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Thermodynamic Considerations of Protein Reactions. III. Kinetics of Protein Denaturation[1,2]

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A model is used as a basis for the development of a theory of the kinetics of protein denaturation. The activation process involves the rupture of a critical number of side-chain hydrogen bonds, and the rate of denaturation depends on the concentration of the molecules in which these side-chain hydrogen bonds are ruptured. In passing from the activated to the denatured state, the system is assumed to pass through an intermediate state in which the helical backbone chains have acquired sufficient freedom to be able to move with respect to each other. Expressions are obtained for the rate constant and for the thermodynamic parameters for the activation process for thermal denaturation (under conditions where the rate is independent of *p*H), for *p*H-dependent denaturation and for urea denaturation. Criteria are also developed for assessing the strength of the side-chain hydrogen bonds. The application of this theory to experimental data on a system, for which this is a valid model, can provide information about the hydrogen bonds which help maintain the native configurations of globular proteins.

Introduction

In previous papers consideration was given to the influence of polar side-chain hydrogen bonding on the binding of protons and other ions by proteins[4] and also on the stability of primary valence bonds in proteins.[5,6] This paper is an extension of the earlier work to the problem of the kinetics of protein denaturation. The theory is developed in terms of interactions of individual groups in a protein molecule.

Previous theories of protein denaturation have been presented by Mirsky and Pauling,[7] Steinhardt[8] and Levy and Benaglia.[9] The absolute reaction rate theory was first applied to denaturation by Stearn and Eyring[10] and later used in discussions of denaturation by Kauzmann[11] and by Lumry and Eyring.[12]

In this paper we shall *assume* that *the only barrier to the transition from the native to the activated state is the presence in the native state of several intramolecular hydrogen bonds between polar R groups which must be simultaneously ruptured in the activated state.* This is not to imply that in real cases other stabilizations are not also disrupted or that all activations require rupture of tertiary hydrogen bonds. The model is presented because it is a relatively simple one and because it formally accounts for the variation of the thermodynamic parameters of activation with *p*H, temperature and urea concentration.

"Denaturation" will be taken to mean a change in protein conformation of such a type that it is convenient to denote a series of conformational states as native and the rest as denatured. This implies a fairly discrete difference between native and denatured states. Furthermore the "denaturations" to be considered here (a) proceed without rupture of any primary valence bonds, (b) proceed at a relatively slow, measurable rate, (c) are strictly unimolecular, (d) proceed over the whole time course of the reaction by simple first order kinetics. This operating definition clearly excludes a great number of protein phenomena from consideration; it is nonetheless hoped that there is a sufficient number of processes which satisfies it to make the model of general interest.

Basis for the Model

In coming to a decision on the model for the activation process three criteria must be satisfied: (a) the interactions postulated must actually exist, (b) the rupture of these interactions must be a logical slow step in the process, (c) the model must account, in terms of reasonable parameters, for the dependence of the rate of denaturation on such variables as *p*H, temperature, urea concentration, etc. The following comments can be made about these criteria: (a) While the existence of an appreciable number of intramolecular hydrogen bonds between polar R groups of proteins is still not conclusively established, a considerable body of evidence, discussed by us on several occasions,[4,5,13-20] argues strongly in its favor. In the remainder of this paper the existence of hy-

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drogen bonds between polar R groups will be assumed. (b) The nature of the final state of denatured proteins is still unknown. The current view favors the notion that the polypeptide chains of the denatured protein are in random configurations, *i.e.*, there is essentially no helical content in the denatured protein. Since helices lacking side-chain interactions are likely to be unstable in water, a logical rate determining step is the breakdown of side-chain stabilization, *i.e.*, we assume the activation step to involve the rupture of side-chain hydrogen bonds without the disruption of the helix. (c) This point is discussed in the remainder of the paper.

Theory

As in the previous papers[4,5] the native protein is considered as an assembly of organized (helical) peptide fragments held rigidly with respect to each other by disulfide bonds and side-chain hydrogen bonds. In those proteins where the side-chain hydrogen bonds are broken, disorganization sets in very rapidly—probably on a time scale of a single molecular vibration. The activation process is envisaged as occurring in two steps: (1) When the native protein is subjected to some stress, such as a change in temperature, pressure, *p*H, urea concentration, etc., the native molecules *instantaneously* equilibrate with the new environment by ionization, rupture or formation of intramolecular hydrogen bonds, binding, etc. (2) Among the re-equilibrated *native* molecules there exists a small concentration ($C^{\ddagger}$) of molecules in which all side-chain hydrogen bonds, responsible for stabilization of the native molecule, are broken. These molecules are considered to be in the activated state.[21] The over-all process takes a finite time since $C^{\ddagger}$ is very small and is replenished only when some molecules pass over the barrier corresponding to the activated state.[21]

The transition state theory yields

$$\frac{dC_D}{dt} = -\frac{dC_N}{dt} = \frac{kT}{h} C^{\ddagger} \qquad \text{(III-1)}$$

assuming unit probability of crossing the barrier. Here C_D and C_N are the concentrations of denatured and native molecules, respectively, at time t, T is the absolute temperature and k and h are Boltzmann's and Planck's constants, respectively.[22]

(21) In the native and activated states the helices are assumed to be held rigidly with respect to each other. After passing over the barrier, corresponding to the activated state, the molecules can rapidly attain a new state in which the helices have acquired the ability to move and can take up different relative positions with respect to each other than existed in the native and activated states. Subsequent passage to the randomly-coiled denatured state proceeds at a rate depending on the free energy of activation for unwinding the free helix. Since, however, the second process is much faster than the first, only the first (rate determining) step is considered.

(22) An alternative formulation can be given in terms of an equilibrium between native molecules, N, and an intermediate state, A, in which the side-chain hydrogen bonds are ruptured. Molecules in state A then pass to the denatured state, D, *e.g.* by a helix-random coil transition. In our treatment in the text we have referred to molecules in state A as being in the transition state. However, in this alternative formulation, state A is simply an intermediate one in equilibrium with state N

$$N \underset{}{\overset{K}{\rightleftharpoons}} A \xrightarrow{k'} D$$

The rate constant for this process is $k'K$ which is also

$$\frac{kT}{h} \exp(-\Delta H^{\ddagger}/RT - \Delta H^{0}/RT) \exp(\Delta S^{\ddagger}/R + \Delta S^{0}/R)$$

where ΔH^0 and ΔS^0 correspond to the equilibrium N $\rightleftharpoons$ A, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are activation parameters corresponding to k'. If $\Delta H^{\ddagger}$ is very small compared to ΔH^0 and $\Delta S^{\ddagger}$ very small compared to ΔS^0, then the mathematics for this alternative formulation will be identical with that presented in the text. Therefore, in the text, we shall retain our formulation, in terms of which the intermediate A is in the transition state.

(23) It is of interest that very few data are available in the literature on $\Delta H^{\ddagger}$ and $\Delta F^{\ddagger}$ at *p*H minima and plateaus. Most authors hold *p*H constant and report thermodynamic data at some arbitrary *p*H. Such data are quite difficult to interpret and, in the absence of additional information, frequently useless.

