Thermodynamics of Conformational Changes of Proteins.
I. *p*H-dependent Denaturation of Muramidase*

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Muramidase (formerly called lysozyme) undergoes a reversible thermal denaturation which is sharply *p*H dependent. The transition temperature is lowered from approximately 75° at *p*H 3 to 45° at *p*H 1.1. Denaturation was measured by viscosity, and optical rotation at 436 mμ. Both methods give identical results and therefore measure the same conformational changes. The $\Delta H°$ for the denaturation process is independent of *p*H and it is 66 ± 3 kcal. Neither $\Delta H°$ nor transition temperature was significantly affected by 0.02–0.15 ionic strength. Theoretical criteria by which the mechanism of denaturation can be analyzed are presented, and the results indicate that the denaturation process is intimately associated with three carboxyl groups known to have abnormally high ionization constants. The pK_a values of the groups are calculated to be in the range of pK_a 1.4–1.8 in the native molecule, and this value is increased to 3.4 in the denatured form. The effect of *p*H on denaturation and the normalization of the three carboxyls is found to be mainly of entropic origin.

The unusual stability of the tertiary structure of muramidase to extremes of *p*H at room temperature has been shown previously in a number of studies (Tanford and Wagner, 1954; Jirgensons, 1958; Donovan *et al.*, 1961). However, at higher temperatures muramidase undergoes a sharply *p*H-dependent reversible change of configuration. This paper describes studies by viscosity and optical rotation of the effect of *p*H on the thermal denaturation of muramidase. The results of both methods are parallel. The transition temperature is a marked function of *p*H between *p*H 2 and 3. Upon denaturation, the three carboxyl groups shown previously to have abnormally low pK_a values (Donovan *et al.*, 1960) at room temperature become normalized. A theoretical examination of the mechanism of such *p*H-dependent transitions provides criteria for obtaining information regarding the groups associated with such transitions and the number and kinds of the protein forms present in equilibrium.

Experimental

Materials.—Crystalline muramidase from hens' egg white was prepared as described previously (Sophianopoulos *et al.*, 1962). A few experiments were performed with commercial muramidase (Armour, lot D638040) and gave essentially identical results. However, small amounts of insoluble material were present in the commercial sample and, as will be described in a later communication, lot D638040 contains an appreciable amount of either buffer salts or other dialyzable impurities and it was not used for any reported results, except to check the results with some other published studies carried out with the same lot.

All other reagents used were commercial preparations of analytical reagent grade and were used without further purification. Distilled water was passed through a column of Amberlite MB-1 (Mallinckrodt, analytical reagent grade) and had a resistance of approximately 2 × 10^6 ohms.

Protein solutions were prepared by weight and the volume was calculated from density data. The partial specific volume of muramidase used was 0.703 ml/g (Sophianopoulos *et al.*, 1962). Potassium chloride and hydrochloric acid were used to establish the *p*H and ionic strength. The ionic strength was taken to be numerically equal to the molar concentration of chloride ions. The *p*H was measured with a Radiometer PHM4 *p*H meter at 25 ± 1°. The protein concentrations were 2.4–2.5% in most instances, and 1.5% in a few, as is pointed out under Results. Most protein solutions were used immediately after preparation or, in general, no more than 3 days after preparation. They were stored in a refrigerator at 2–3°. In cases where time-dependent irreversible changes were measured, solutions were held up to a week.

Viscosity Measurements.—Viscosity measurements were carried out by the method and apparatus described elsewhere (Holcomb and Van Holde, 1962). Average deviations were calculated for measurements where deviations were expected to be the largest, and in these cases the average deviation did not exceed 0.012 cc/g. With the thermostat system described by Holcomb and Van Holde (1962) and by circulating the protein and solvent solutions during temperature equilibration, thermal equilibrium could be established within 15 minutes, so that exposure of protein solutions to high temperatures was kept to a minimum. Because of the long flow-time of the viscometers (approximately 300 seconds at room temperature), no kinetic-energy corrections were made. The reported viscosity is kinematic viscosity; density corrections were made to a few representative data, the $\Delta H°$ values were increased by 1 kcal, and the T_{tr} was decreased by 0.1°. Since the correction was small, no such further corrections were made.

