point of the water, and less will be frozen out. We find that salt and alcohol both affect denaturation kinetics in the same way; both lower the freezing point of water and prevent its "freezing out." This effect is seen in the progressive increase of $\Delta S^{\ddagger}$ with increasing salt or alcohol concentration. The heat of freezing of this water is also lost, and $\Delta H^{\ddagger}$ is seen to go up with increasing salt and alcohol concentration.

Urea would, of course, have the same effect on the water molecules as would salt or alcohol, and yet its effect on the kinetics of denaturation is in the opposite direction. This probably means that the solvent frozen out is no longer water but urea, and that the bonding of urea to the activated complex is very strong. This is reflected in the large negative entropy of activation. In the absence of urea, at pH about 6, $\Delta S^{\ddagger}$ is about 300 units for the denaturation of egg albumin, whereas in concentrated urea solution at the same pH it is -90 units. There is thus a decrease of about 400 entropy units when water is replaced by urea as solvent. The net heat of binding the urea is also much greater than that for water. Thus $\Delta H^{\ddagger}$ for this same denaturation in water is about 135,000 cal., while in concentrated urea solution it is about -6000 cal., so that the net binding of urea is measured by a ΔH about 140,000 cal. more than is that of water.

This tendency of urea to form stronger bonds than water is to be ex-

pected when it is realized that the dipole moment of urea is about 8.6 Debye units (6) and that an 8 molal solution has a dielectric constant of about 108 at 0°C. (41).

A quantitative theory can be applied to take account of the change in activity coefficients with change in dielectric constant of the solvent. Solvent effects occur, however, which are independent of dielectric constant.

SOLVATION AND THE STRUCTURE OF PROTEINS

To make a hole in a liquid the size of the molecule requires the same amount of heat as to vaporize the molecule. To do both simultaneously requires twice the heat of vaporization. This fact is of interest in connection with theories of protein structure. Thus if one assumes space-enclosing structures such as those postulated by Wrinch (40), large quantities of solvent will be enclosed (*1*) in the interior of the molecule and (*2*) in the space between the protein molecules of a crystal. Spheres arranged in simple cubic structure have as much empty space between the spheres as the spheres themselves occupy. Actual structures which occur in crystals are never quite as loose as this. The unoccupied space for spherical molecules is ordinarily of the order of one-quarter of the occupied space. Although in ordinary crystals this space remains empty, protein molecules are so large that the solvent fills the lacuna. Steinhardt's (36) solubility results for pepsin give values which depend on the relative amounts of solid and solvent; this leads him to the conclusion that solid pepsin must be regarded as at least a two-component system. This would be expected if pepsin molecules were even approximately spherical.

Removal of the solvent from crystalline proteins by drying or by introducing it into a second solvent unable to replace the first should cause those molecules having large lacunas to collapse. The degree of instability is readily estimated from the above considerations if the size of the holes be known. Such a collapse of an enzyme would probably denature and inactivate it. Unfortunately the available experimental material does not justify a more detailed discussion at this time.

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