In order to calculate $C^{\ddagger}$ we recall[5] that the probabilities that specified single, cooperative or double hydrogen bonds[6] exist are x_{ij}, x_{rs} and x_{lm}, respectively. Thus the probability that these bonds do not exist is $(1 - x_{ij})$, $(1 - x_{rs})$ and $(1 - x_{lm})$, respectively. We assume further, as was done in previous papers, that the bonds are independent, *i.e.*, that the existence of a given bond does not affect the probability of formation of another bond in the same molecule. Thus, the probability that several specified hydrogen bonds *do not exist* in a given molecule is simply the product of the individual probabilities for their absence. Thus

$$C^{\ddagger} = C_N \prod (1 - x_{ij}) \prod (1 - x_{rs}) \prod (1 - x_{lm}) \qquad \text{(III-2)}$$

where the products are taken over all of the different kinds of hydrogen bonds. From equations III-1 and III-2 the first order rate constant for denaturation, k_1, becomes

$$k_1 = \frac{kT}{h} \prod (1 - x_{ij}) \prod (1 - x_{rs}) \prod (1 - x_{lm}) \qquad \text{(III-3)}$$

Equation III-3 describes all denaturation processes compatible with our model. However, since x_{ij}, x_{rs} and x_{lm} depend on several variables such as temperature, *p*H, and urea concentration, we can investigate these individual functions by studying the effect of several variables on the rate of denaturation. As far as possible we should like to vary these one at a time.

Thermal Denaturation

In aqueous solutions the denaturing effects of *p*H and temperature are so closely connected that only rarely can denaturation processes be considered as purely thermal. However, all plots of the rate of denaturation *vs.* *p*H show at least one minimum and frequently some plateaus. At the *p*H of such minima, especially if they are broad, and on the broad plateau portions, we can make the fairly justifiable assumption that x_{ij}'s, x_{rs}'s and x_{lm}'s are independent of *p*H. Thus all the terms in these functions concerned with hydrogen ion dependence can be dropped.[23]

If we restrict ourselves further to a case where only heterologous single hydrogen bonds[6] are involved then, from equation II-17

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij}} \qquad \text{(III-4)}$$

and

$$k_1 = \frac{kT}{h} \prod \left(\frac{1}{1 + K_{ij}}\right) \qquad \text{(III-5)}$$

By analogy with equations I-38 to I-41

$$\Delta F^{\pm} = RT \sum \ln (1 + K_{ij}) \qquad \text{(III-6)}$$

$$\Delta H^{\pm} = - \sum \frac{K_{ij}}{1 + K_{ij}} \Delta H^{0}_{ij} \qquad \text{(III-7)}$$

$$\Delta S^{\pm} = - R \sum \ln (1 + K_{ij}) - \frac{1}{T} \sum \frac{K_{ij}}{1 + K_{ij}} \Delta H^{0}_{ij} \qquad \text{(III-8)}$$

where $\Delta F^{\pm}$, $\Delta H^{\pm}$ and $\Delta S^{\pm}$ are the standard changes in free energy, enthalpy and entropy, respectively, for the activation process, and ΔH^{0}_{ij} is the enthalpy of formation of the ij^{th} hydrogen bond. These equations predict large positive values for $\Delta F^{\pm}$, $\Delta H^{\pm}$ and $\Delta S^{\pm}$, which are most frequently found for protein denaturation. The large positive $\Delta S^{\pm}$ arises here from the rupture of side-chain hydrogen bonds and the resulting freedom of torsional oscillations in the side-chain groups (see paper I).[4] Thus, in our model, the $\Delta S^{\pm}$ is primarily due to the greatly increased number of energy levels available to the activated molecule rather than, as in other models, to the increased number of spatial configurations in the activated state.

In order to simplify further discussion, we shall assume temporarily that all K_{ij}'s and ΔH^{0}_{ij}'s are equal. While this is clearly a gross oversimplification it helps to reduce the resulting expressions to manageable size. With this approximation equations III-6 to III-8 become

$$\Delta F^{\pm} = nRT \ln (1 + K_{ij}) \qquad \text{(III-9)}$$

$$\Delta H^{\pm} = - \frac{nK_{ij}}{1 + K_{ij}} \Delta H^{0}_{ij} \qquad \text{(III-10)}$$

$$\Delta S^{\pm} = -nR \ln (1 + K_{ij}) - \frac{nK_{ij}}{1 + K_{ij}} \frac{\Delta H^{0}_{ij}}{T} \qquad \text{(III-11)}$$

First Criterion for the Strength of Bonding.— The measurement of the rate of a given pH-independent, thermal denaturation and of its dependence on temperature yields only two independent experimental quantities $\Delta F^{\pm}$ and $\Delta H^{\pm}$. On the other hand, it is seen from equations III-9 and III-10 that even in a simple case, where all the hydrogen bonds are assumed equivalent, there are three independent parameters, namely n, K_{ij} and ΔH^{0}_{ij}, which remain to be determined.

It is clear that the problem cannot be solved until an arbitrary assumption is made about the size of one of the parameters. It appears to us to be most reasonable to fix the value of ΔH^{0}_{ij}, since neither n nor K_{ij} can be regarded as universal properties of all proteins and of all hydrogen bonds.

As will be shown below, granting that ΔH^{0}_{ij} should be the same for all denaturations studied, the assignment of ΔH^{0}_{ij} as -6 kcal./mole and ΔH^{0}_{lm} and ΔH^{0}_{rs} as -12 kcal./mole is not entirely arbitrary[4,5,13,17,18] but is based on some evidence from the field of denaturation as well as from previous studies. Consider a function $\frac{-\Delta H^{\pm} RT}{\Delta F^{\pm} \Delta H^{0}_{ij}}$ which, from equations III-9 and III-10, is

$$\frac{-\Delta H^{\pm} RT}{\Delta F^{\pm} \Delta H^{0}_{ij}} = \frac{K_{ij}}{(1 + K_{ij}) \ln (1 + K_{ij})} \equiv g (K_{ij}) \qquad \text{(III-12)}$$

where $g (K_{ij})$ is a function of K_{ij}. Note that this process eliminates n. Thus, if a value of ΔH^{0}_{ij} is assigned we can read the value of K_{ij} directly from the experimental data and a table of $g (K_{ij})$ *vs.* K_{ij} (Table I). It is further worth noting that,

TABLE I

VALUES OF $g (K_{ij})$

K_{ij} (or K_{rs})	$g (K_{ij})$ or $g (K_{rs})$
0	1
0.5	0.828
1	.725
3	.543
9	.391
99	.215
999	.145
∞	0

since $\ln (1 + K_{ij})$ increases more rapidly than $K_{ij}/(1 + K_{ij})$, $g (K_{ij})$ is a monotonically decreasing function of K_{ij}. Since $g (K_{ij})$ varies only between zero and unity this imposes some definite restrictions on the possible values of the left hand side of equation III-12.