Optical Rotation.—Optical-rotation measurements were performed with a Schmidt and Haensch visual polarimeter at a wavelength of 436 mμ, using a Bausch and Lomb grating filter. A 1-dm all Pyrex water-jacketed polarimeter tube was used. The temperature was kept constant with a Haake Model FSe thermostat. The precison was 1.5–2% of the total rotation change. Measurements at the sodium D line gave similar results; the values were corrected for changes of protein concentration due to changes of solution density with temperature.

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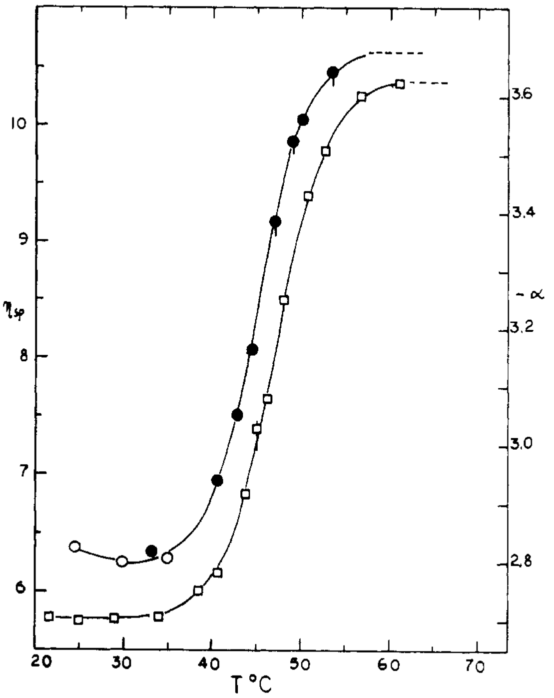

FIG. 1.—Variation of specific viscosity (in cc/g), and levorotation with temperature. *Circles:* specific viscosity of a solution of 2.50 g of muramidase per 100 ml of solution, *p*H = 1.08 (at 25°), ionic strength 0.15; ●, measurements on heating for the first time; ○, measurements on cooling the same solution; ●, measurements on heating the same solution after 24 hours at 25°. *Squares:* levorotation at 436 mμ of a solution of 2.48 g of muramidase/100 ml of solution, *p*H = 1.55 (at 25°), ionic strength 0.055; □, measurements on heating; □, measurement on cooling.

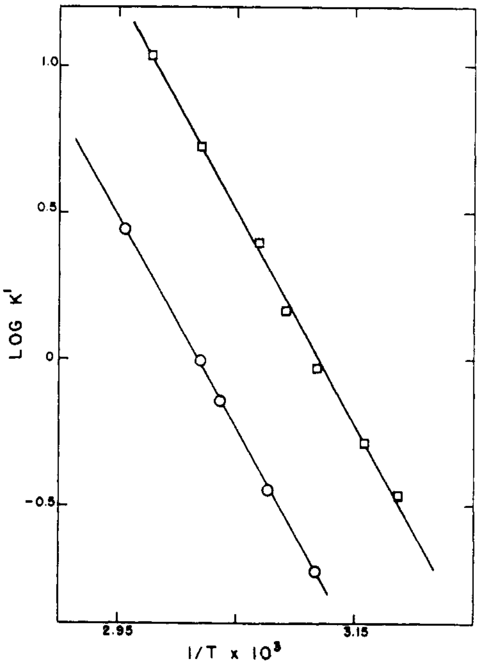

FIG. 2.—Representative Van't Hoff plots for the reversible thermal denaturation. The K' was measured by equation (3) (see Results). ○, K' from specific viscosity measurements of a solution of 2.50 g of muramidase/100 ml of solution, *p*H = 2.39 (at 25°), ionic strength 0.02; □, K' from specific viscosity measurements of a solution of 2.50 g of muramidase/100 ml of solution, *p*H = 2.23, ionic strength 0.15.

RESULTS

Calculation of the Fraction of Protein in the Denatured Form and of Apparent Equilibrium Constants.—The method used to calculate the fractions of native and reversibly denatured protein from viscosity and optical-rotation experiments have been given in detail elsewhere (Holcomb and Van Holde, 1962). In summary, the values of reduced viscosity and optical rotation reach limiting values at low and at high temperatures, as shown in Figure 1. If it is assumed that the limiting values at low temperatures are characteristic of the native form of the protein and the high values are those of the denatured form, and if it is assumed that these values are directly proportional to the concentration of each species at all temperatures, then the fraction of the denatured form is given by

$$f_d = \frac{C_d}{C_t} = \frac{C_d}{C_n + C_d} = 1 - f_n \qquad (1)$$

where C_n and C_d are the concentrations of the native and denatured forms, respectively, and C_t is the total protein concentration. The foregoing is an oversimplification, but the various additional conditions under which the above relation holds will be discussed. Experimentally, f_d can be calculated by the relation:

$$f_d = (y_t - y_n)/(y_d - y_n) \qquad (2)$$

where y refers to values of either viscosity or optical rotation; y_t refers to values measured at any temperature T, and y_n and y_d are the limiting values obtained at low and high temperatures, respectively.

If it is assumed that the reaction can be described by the relation $P \rightleftharpoons P_d$, then an "apparent" equilibrium constant can be calculated:

$$K' = \frac{C_d}{C_n} = \frac{f_d}{1 - f_d} \qquad (3)$$

Further, the standard enthalpy of the "reaction" can be calculated from van't Hoff plots of ln K' versus $1/T$. Representative graphs are shown in Figure 2.

As shown in Figure 1, at temperatures before the onset of denaturation, the optical rotation remained constant. A small decrease in viscosity was noted with increasing temperature, and before temperatures were reached at which the viscosity increased because of denaturation. This decrease was probably caused by reduction of electroviscous effects with increasing temperature. The minimum of the experimental curve was taken as the viscosity value for the native form. At high temperatures, especially above 75°, the establishment of the limiting values was somewhat more difficult because of the onset of time-dependent irreversible changes of the protein. Reliable values were established in one or more of the following ways: first, by keeping the protein at high temperatures for as little time as possible; second, by heating protein samples to high temperatures, without carrying out measurements at many intermediate temperatures; third, correcting the values at high temperatures by knowing the extent of irreversible denaturation at high temperatures from independent kinetic studies. The kinetics of the irreversible denaturation of muramidase will be the subject of another publication; but it may be pointed out that in the temperature range used in this study, the extent of irreversible denaturation is small. Also the change of the values of viscosity and optical rotation upon irreversible denaturation is small. In a sense, the irreversible changes are rather deceiving because neither the viscosity nor the optical rotation changes appreciably for some time although "irreversibility," as judged by the same methods upon cooling the sample, may have reached a value of over 50%.

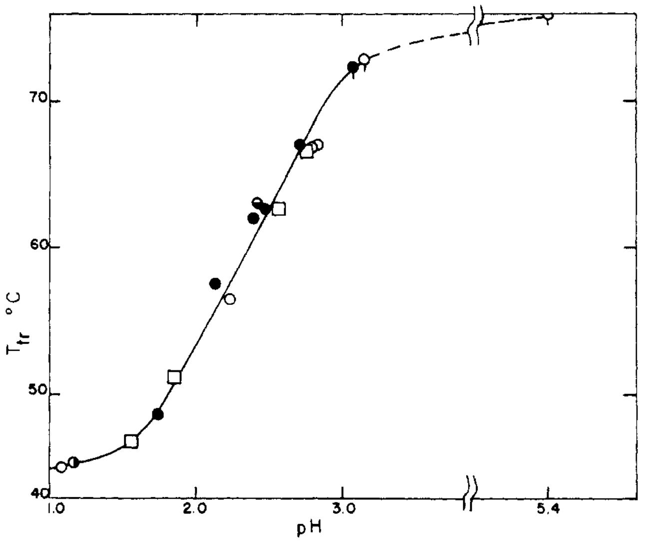

FIG. 3.—Variation of the transition temperature of the reversible denaturation with *p*H. The concentrations referred to below ($P\%$) are in grams of protein per 100 ml of solution. The points with a vertical bar (above 70°) are somewhat uncertain (see text). ●, from η_{sp} measurements, $P\% = 2.5$, 0.02 ionic strength; ○, from η_{sp} measurements, $P\% = 2.5$, 0.15 ionic strength; ◑, from η_{sp} measurements, $P\% = 1.5$, 0.15 ionic strength; ◓, from η_{sp} measurements, $P\% = 1.5$, 0.02 ionic strength; □, from optical rotation at 436 mμ, $P\% = 2.5$, 0.02–0.055 ionic strength.