$$0.00 < \frac{-\Delta H^{\pm} RT}{\Delta F^{\pm} \Delta H^{0}_{ij}} < 1.00 \qquad \text{(III-13)}$$

Since $\Delta F^{\pm} > 0$ for all reactions of measurable rate, the right hand side of the inequality can be rearranged to yield

$$- \frac{\Delta H^{\pm}}{\Delta H^{0}_{ij}} < \frac{\Delta F^{\pm}}{RT} \qquad \text{(III-14)}$$

This inequality implies that ΔH^{0}_{ij} is not quite arbitrary but that it has a minimal value related to the maximal $\Delta H^{\pm}$ that we wish to include in our model. Since

$$\frac{\Delta F^{\pm}}{RT} = \ln \frac{kT}{hk_1} \qquad \text{(III-15)}$$

and since the rate constant k_1 must lie in the approximate range of 10^{-2} to 10^{-5} sec.$^{-1}$ in order to be conveniently observable, $\Delta F^{\pm}/RT$ must lie between roughly 34 for the fastest observable reactions and about 41 for the slowest. Therefore

$$- \frac{\Delta H^{\pm}}{\Delta H^{0}_{ij}} < 41 \qquad \text{(III-16)}$$

for observable reactions. Thus $\Delta H^{\pm}_{max} = -41\ \Delta H^{0}_{ij}$. The value of -6 kcal./mole for ΔH^{0}_{ij} restricts us to $\Delta H^{\pm}_{max}$ of 246 kcal./mole, a value that does not appear to be excessively small. On the other hand, a choice of $\Delta H^{0}_{ij} = -3$ kcal./mole would definitely be too restrictive since several $\Delta H^{\pm}$ values of the order of 150 kcal./mole are known.[24]

It is clear that the inequality (III-16) is not restrictive enough, since a greater restriction can be placed due to the fact that bonds with $K_{ij} = 0$ do not exist. For any finite K_{ij}, $g (K_{ij}) < 1.00$ and a more stringent inequality should hold.

After the value of $g (K_{ij})$ is determined. K_{ij} is ascertained from equation III-12; we must then examine the reasonability of this value. Heter-

(24) No literature data are quoted to support this statement since the arguments above apply only to pH-independent, thermal denaturation and the number of studies of $\Delta H^{\pm}$ as function of pH is too small to support this contention. On the other hand, the large number of known $\Delta H^{\pm}$'s in the 100 to 200 kcal./mole range for pH-dependent denaturation strongly supports our contention.

ologous, single hydrogen bonds are quite weak; thus large values of K_{ij} are unreasonable. It is clear from considerations of the previous papers[4,5] that K_{ij}'s should be of the order of unity and certainly no greater than 100. (The latter corresponds roughly to $\Delta S^0_{ij} = -10$ e.u., a very small value.) However, in many denaturations, especially of low-molecular-weight proteins, $\Delta H^{\ddagger}$ values range between 40–60 kcal./mole. This leads to very small g (K_{ij}) and therefore very large (unreasonably large) K_{ij}'s. This observation simply indicates that in such cases single hydrogen bonds do not break independently as was assumed before but that the rupture of one such bond strongly implies rupture of another (or of several others).

The simplest example of such a cooperative scheme are homologous double carboxyl. . .carboxyl bonds or cooperative double bonds with two donors hydrogen-bonded to the same acceptor, *e.g.*, two tyrosyls hydrogen-bonded to one carboxylate ion. Such interactions lead to considerably stronger bonding, since the enthalpy of formation ΔH^0_{rs} is about twice ΔH^0_{ij}, but the entropy loss is only roughly 3/2 ΔS^0_{ij}. The equilibrium constant for such interactions could range between 10^2 and 10^4. If such interactions (and only such interactions) are assumed then g (K_{rs}) can be obtained from

$$g\,(K_{rs}) = \frac{-\Delta H^{\ddagger} RT}{\Delta F^{\ddagger}\ \Delta H^0_{rs}} = \frac{g\,(K_{ij})}{2} \qquad \text{(III-17)}$$

Since $\Delta H^0_{rs} = 2\ \Delta H^0_{ij}$ it is necessary to decide whether double interactions or single interactions are predominantly present in applying equations of the type (III-12) and (III-17). This is done by checking the reasonability of the values obtained for K_{ij} and K_{rs} and then applying the appropriate relationship. It follows that for denaturations with small $\Delta H^{\ddagger}$ (say below 60 kcal./mole) double interactions will be predominantly involved, while in cases of large $\Delta H^{\ddagger}$ single hydrogen bonds will predominate. Situations must, of course, occur where both double and single interactions are of importance. Such situations can be treated in detail only by returning to the original equations; however even then the rough calculations outlined above should serve as a guide. Similar considerations apply to homologous, double, carboxyl. . .carboxyl hydrogen bonds since $\Delta H^0_{rs} \sim \Delta H^0_{lm}$, but such bonds should be even stronger than the coöperative one.[4]

Once the choice between double or single interactions has been made and the value of K_{ij} or K_{rs} determined, the value of n, the number of hydrogen bonds which must be absent in the activated protein, can be determined by solving equation (III-9) or (III-10). The resulting answer should be an integer. It is very unlikely to turn out to be integral due to the arbitrary and almost certainly incorrect assumption that all K_{ij}'s are equal.

The general conclusion that can be made is that denaturations with small $\Delta H^{\ddagger}$ involve the rupture of very few (n small) very strong (K_{ij} or K_{rs} large) and probably coöperative bonds. Processes with large $\Delta H^{\ddagger}$ involve the rupture of very many (n large) weak (K_{ij} small) non-cooperative bonds. Clearly, denaturations of low molecular weight proteins fall in the former class and of high molecular weight in the latter. This is not at all meant to imply that the hydrogen bonds in small proteins are stronger than in large ones but only that these situations lead to denaturations proceeding at observable rates.

Second Criterion for the Strength of Bonding. It is obvious from the foregoing that, while the $\Delta H^{\ddagger}/\Delta F^{\ddagger}$ ratio provides valuable information about the strength of the bonds ruptured, more data are needed. Such data can be obtained from the study of $\Delta C^{\ddagger}_p$ for a *pH-independent*, thermal denaturation process. Since the measurement of $\Delta H^{\ddagger}$ is already quite difficult, we may at first despair of any chance of measuring $\Delta C^{\ddagger}_p$. This, however, need not be so provided that $\Delta C^{\ddagger}_p$ is large enough and the plot of ln k_i *vs.* $1/T$ has appreciable curvature.

$\Delta C^{\ddagger}_p$ can be obtained from equation III-10, as

$$\Delta C^{\ddagger}_p = \left(\frac{\partial \Delta H^{\ddagger}}{\partial T}\right)_p = -\frac{n(\Delta H^0_{ij})^2 K_{ij}}{RT^2\,(1 + K_{ij})^2} - \frac{nK_{ij}}{1 + K_{ij}}\left(\frac{\partial \Delta H^0_{ij}}{\partial T}\right)_p \qquad \text{(III-18)}$$

The first term arises from the decrease of K_{ij} as the temperature increases, *i.e.* at higher temperatures fewer hydrogen bonds have to be broken, and $\Delta H^{\ddagger}$ is lower. The second term opposes the first (evaluated approximately in the Appendix) since ΔH^0_{ij} becomes more negative with increasing temperature.