In all studies reported here, reversibility was always tested by making measurements at at least two temperatures upon cooling. Values where irreversibility of over 5% was found were not used or corrected for use.

It has been pointed out previously in studies with ribonuclease (Holcomb and Van Holde, 1962) that values of intrinsic rather than reduced viscosity are more reliable in representing the concentration of the native and denatured forms of the protein. Although the intrinsic viscosity was not measured over the entire *p*H range of the transition, studies were carried out at 1.5 g/100 ml protein concentration at the two *p*H extremes and at an intermediate *p*H. The values of T_{tr} and $\Delta H°$ obtained were, within experimental error, of the same magnitude as those obtained at 2.5 g/100 ml concentration. The results are shown in Figure 3. Thus values of reduced viscosity may be considered a reliable measure of the denatured form, in this case. One reason may be that the conformational change brought about upon denaturation is not extensive. The results suggest that the concentration dependence of the reduced viscosity of the denatured form may be similar to that of the native form.

Graphs of $\ln K'$ versus $1/T$ need not be linear. They are not linear in the studies with ribonuclease (Holcomb and Van Holde, 1962; Hermans and Scheraga, 1961a). In this study, however, such plots were linear within experimental error. Within each experiment, values of $\Delta H°$ within ± 2 kcal were obtained with values of f_d from 0.10 to 0.95. This is an entirely adequate range of f_d to establish linearity of the plots. The values of $\Delta H°$ were obtained both graphically and by the least squares method in each case. The transition temperature, T_{tr}, is commonly taken as the temperature at which $K' = 1$. A graph of the transition temperature versus *p*H, obtained under a variety of conditions, is shown in Figure 3. As can be seen from Figure 3, there is no significant difference between T_{tr} obtained at 1.5 g/100 ml and 2.5 g/100 ml protein concentration. Also, no significant difference is observed when T_{tr} is measured by viscosity or optical rotation. This implies that both methods measure the same process. A previously published T_{tr} obtained by differential spectrophotometry (Foss, 1960) at a single *p*H (2.2) is in fair agreement with our results. It seems that, at least at *p*H 2.2, all three methods measure the same conformational change. Our optical rotation results at *p*H 2.2 are also in agreement with those obtained in another laboratory[1] after reinvestigation of the initially reported value (Foss, 1960).

Ionic strength from 0.02 to 0.15 seems to have no effect on either the $\Delta H°$ or the T_{tr}. Such a change of ionic strength has a pronounced effect on the titration curves of most proteins. The significance of lack of ionic strength effects will be discussed further.

Theoretical Considerations.—Various theories which have been formulated to interpret conformational changes in proteins have been summarized in a recent review (Scheraga, 1963). Here we shall be concerned mainly with the effect of *p*H on reversible conformational changes and the establishment of the relationship of the experimentally determined K', the so-called equilibrium constant, to theory.

Experimentally, K' represents the following ratio:

$$K' = \frac{{}^{d}[\mathrm{A} + \mathrm{B} + \cdots + \mathrm{Z}]}{[\mathrm{A} + \mathrm{B} + \cdots + \mathrm{Z}]} \tag{4}$$

where A, B, . . . represent various protein species and the superscript *d* on the left of the bracket indicates that all the species in the bracket are in the denatured form. A superscript is omitted from the species in the native form. This practice will be followed in this article. A, B, . . . would represent the concentrations of the particular species only if every species in the denatured (or the native) form had the same specific value for the quantity measured such as reduced viscosity, specific levorotation, or others. In this case, equation (4) could be rewritten as:

$$K' = \frac{{}^{d}[(\mathrm{A}) + (\mathrm{B}) + \cdots (\mathrm{Z})]}{[(\mathrm{A}) + (\mathrm{B}) + \cdots (\mathrm{Z})]} \tag{4a}$$

We shall assume that relation (4a) holds in this study. The experimental K' may describe not an essentially single-step process, but rather a multiple-step one. A number of such mechanisms have been considered in detail elsewhere (Holcomb and Van Holde, 1962). In another series of studies (Hermans and Scheraga, 1961a,b) a mechanism somewhat related to the one presented here has been given. It has been pointed out previously (Tanford, 1961a) that in a *p*H-dependent isomerization the values of the ionization constants of the groups affected by the isomerization must be different in the two forms of the protein, on thermodynamic grounds. Reversible denaturation may be considered in a similar manner; the case is rather simple if no other *p*H-dependent changes, such as isomerization or association, occur simultaneously. If the number of ionizable groups involved in the denaturation process is limited to three, then the experimental K' is given by:

$$K = \frac{{}^{d}(P)_t}{(P)_t} = \frac{{}^{d}[(\mathrm{PH_1H_2H_3}) + (\mathrm{PH_1H_2}) + (\mathrm{PH_1H_3}) + (\mathrm{PH_2H_3}) + (\mathrm{PH_1}) + (\mathrm{PH_2}) + (\mathrm{PH_3}) + (\mathrm{P})]}{[(\mathrm{PH_1H_2H_3}) + (\mathrm{PH_1H_2}) + (\mathrm{PH_1H_3}) + (\mathrm{PH_2H_3}) + (\mathrm{PH_1}) + (\mathrm{PH_2}) + (\mathrm{PH_3}) + (\mathrm{P})]} \tag{5}$$

where ${}^{d}(P)_t$ and $(P)_t$ refer to the total concentration of the denatured and native species, respectively. The subscripts of the H refer to the particular ionizable groups designated by 1, 2, and 3 which are protonated;

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[1] Personal communication of J. Foss to one of us (A. J. S.), February, 1963.

for example, for the species PH_2H_3 the groups 2 and 3 are protonated, but group 1 is not. For each protein form, denatured or native, there are twelve possible microscopic constants relating the ionization equilibria of the eight species. In the scheme below, only three such intrinsic constants are shown:

$$\begin{tikzcd}
{}^d\mathrm{PH_1H_2H_3} \arrow[r, shift left, "{}^dk_1"] \arrow[d, shift left] & {}^d\mathrm{PH_2H_3} \arrow[l, shift left] \arrow[r, shift left, "{}^dk_{12}"] \arrow[d, shift left] & {}^d\mathrm{PH_3} \arrow[l, shift left] \arrow[r, shift left, "{}^dk_{123}"] \arrow[d, shift left] & {}^d\mathrm{P} \arrow[l, shift left] \arrow[d, shift left] \\
\mathrm{PH_1H_2H_3} \arrow[u, shift left, "K_{0D}"] \arrow[r, shift left, "k_1"] & \mathrm{PH_2H_3} \arrow[u, shift left, "K_{1D}"] \arrow[l, shift left] \arrow[r, shift left, "k_{12}"] & \mathrm{PH_3} \arrow[u, shift left, "K_{2D}"] \arrow[l, shift left] \arrow[r, shift left, "k_{123}"] & \mathrm{P} \arrow[u, shift left, "K_{3D}"] \arrow[l, shift left]
\end{tikzcd} \tag{M6}$$

Examples of microscopic dissociation equilibria are:

$$k_2 = \frac{(\mathrm{PH_1H_3})(\mathrm{H})}{(\mathrm{PH_1H_2H_3})}, \qquad k_{13} = \frac{(\mathrm{PH_1})(\mathrm{H})}{(\mathrm{PH_1H_3})} \tag{7}$$

The denaturation constants carry a capital D subscript and in addition a number as subscript which indicates the number of protons dissociated from the molecule. One might use appropriate subscripts, as in the microscopic ionization constants, to write "microscopic" denaturation constants. However, we shall mainly be concerned with the two constants K_{0_D} and K_{3_D}:

$$K_{0D} = \frac{{}^d(\mathrm{PH_1H_2H_3})}{(\mathrm{PH_1H_2H_3})}, \qquad K_{3D} = \frac{{}^d(\mathrm{P})}{(\mathrm{P})} \tag{8}$$

and, therefore, such cumbersome additional notation has been avoided in this case.