The first term at first increases and then decreases with increasing values of K_{ij}, reaching a maximum of $\frac{-n\,(\Delta H^0_{ij})^2}{4RT^2}$ (or $-50n$ cal./mole/deg.) for $K_{ij} = 1$, a highly probable value. The second opposing term, which is quite difficult to evaluate exactly, increases monotonically with K_{ij}. At $K_{ij} = 1$ it lies between $5n$ and $8n$ cal./mole/deg. (see Appendix). It is thus seen that for small K_{ij} the contribution to $\Delta C^{\ddagger}_p$ is about -40 to -45 cal./mole/deg. per hydrogen bond. This is a relatively small contribution and can be experimentally detected only when n is large. It can also be seen that when K_{ij} or K_{rs} is large the contribution to $\Delta C^{\ddagger}_p$ becomes positive; however it is probably never greater than $+15$ cal./mole/deg. Again large n's would be required to see this contribution. It was already stated that observable denaturations involve either large n's and small K_{ij}'s or small n's and large K_{ij}'s or K_{rs}'s. But large n's are required to observe a measurable $\Delta C^{\ddagger}_p$. Thus, for denaturations involving strong interaction (small n) $\Delta C^{\ddagger}_p$ should be essentially zero; for denaturations involving weak interactions (large n) $\Delta C^{\ddagger}_p$ should be negative. Large positive $\Delta C^{\ddagger}_p$ *cannot be observed* on the basis of our model for *pH independent*, thermal denaturations.

If $\Delta C^{\ddagger}_p$ is found to be large and negative this can be taken as proof that K_{ij} is small. In such a case equation III-18 is well approximated by

$$\Delta C^{\ddagger}_p = -\frac{n(\Delta H^0_{ij})^2 K_{ij}}{RT^2\,(1 + K_{ij})^2} \qquad \text{(III-19)}$$

Combination with equation III-10 yields

$$\frac{\Delta C^{\ddagger}_p}{\Delta H^{\ddagger}} = \frac{\Delta H^0_{ij}}{RT^2\,(1 + K_{ij})} \qquad \text{(III-20)}$$

This relation is of little use in evaluating small K_{ij}'s, but it provides an excellent criterion for checking the assumption that ΔH^0_{ij} is -6 kcal./mole.

*p*H-Dependent Denaturation

We shall now consider the breaking of hydrogen bonds in a *p*H region where either some donors or some acceptors or both can dissociate protons. We shall treat first a simple case, where only heterologous single bonds are broken and then treat a complex situation in which double bonds are broken.

Simple Case.—For heterologous single hydrogen bonds the expression for x_{ij} is given by equation II-7.

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \quad \text{(III-21)}$$

so that

$$1 - x_{ij} = \frac{1 + K_1/[H^+] + [H^+]/K_2}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \quad \text{(III-22)}$$

where K_1 and K_2 are the ionization constants of the donor and acceptor, respectively, *in the absence of hydrogen bonding*, and $[H^+]$ is the hydrogen ion activity. The rate constant for denaturation is given by

$$k_1 = \frac{kT}{h} \prod \left(\frac{1 + K_1/[H^+] + [H^+]/K_2}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \right) \quad \text{(III-23)}$$

Equation III-23 shows the dependence of the rate constant on *p*H for this simple case. Electrostatic effects have been neglected. The constants K_1 and K_2 can be compared directly with those for model compounds in order to identify the groups involved in the activation process.

A frequently used method of representing kinetic data is to plot log k_1 *vs.* *p*H at a series of temperatures. Therefore, it is of interest to express the slope of such an experimental curve in terms[25] of equation III-23.

$$\frac{d \log k_1}{d\, pH} = -[H^+] \frac{d \ln k_1}{d[H^+]} = \sum \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \cdot \frac{K_1/[H^+] - [H^+]/K_2}{1 + K_1/[H^+] + [H^+]/K_2} = \sum x_{ij} \frac{K_1/[H^+] - [H^+]/K_2}{1 + K_1/[H^+] + [H^+]/K_2} = \sum q^{\mp}_{ij} \quad \text{(III-24)}$$

The quantity $\sum q^{\mp}_{ij}$ is the apparent order of the reaction with respect to the hydrogen ion. The reaction will be zero order in hydrogen ion, and the rate will be minimal, when $\sum q^{\mp}_{ij} = 0$. Unfortunately, the detailed solution for the minimum in the log k_1 *vs.* *p*H curve for several varying K_{ij}'s, K_1's and K_2's is very complex. The only general statement that can be made is that if there is at least one donor and one acceptor there will be at least one solution.[26] If all K_1's are equal and all K_2's are also equal (with K_{ij}'s arbitrary) the solution becomes trivial

$$[H^+] = \sqrt{K_1 K_2} \quad \text{(III-25)}$$

i.e., the log k_1 *vs.* *p*H curve will have a minimum at a *p*H given by equation III-25. Thus, when K_1 and K_2 are ultimately determined, they may be checked by equation III-25.

At *p*H's other than that at which the minimum rate occurs, one or the other of the terms $K_1/[H^+]$ or $[H^+]/K_2$ will be the predominant one. Hence, equation III-24 can be simplified to

$$\sum q^{\mp}_{ij} = -\sum \frac{K_{ij}}{1 + K_{ij} + [H^+]/K_2} \cdot \frac{[H^+]/K_2}{1 + [H^+]/K_2} \quad \text{(III-26)}$$

in the acid range ($[H^+] \sim K_2$), and to

$$\sum q^{\mp}_{ij} = \sum \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+]} \cdot \frac{K_1/[H^+]}{1 + K_1/[H^+]} \quad \text{(III-27)}$$

in the basic range ($[H^+] \sim K_1$). The slope, $\sum q^{\mp}_{ij}$, will be negative in the acid range and positive in the basic range.

The maxima in these slopes (*i.e.* where the rate is most *p*H-dependent) are given by the equations:

$$\sum \frac{[([H^+]/K_2)^2 - (1 + K_{ij})][H^+]/K_2}{(1 + K_{ij} + [H^+]/K_2)(1 + [H^+]/K_2)} = 0 \quad \text{(III-28)}$$

and

$$\sum \frac{[(K_1/[H^+])^2 - (1 + K_{ij})]K_1/[H^+]}{(1 + K_{ij} + K_1/[H^+])(1 + K_1/[H^+])} = 0 \quad \text{(III-29)}$$

Again, the solutions are complex unless all K_{ij}'s, K_1's and K_2's are equal. In such a case

$$[H^+]/K_2 = \sqrt{1 + K_{ij}} \quad \text{(III-30)}$$

and

$$K_1/[H^+] = \sqrt{1 + K_{ij}} \quad \text{(III-31)}$$

The value of the maximum slope is

$$\left(\sum q^{\mp}_{ij}\right)_{max} = \frac{nK_{ij}}{(1 + \sqrt{1 + K_{ij}})^2} \quad \text{(III-32)}$$

Equation III-32 provides another condition for the choice of the number and strength of hydrogen bonds involved in denaturation.

It may be mentioned that the foregoing theory is essentially the same as that used to treat the polymerization of fibrin monomer.[13,25] This polymerization involves the formation of intermolecular hydrogen bonds and is formally the opposite process of the activation process in which hydrogen bonds are broken. In the polymerization process q protons are liberated (or taken up, depending on the *p*H) per ij^{th} hydrogen bond formed.

Considering equation III-32, the values of $(\sum q^{\mp}_{ij})_{max}$ as a function of K_{ij} (or K_{rs}) are listed in Table II. It is worth noting that the rupture of a single hydrogen bond does not lead to a slope of unity on a plot of log k_1 *vs.* *p*H but to much smaller values (see Table II) unless the hydrogen bond is very strong. Thus a single prototropic step in former theories[8,9] need not correspond to any definite number of hydrogen bonds. In small proteins where, as pointed out previously, there will

(25) Equation III-24 is essentially the same as equation 8 used in the theory of polymerization of fibrin monomer.[13] Similarly for the approximate forms of equation III-24 which follow.

(26) It is very unlikely that more than one solution exists. For example, consider a situation where $\Sigma q^{\mp}_{ij} = q_1 + q_2$ and both q_1 and q_2 are of the form shown in Fig. 3 of ref. 13. Imagine the curve for q_2 to be shifted slightly toward higher *p*H with respect to the curve for q_1. Then, for $\Sigma q^{\mp}_{ij}$ to have more than one zero q_1 would have to be negative when $dq_2/d\,pH$ is still positive. Since this is an unlikely situation, it is unlikely that there will be more than one zero. If there is more than one zero in the general case, then there are 3, 5, 7, etc. (*i.e.*, an odd number of zeros).

TABLE II

VALUES OF $(\Sigma q^{\mp}_{1j})_{max}$ PER ij^{th} HYDROGEN BOND BROKEN

K_{1j} (or K_{rs})	$(\Sigma q^{\mp}_{ij})_{max}$
1	0.17
3	.33
8	.50
15	.60
99	.81
899	.94

be a rupture of a few strong bonds the single prototropic step idea may be a good approximation. It can be further seen, both from equation III-32 and Table II, that $(\sum q^{\mp}_{ij})_{max}$ is very sensitive to K_{ij} when K_{ij} is small and then becomes essentially independent of K_{ij} for large K_{ij} (or K_{rs}). A difference of 10% in the slope of a log k_1 *vs.* *p*H plot is hard to detect experimentally, and yet it corresponds to a tenfold change in K_{rs} at high K_{rs}. Similarly for the *p*H at which $(\sum q^{\mp}_{ij})_{max}$ occurs. From equations III-30 and III-31, for large K_{rs}

$$(pH)_{(\Sigma q^{\mp}_{ij})_{max}} = pK_1 + (\log K_{ij})/2 \quad \text{(III-33)}$$

$$= pK_2 - (\log K_{ij})/2 \quad \text{(III-34)}$$

Equations III-33 and III-34 suggest that a tenfold change in K_{ij} corresponds to a *p*H change of 0.5. However, for large K_{ij}, the value of $\sum q^{\mp}_{ij}$ remains close to unity over a large *p*H range and, therefore, the *p*H of the maximum is very difficult to locate.

We can obtain the heat of activation by differentiation of ln k_1 (from equation III-23) with respect to T at constant $[H^+]$.

$$\Delta H^{\mp} = \sum x_{ij}\left[-\Delta H^0_{ij} + \frac{(K_1/[H^+])\Delta H_1^0 - ([H^+]/K_2)\Delta H_2^0}{1 + K_1/[H^+] + [H^+]/K_2}\right] \quad \text{(III-35)}$$

At low *p*H, the ionizing group involved in denaturation is the carboxyl group, for which ΔH_2^0 is very small. Also $K_1/[H^+]$ is essentially zero, making the second term in the square brackets zero. From equation III-21 it can be seen that x_{ij} decreases as the *p*H is decreased. Therefore, at low *p*H, $\Delta H^{\mp}$ decreases as the *p*H decreases. On the other hand, on the alkaline side of the minimum rate the decrease of the $(-x_{ij}\,\Delta H^0_{ij})$ term is compensated by a positive term arising from the ionization of donors. Thus, as we move into the alkaline range $\Delta H^{\mp}$ may remain constant or may even increase to a maximum, even though $\Delta F^{\mp}$ decreases (*i.e.* k_1 is increasing; see equation III-23). According to equation III-12, a combination of a constant or increasing $\Delta H^{\mp}$ with a decreasing $\Delta F^{\mp}$ implies an increase in $g(K_{ij})$ as the *p*H is raised. As already pointed out, a large $g(K_{ij})$ and a large $\Delta H^{\mp}$ correspond to the rupture of many weak bonds in a large protein. Thus, the existence of increasing $\Delta H^{\mp}$ in the *p*H-dependent region, giving rise to a large $g(K_{ij})$, can be explained by considerably weaker bonds than would be required on the basis of the *p*H-independent treatment.

The condition for a maximum in $\Delta H^{\mp}$ (*i.e.*, the *p*H at which the ratio of the rates at two temperatures is maximal) is obtainable from equation III-35, applied to the alkaline region.

$$\frac{d}{d[H^+]}\left\{\sum \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+]}\left[-\Delta H^0_{ij} + \frac{K_1/[H^+]}{1 + K_1/[H^+]}\Delta H_1^0\right]\right\} = 0 \quad \text{(III-36)}$$

For identical groups this reduces to

$$K_1/[H^+] = \frac{\Delta H^0_{ij}}{\Delta H_1^0 - \Delta H^0_{ij}} \pm \sqrt{\left(\frac{\Delta H^0_{ij}}{\Delta H_1^0 - \Delta H^0_{ij}}\right)^2 + \left(\frac{\Delta H^0_{ij} + \Delta H_1^0(1 + K_{ij})}{\Delta H_1^0 - \Delta H^0_{ij}}\right)} \quad \text{(III-37)}$$

For tyrosyl residues, $\Delta H_1^0 = +6$ kcal./mole and for lysyl residues $\Delta H_1^0 = +12$ kcal./mole. Thus, for tyrosyls

$$K_1/[H^+] = -0.50 + 0.71\sqrt{0.50 + K_{ij}} \quad \text{(III-38)}$$

and for lysyls

$$K_1/[H^+] = -0.33 + 0.82\sqrt{0.67 + K_{ij}} \quad \text{(III-39)}$$

Comparison with equation III-31 shows that the maximum in $\sum q^{\mp}_{ij}$ occurs at a *p*H near where $\Delta H^{\mp}$ is maximal, providing an additional criterion for locating the maxima in both functions.