In the general case where n ionizable groups are involved in the denaturation process, K' can be expressed in terms of either K_{0_D} or K_{3_D} and appropriate expressions which are functions of (H^+) and the ionization constants of the species involved.

$$K' = \frac{{}^d(\mathrm{PH_1} \cdot\cdot\ \mathrm{H}_n)}{(\mathrm{PH_1} \cdot\cdot\ \mathrm{H}_n)} \cdot \frac{{}^df_o\ (1/H)}{f_o\ (1/H)} = K_{0D} \frac{{}^df_o(1/H)}{f_o(1/H)} \tag{9}$$

or

$$K' = K_{nD} \cdot \frac{{}^df_n(H)}{f_n(H)} \tag{9a}$$

where

$$f_o(1/H) = \left[1 + \frac{K_1}{(\mathrm{H}^+)} + \frac{K_1K_2}{(\mathrm{H}^+)^2} + \ldots + \frac{K_1K_2 \ldots K_n}{(\mathrm{H}^+)^n}\right] \tag{10}$$

and ${}^df_o(1/H)$ is an expression identical to (10) but referring to the dissociation constants of the denatured species. Here $K_1, K_2, \ldots$ are the (macroscopic) dissociation constants related to the microscopic constants as follows[2]:

$$\begin{aligned} K_1 &= k_1 + k_2 + k_3 + \cdots \\ K_1K_2 &= k_1k_{12} + k_1k_{13} + k_2k_{23} + \cdots \\ K_1K_2K_3 \cdots &= k_1k_{12} \cdots k_{12\cdots n} \end{aligned} \tag{11}$$

In a similar manner and using the same microscopic constants, $f_n(H)$ is given as:

$$f_n(H) = \left[1 + \frac{(\mathrm{H}^+)}{K_1} + \frac{(\mathrm{H}^+)^2}{K_1K_2} + \cdots \frac{(\mathrm{H}^+)^n}{K_1K_2 \cdots K_n}\right] \tag{10a}$$

where the (macroscopic) dissociation constants are given by:

$$\begin{aligned} \frac{1}{K_1} &= \left(\frac{1}{k_1} + \frac{1}{k_2} + \cdots + \frac{1}{k_n}\right) \\ \frac{1}{K_1K_2} &= \left(\frac{1}{k_1k_{12}} + \frac{1}{k_1k_{13}} + \cdots + \frac{1}{k_{n-1}k_n}\right) \end{aligned} \tag{11a}$$

and so on. Equations such as (10) assume a simple form in the case of n equivalent and independent groups. In this case, the microscopic constants are identical, so that $k_1 = nk$, where k is now a single microscopic constant. In the case (of interest here) where $n = 3$, equation (10) reduces to:

$$f_o(1/H) = \left[1 + \frac{k}{(\mathrm{H}^+)}\right]^3 \tag{12}$$

It should be pointed out that although in titration studies all titratable groups are included, in the foregoing expressions only groups whose dissociation constants change in going from one protein form to the other are included and detectable. Referring to scheme (M6), it is seen that the scheme describes only one of the many possible paths by which the fully protonated native and denatured forms can go to their respective fully unprotonated species. One of several possible relationships between the ionization and denaturation constants is:

$$\frac{K_{0D}}{K_{3D}} = \frac{k_1k_{12}k_{123}}{{}^dk_1{}^dk_{12}{}^dk_{123}} \tag{13}$$

An important deduction can be made from equation (13), namely, that whenever the ratio K_{0_D}/K_{3_D} is independent of temperature (it is independent of pH, by definition), the sum of the enthalpies of ionization of the microscopic constants, chosen successively along a continuous path from $\mathrm{PH}_n \rightarrow \mathrm{P}$ of the native form, is equal to the sum of the enthalpies of ionization of the corresponding constants in the denatured form.

From equations (9) and (10) it is seen that when $(\mathrm{H}^+) \approx \infty$, $K' = K_{0_D}$; and from equations (9a) and (10a), $K' = K_{3_D}$ when $(\mathrm{H}^+) \approx 0$. In our studies the values of $\Delta H°$ found from plots of $\ln K'$ versus $1/T$ were virtually constant throughout the pH range studied. If it is indeed taken that $\Delta H°$ is independent of pH and constant, then ${}^df_o(1/H)/f_o(1/H)$ is independent of temperature at any definite hydrogen-ion concentration. Also:

$$\frac{d \ln K'}{d(1/T)} = \frac{d \ln K_{0D}}{d(1/T)} = \frac{d \ln K_{3D}}{d(1/T)} \tag{14}$$

In other words, the standard enthalpy of denaturation of the fully protonated form is equal to that of the fully unprotonated form, and this is equal to the experimentally determined one from K'. Moreover, the sum of the enthalpies of ionization of groups 1, 2, and 3 in the native form is equal to the sum of the enthalpies of ionization of the same groups in the denatured form.

The values of K_{0D} and K_{3D} can be obtained at any temperature, if the T_{tr} becomes independent of pH at the extremes of pH. Extrapolation may be resorted to, but the values of K_{0D} and K_{3D} become more uncertain as the limiting T_{tr} values are approached. Assuming that the low ($T_{\mathrm{tr}L}$) and high ($T_{\mathrm{tr}H}$) limits of the transition temperature are established, since $\Delta H°$ is independent of both temperature and pH, we may write:

$$\ln \frac{K'_2}{K'_1} = -\frac{\Delta H°}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \tag{15}$$

and since at $T_{\mathrm{tr}L}$ $K' = K_{0D} = 1$ and at $T_{\mathrm{tr}H}$ $K' = K_{3D} = 1$, at any other temperature,

$$\ln K_{0D} = -\frac{\Delta H°}{R}\left(\frac{1}{T} - \frac{1}{T_{\mathrm{tr}L}}\right) \tag{15a}$$

and

$$\ln K_{3D} = -\frac{\Delta H°}{R}\left(\frac{1}{T} - \frac{1}{T_{\mathrm{tr}H}}\right) \tag{15b}$$

Also,

$$\ln \frac{K_{0D}}{K_{3D}} = -\frac{\Delta H°}{R}\left(\frac{1}{T_{\mathrm{tr}L}} - \frac{1}{T_{\mathrm{tr}H}}\right) \tag{15c}$$

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[2] The hydrogen-ion-dependent functions used here are quite similar to the ones derived for the ionization behavior of polyelectrolytes and used in deriving expressions for the titration of proteins and other polyelectrolytes. Such equations, as well as equations for and excellent discussions on electrostatic free energy and the charged sphere model, can be found in texts such as that of Edsall and Wyman (1958) and Tanford (1961b).

From experiments at constant pH the values of ln K' can be obtained at any temperature from van't Hoff plots. From a series of such experiments at a number of pH values, the variation of ln K' with pH can be obtained at any temperature. Figure 4 shows such graphs at four temperatures. Since the values of K_{0D} and K_{3D} can be obtained from equations (15a) and (15b), values of K_{0D} and K_{3D} can be substituted in equations (9) and (9a) to obtain values of ${}^{d}f_o(1/H)/f_o(1/H)$ or ${}^{d}f_n(H)/f_n(H)$ as a function of pH, and the values of the constants can be obtained by approximation methods, as is done with titration studies. If the values of the pH-dependent functions such as $f_o(1/H)$ are independent of temperature, simplified expressions can be derived. First, the temperatures may be limited to T_{tr} so that $1 = K_{0D}[{}^{d}f_o(1/H)/f_o(1/H)]$. If further simplifying assumptions are made, such as are expressed in equation (12), then for the case of three dissociable groups and utilizing only T_{tr} values,

$$1 = K_{0D}^{*}\frac{\left[1 + \frac{{}^{d}k}{(\text{H}^{+})}\right]^{3}}{\left[1 + \frac{k}{(\text{H}^{+})}\right]^{3}} \qquad (16)$$

By utilizing the simplifying assumption that $K = k_1 = k_{12} \cdots$ equation (13) becomes:

$$\frac{K_{0D}^{1/3}}{K_{3D}^{1/3}} = \frac{k}{{}^{d}k} \qquad (13\text{a})$$

Taking the third root of equation (16), substituting into it equation (13a) and rearranging, the following expression results:

$$\frac{(K_{0D}^{*1/3} - 1)}{(1 - K_{3D}^{*1/3})} = \frac{k}{(\text{H}^{+})} \qquad (16\text{a})$$