Complex Case.—It is possible that some of the hydrogen bonds broken are of a more complicated type than the heterologous single bonds just considered. As an example of such a complex situation we shall consider a homologous double bond, the carboxyl. . .carboxyl acetic acid dimer type bond. For such a bond (see Appendix II of paper I[4]), an approximate expression for x_{1m}, neglecting K_{ij}, is

$$x_{1m} = \frac{K_{1m}}{1 + (K_2/[H^+])^2 + K_{1m}} \quad \text{(III-40)}$$

therefore

$$(1 - x_{1m}) = \frac{1 + (K_2/[H^+])^2}{1 + (K_2/[H^+])^2 + K_{1m}} \quad \text{(III-41)}$$

For a reaction involving the rupture of these double bonds, as well as heterologous single bonds, this expression for $(1 - x_{1m})$ must be substituted into equation III-3 to obtain the rate constant.

At very low *p*H it is difficult to ionize a COOH group if it is involved in a double bond.[4] Therefore, if the activation process involves the rupture of such double bonds, the rate of denaturation would decrease as the *p*H is lowered. If denaturation also involves the rupture of several heterologous single bonds, whose breakdown will be speeded up by lowering the *p*H, the two effects will be superimposed. In such a case the rate of denaturation may have two fairly widely separated minima.

The expression for $\sum q^{\mp}_{1m}$ is

$$\sum q^{\mp}_{1m} = 2\sum x_{1m}\frac{(K_2/[H^+])^2}{1 + (K_2/[H^+])^2} \quad \text{(III-42)}$$

If all K_{1m}'s and K_2's are equal, the maximum of this function occurs at

$$K_2/[H^+] = (1 + K_{1m})^{1/4} \quad \text{(III-43)}$$

Even for very large K_{1m}, the value of $K_2/[H^+]$ is of the order of unity.

Urea Denaturation

To treat urea denaturation with the same theory it is necessary to assume that urea molecules compete for the hydrogen bonding sites and thus decrease the

probability of formation of intramolecular hydrogen bonds between the R groups. Again, we consider that the initial binding of urea to the native molecule is instantaneous and that the activation process is due to the simultaneous breakage of n hydrogen bonds.

We can define two equilibrium constants for binding of a urea molecule to the donor or acceptor, respectively

$$K_{DH...U} = \frac{P_{DH...U,A}}{P_{DH,A}[U]} \qquad \text{(III-44)}$$

and

$$K_{A...U} = \frac{P_{DH,U...A}}{P_{DH,A}[U]} \qquad \text{(III-45)}$$

where the P's stand for concentration fractions of the given species,[4] and [U] is the urea activity.

We shall treat heterologous single bonds in the pH-independent region (*i.e.* all donors in the form DH, and all acceptors in the form A). For this case

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_{DH...U}[U] + K_{A...U}[U]} \qquad \text{(III-46)}$$

Lacking information on the magnitudes of $K_{DH...U}$ and $K_{A...U}$ but recognizing that they are probably not equal, we shall very arbitrarily neglect the smaller one and write simply K_U for the larger one. The determination of K_U is discussed below. This approximation will certainly have to be modified when the theory is applied to experimental data on urea denaturation. With this approximation, equation III-3 becomes

$$k_1 = \frac{kT}{h} \prod \left(\frac{1 + K_U[U]}{1 + K_{ij} + K_U[U]} \right) \qquad \text{(III-47)}$$

From equation III-47 it follows that k_1 increases as [U] increases.

If K_U is known at any temperature then, in conjunction with equation III-47, we can determine the apparent order, $n^{\pm}$, with respect to [U].

$$n^{\pm} = \frac{d \ln k_1}{d \ln [U]} = \sum x_{ij} \frac{K_U[U]}{1 + K_U[U]} \qquad \text{(III-48)}$$

Equation III-48 is analogous to equation III-27. The maximum value of $n^{\pm}$ will occur when

$$K_U[U] = \sqrt{1 + K_{ij}} \qquad \text{(III-49)}$$

the value of $n^{\pm}_{max}$ being

$$n^{\pm}_{max} = \frac{nK_{1j}}{(1 + \sqrt{1 + K_{ij}})^2} \qquad \text{(III-50)}$$

If such a condition is experimentally attainable, information about K_U and K_{ij} is provided.

The heat of activation can be obtained by differentiating ln k_1 of equation III-47 with respect to T at constant [U].

$$\Delta H^{\pm} = \sum x_{ij} \left[\frac{K_U[U](\Delta H^0_U - \Delta H^0_{ij}) - \Delta H^0_{ij}}{1 + K_U[U]} \right] \qquad \text{(III-51)}$$

where ΔH^0_U is the heat of formation of the urea-R group complex.

It is well known that the urea denaturation of certain proteins is characterized by a negative[27] $\Delta H^{\pm}$, while others exhibit a positive[28] $\Delta H^{\pm}$. In the case of tobacco mosaic virus $\Delta H^{\pm}$ is negative below a certain temperature and positive above it.[29] Ovalbumin behaves in a similar manner.[30] Equation III-51 predicts such a behavior with an inversion temperature T_i at $\Delta H^{\pm} = 0$.

If all K_U's, K_{ij}'s, ΔH^0_{ij}'s and ΔH^0_U's are the same then, according to equation III-51

$$K_U[U] = \frac{\Delta H^0_{ij}}{\Delta H^0_U - \Delta H^0_{ij}} \qquad \text{(III-52)}$$

at T_i. Re-writing this equation as

$$\frac{K_U[U]}{1 + K_U[U]} = \frac{\Delta H^0_{ij}}{\Delta H^0_U} \qquad \text{(III-53)}$$

we see that

$$|\Delta H^0_{ij}| < |\Delta H^0_U| \qquad \text{(III-54)}$$

no matter what the value of K_U is. Since K_U decreases with increasing temperature and since the inequality of equation III-54 holds, $\Delta H^{\pm}$ will be negative below T_i and positive above T_i, as observed.[29,30]

The equations above can be best utilized by doing either of the two equivalent experiments: (a) for several urea concentrations determine the temperature, T_i, where $\Delta H^{\pm} = 0$, (b) for several temperatures determine the urea concentration, $[U]_i$, where $\Delta H^{\pm} = 0$. Since

$$[U]_i = \frac{\Delta H^0_{ij}}{(\Delta H^0_U - \Delta H^0_{ij})K_U} \qquad \text{(III-55)}$$

and, since it is reasonable to assume that the ratio $\frac{\Delta H^0_{ij}}{\Delta H^0_U - \Delta H^0_{ij}}$ is essentially independent of temperature, we can write

$$\frac{d \ln [U]_i}{dT} = -\frac{\Delta H^0_U}{RT^2} \qquad \text{(III-56)}$$

Thus, a plot of ln $[U]_i$ *vs.* $1/T$ should yield a straight line with a slope $\Delta H^0_U/R$. Knowing ΔH^0_U (and ΔH^0_{ij}) the value of K_U (at T_i) can be determined from equation (III-55).

There is another consequence of equation (III-51) and inequality (III-54). Consider an experiment where $\Delta H^{\pm}$ is measured at constant temperature as a function of urea concentration [U]. With no urea $\Delta H^{\pm}$ is large and positive (given by equation (III-7)). Addition of urea will at first make $\Delta H^{\pm}$ fall with increasing [U]. As the urea concentration is sufficiently increased $\Delta H^{\pm}$ will become negative. However it is clear by inspection of equation (III-51) that, for infinite [U], $\Delta H^{\pm}$ must approach zero as a limit. Thus $\Delta H^{\pm}$, plotted as a function of [U], must have a minimum ($\Delta H^{\pm}$ must be negative when this minimum occurs). It is by no means clear that the urea concentration where this minimum occurs will be experimentally attainable for all proteins or for all temperatures. It appears that the minimum could be best obtained at low temperatures where relatively low urea concentrations are required to produce a negative $\Delta H^{\pm}$. The condition for the minimum is given by

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

(28) E. Mihalyi, *Acta Chem. Scand.*, **4**, 317 (1950).

(29) M. A. Lauffer, THIS JOURNAL, **65**, 1793 (1943).

(30) R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953).

$$[U]_{min} = \frac{\Delta H^0_{ij}}{(\Delta H^0_U - \Delta H^0_{ij})K_U}\left[1 + \sqrt{1 + \frac{\Delta H^0_U - \Delta H^0_{ij}}{\Delta H^0_{ij}}(2 + K_{ij}) + \left(\frac{\Delta H^0_U - \Delta H^0_{ij}}{\Delta H^0_{ij}}\right)^2 (1 + K_{ij})}\right] \quad \text{(III-57)}$$

It is worth noting that, according to equation (III-55), the multiplying factor in equation III-57 is simply $[U]_i$, the urea concentration required to make $\Delta H^{\ddagger} = 0$ at constant T. Thus if $[U]_i$ is known for a given T, $[U]_{min}$ can be used to evaluate the term under the square root and thus K_{ij}. It appears that $[U]_{min}$ can be reached only when K_{ij} is small.

Comparison with Current Theories

It is of interest to point out the difference between the theory presented here and that proposed by Steinhardt[8] and extended by Levy and Benaglia.[9] The former theories are based on two postulates[9]: "(1) The protein is a polyvalent acid and base (in the Brönsted sense) which by association or dissociation of protons may assume many ionic forms, the activities of which are related to one another by mass action equations involving $[H^+]$; (2) a number of the prototropic changes are accompanied by changes in the stability which are reflected in the rate of production of an insoluble product. The stability may be increased or decreased by dissociation of protons." We retain these postulates. The difference between our approach and the former one is threefold: (1) we take into account the effect of hydrogen bonding on the ionization constants; (2) assuming the validity of our model, we express our rate equations in terms of accessible thermodynamic parameters (K_{ij}, K_1, K_2, K_U, ΔH^0_{ij}, ΔH^0_1, ΔH^0_2, ΔH^0_U, etc.), whereas the former theories make use of rate constants k_1, k_2, k_3, etc. for the denaturation of the protein in the various states of ionization. The constants k_1, k_2, k_2, etc. and the various ionization constants K_1, K_2, K_3, etc. of Levy and Benaglia[9] are usually not determinable, making it difficult to identify the groups involved. (3) Complex hydrogen bonding situations do not appear in the former theories. Also, in our treatment, a large negative value of ΔH^0_{ij} has been justified.

As a consequence of the above similarities and differences, the equations of Levy and Benaglia, used to interpret the rate of denaturation data over a wide range of pH and temperature, are formally similar to equation III-23. However, they have no equation similar to our equation III-3 for other than heterologous single bonds. Therefore, we have to re-examine the conclusion of the former theories that the number of hydrogen bonds broken between ionizable groups during the denaturation process is equal to the highest power of $[H^+]$ used in the expression for k_1. Even if activation involves only heterologous single bonds, then each bond contributes to the expression for k_1 terms with fractional powers in $[H^+]$ (see Table II). If other types of bonds, such as homologous double bonds, are involved, then still other powers of $[H^+]$ can appear for each bond broken (see equation III-41). These effects are not included in the former theories.

In summary, we have proposed a theory giving rise to equations which, in various combinations, should account for the thermodynamic data for the activation process (for reactions for which our model is applicable). Thus, we can obtain an insight into the reaction in terms of the specific side-chain groups involved. In other words, denaturation studies could be useful, along with the methods indicated in papers I[4] and II[5], for the location of internal hydrogen bonds between specific side-chain groups.

Appendix

Evaluation of $(\Delta C_p^0)_{ij}$.—When a hydrogen bond is formed the torsional oscillations around single bonds in the side chains are eliminated. These torsional oscillations in the free side chains lead to very closely spaced energy levels compared to kT and thus are at equipartition. The heat capacity corresponding to this motion is R per single torsional oscillation and therefore its contribution to $(\Delta C_p^0)_{ij}$ should be $-\nu_{ij}R$, where ν_{ij} is the number of oscillations frozen out in the formation of the ij^{th} hydrogen bond. The value of ν_{ij} will generally vary from bond to bond, but it should be quite close to 5 for single bonds, 6 for carboxyl. . .-carboxyl double bonds and 8 for coöperative double bonds.

Another contribution to $(\Delta C_p^0)_{ij}$ should be considered. Since most of the rotations frozen out in the formation of intramolecular hydrogen bonds are around carbon–carbon bonds and since the carbons in question are generally involved in tetrahedral bonding, we should expect three angular potential energy minima. When the side chain group is not involved in a hydrogen bond, it can redistribute itself between these three minima. On the other hand, when the hydrogen bond is formed this distribution is no longer possible. Since the substituents on the carbon atoms in a side chain are not all the same, we should expect the three minima to be unequal. Thus a redistribution due to an increase in temperature will lead to an increase in the heat content of the non-hydrogen bonded species and thus to a negative contribution to $(\Delta C_p^0)_{ij}$.

A complete evaluation of this contribution is impossible since the differences between the various minima are unknown. On the other hand, the maximal possible contribution is relatively easy to obtain. Let us assume for the sake of simplicity that of the three minima two are equal. Two cases are then possible: either the lower state is degenerate and the higher state is not or the higher state is degenerate and the lower state is not. It is immediately clear that a higher C_p contribution is possible in the second case since more energy is available due to redistribution. If we call the energy difference between the higher and the lower state E_i, then the energy due to redistribution is

$$E = H = \frac{2E_i\, e^{-E_i/RT}}{1 + 2e^{-E_i/RT}} = 2E_i\,(e^{E_i/RT} + 2)^{-1}$$

Differentiation with respect to temperature yields the C_p contribution

$$\left(\frac{\partial H}{\partial T}\right)_p = C_p = \frac{2E_i^2}{RT^2}\, e^{E_i/RT}\,(e^{E_i/RT} + 2)^{-2}$$

To evaluate the maximal possible C_p contribution we need only to differentiate C_p with respect to E_i at constant T. Setting this equal to zero will yield E_i which will make C_p maximal.

Let

$$\frac{E_i}{RT} = x$$

Then

$$C_p = 2R\,x^2\,e^x\,(e^x + 2)^{-2}$$

$$\frac{dC_p}{dx} = (e^x + 2)^{-2}\,[4Rxe^x + 2Rx^2e^x] - 4Rx^2e^x\,(e^x + 2)^{-3}e^x = 0$$

Therefore

$$2 + x = \frac{2\,x\,e^x}{e^x + 2}$$

$$2\,e^x + xe^x + 4 + 2x = 2xe^x$$

$$2\,(x + 2) = (x - 2)e^x$$

The equation can be solved graphically, the solution being $x = 2.65$. Since $x = E_i/RT$ and since denaturation

measurements are generally done in the temperature interval of 300 to 350°K., E_i should lie between 1600 and 1800 cal./mole.

Introduction of $x = 2.65$ into the heat capacity expression yields

$$(C_p)_{max} \cong 0.75R$$

Thus we may expect an additional negative contribution to $(\Delta C_p^0)_{ij}$ between zero and $0.75R$. It is, of course, highly unlikely that the potential energy minima will be such as to yield the maximum C_p for all hydrogen bonds involved and for all bonds in a given side group. The only rigorous statement that can be made is

$$-1.00\ \nu_{ij}\ R > (\Delta C_p^0)_{ij} > -1.75\ \nu_{ij}\ R$$

We should expect $(\Delta C_p^0)_{ij}$ to lie closer to the less negative value.

[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS]

The Enzymic Kinetics of Carbonic Anhydrase from Bovine and Human Erythrocytes

BY HOWARD DEVOE AND G. B. KISTIAKOWSKY

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The rates of the carbonic anhydrase-catalyzed hydration of carbon dioxide and the reverse dehydration of bicarbonate ion were measured as a function of substrate concentration to yield information about the enzymic mechanism. The measurements were made at 0.5° and neutral *p*H by following, in a "stopped flow" apparatus, optical densities of reaction solutions containing *p*-nitrophenol indicator and dilute phosphate buffer. Carbonic anhydrases from both bovine and human erythrocytes were studied which contained 0.27 and 0.28% of zinc, respectively. The bovine enzyme requires peptone to stabilize the enzymic activity of dilute solutions, is not affected by *p*-nitrophenol and is inhibited to varying extents by different neutral salts. Its hydration and dehydration kinetics obey the Michaelis–Menten equation. A simple mechanism which agrees with the *p*H dependence of the kinetics requires two adjacent sites on the enzyme molecule: one to bind a hydroxide ion and the other to bind the substrates CO_2 and HCO_3^-. The human enzyme differs from the bovine enzyme in not requiring peptone for stabilization, in being activated by *p*-nitrophenol and in being inhibited less specifically by neutral salts. Its dehydration kinetics obey the Michaelis–Menten equation but its hydration kinetics do not; this may be interpreted as due to activation of the enzyme by CO_2.

Introduction

The physical and chemical properties of carbonic anhydrase, which at neutral *p*H catalyzes the reaction

$$CO_2(aq.) + H_2O = HCO_3^- + H^+ \qquad (1)$$

have been studied by a number of authors.[1] The kinetics of the hydration reaction with purified horse erythrocyte carbonic anhydrase have been studied by Kiese,[2] the hydration and dehydration reactions with a crude preparation of bovine erythrocyte carbonic anhydrase by Roughton and Booth[3] and the hydration reaction with highly purified human erythrocyte carbonic anhydrase by Davis.[4,5] These workers claim that the dependence of the catalyzed rate on the concentration of substrate (CO_2 or HCO_3^-) follows Michaelis–Menten kinetics and report different values for the hydration Michaelis constants of the three enzyme preparations.

The value of the equilibrium constant of reaction 1 is such that at neutral *p*H the reaction can be run in either direction with equal convenience. The present work took advantage of this to study the kinetics of the enzymic catalysis in both directions with the same enzyme concentration and comparable solution compositions. Carbonic anhydrases from both bovine and human erythrocytes were studied, using a "stopped flow" apparatus[6] to rapidly mix the reactants and low temperature (0.5°) to minimize the non-enzymic reaction.

(1) See F. J. W. Roughton and A. M. Clark, in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1951, Ch. 43.

(2) M. Kiese, *Biochem. Z.*, **307**, 400 (1941).

(3) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 319 (1946).

(4) R. P. Davis, THIS JOURNAL, **80**, 5209 (1958).

(5) R. P. Davis, *ibid.*, **81**, 5674 (1959).

(6) F. J. W. Roughton and B. Chance, in S. L. Friess and A. Weissberger, "Technique of Organic Chemistry," Vol. VIII, Interscience Publishers, Inc., New York, N. Y., 1953, Ch. 10.

Experimental

Method.—The reaction was followed by measuring the rate of *p*H change of the weakly buffered reaction solution.

An attempt to measure the enzymic reaction with a glass electrode incorporated in the flow apparatus failed, because the apparent rate in a run was greater the longer the electrode had previously contacted an enzyme solution. Experimentation made it clear that enzyme adsorption on the glass membrane of the electrode caused the difficulty, apparently by increasing the local enzyme concentration at the membrane.[7] This finding throws some doubt on the accuracy of Davis'[4,5] carbonic anhydrase rate measurements, since they were made with a glass electrode which was pre-equilibrated with enzyme solution.

The method finally adopted was the photometric measurement of the optical density of *p*-nitrophenol included as an indicator in the reaction solution. The flow apparatus was thermostated at 0.50 ± 0.02°. One of the reactant solutions was the substrate solution, either aqueous CO_2 or aqueous $KHCO_3$. A CO_2 solution was prepared by bubbling a gaseous mixture of CO_2 and N_2 for at least 20 min. through water at the temperature of the flow apparatus. The other reactant solution contained the phosphate buffer, enzyme, indicator and any other materials included in the runs; each batch was used for a series of about eight runs.

About 5 ml. of both reactant solutions were simultaneously discharged from separate glass syringes into a 2 mm. bore glass capillary tube, where turbulent flow mixed the solutions in a 1:1 ratio. The flow was stopped suddenly after about 1 sec., leaving mixed reaction solution at rest in the observation cell of the photometer. During the initial 10 sec. of the run, the fraction of the incident light (in the 400 mμ wave length region) transmitted by the observation cell was measured with a phototube. A photographic record of the signal as a function of time was made from an oscillograph display and was used to determine the initial *p*H and the initial reaction rate.[8]

The values given for the enzymic reaction rate, v_{enz}, have been corrected for the non-enzymic rate calculated from published values of the hydration[9] and dehydration[10] rate

(7) We wish to thank Professor F. J. W. Roughton for alerting us to the possibility that a glass electrode might be incapable of measuring reaction rates accurately in the presence of protein.

(8) Details are given in the Ph.D. thesis of H. DeVoe, Harvard University, 1960.

(9) B. R. W. Pinsent and F. J. W. Roughton, *Trans. Faraday Soc.*, **47**, 263 (1951).

(10) J. A. Sirs, *ibid.*, **54**, 207 (1958).