One may graph the expression on the left of equation (16a) versus $1/H$, or equation (16a) may be expressed in logarithmic form:

$$\log\frac{(K_{0D}^{*1/3} - 1)}{(1 - K_{3D}^{*1/3})} = -pk + p\text{H} \qquad (16\text{b})$$

where $pk = -\log k$. In equation (16) through (16b), K_{0D}^{*} and K_{3D}^{*} have been marked with an asterisk to emphasize that in this case, their values are those at the particular *transition* temperature at any pH. It should be noted that similar expressions for two or any other number of ionizable groups can be derived, provided the same simplifying assumptions are made.

Another way of obtaining information regarding the number and kind of ionizable groups involved is the following. Equation (9) combined with equation (10) implies that there exists equilibrium between all species involved, so that equation (9) can be differentiated with respect to ln H to give immediately:

$$\frac{d \ln K'}{d \ln H} = r_n - r_d \qquad (17)$$

where

$$r_n = \frac{d f_o(1/H)}{d \ln H}$$

$$= \frac{\left[\frac{K_1}{(\text{H}^{+})} + \frac{2K_1K_2}{(\text{H}^{+})^2} + \frac{3K_1K_2K_3}{(\text{H}^{+})^3} + \cdots + \frac{nK_1K_2\cdots K_n}{(\text{H}^{+})^n}\right]}{\left[1 + \frac{K_1}{(\text{H}^{+})} + \frac{K_1K_2}{(\text{H}^{+})^2} + \cdots + \frac{K_1K_2\cdots K_n}{(\text{H}^{+})^n}\right]} \qquad (18)$$

Here r_d is given by an expression identical to (18), involving the dissociation constants of the groups of the denatured species; r_n and r_d are the number of moles of protons removed per mole of the particular protein species, native and denatured, respectively. An expression similar to equation (18) has been given previously (Harris and Rice, 1954; Hermans and Scheraga, 1961b). The value of $(r_n - r_d)$ can be obtained from graphs such as in Figure 4. It is rather difficult to obtain accurate $r_n - r_d$ values from such plots, but they are reliable enough to be used in conjunction with other methods discussed. An important piece of information from such graphs is that the maximum slope $(r_n - r_d)$ of such graphs is equal to the minimum number of ionizable groups involved. The maximum slope obtained from Figure 4 is 2.76, thus the minimum number of groups involved is three.

Electrostatic-Charge Effects.—The change of the apparent ionization constants can be attributed in whole or in part to changes in electrostatic interactions upon denaturation. The charged-sphere model of Linderstrom-Lang will be the only one examined here.[3] We shall consider a polyelectrolyte containing groups which in their acidic form are uncharged, such as carboxyls, and also groups which are cationic in their acidic form, such as ammonium groups; it will be assumed also that the titration regions of the two kinds of groups do not overlap, so that when one kind, such as the carboxyls, is titrated, the cationic groups contribute a constant charge c to the protein. The resulting expressions will not be elaborated upon since, for the derivations, ready reference can be made.[2] It will be stated simply that the macroscopic dissociation constants as those in equation (11) are now, for example:

$$K_1 = nk_{\text{int}} = \frac{(\Sigma\text{P}^{-})(\text{H}^{+})}{(\text{PH})}\cdot\exp w\cdot[(c - 1)^2 - c^2] \qquad (19)$$

and the function of $f_o(1/H)$ of equation (10) becomes:

$$f_o(1/H) = \sum_{h=0}^{n}\frac{n!}{(n - h)!n!}\cdot\frac{k^{h}{}_{\text{int}}}{(\text{H}^{+})^{h}}\cdot\exp w[c^2 - (c - h)^2] \qquad (20)$$

A similar expression can be written for ${}^{d}f_o(1/H)$ which contains the parameters appropriate for the denatured form. In equations (19) and (20), c is a fixed charge due to the cationic groups, n is the total number of titratable groups with the same intrinsic ionization constant k_{int}, h is a variable negative charge equal to the number of groups which have lost protons in a particular species, and the electrostatic parameter w is defined by:

$$w = \frac{N\epsilon^2}{2DRT}\cdot\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (21)$$

where b is the radius of the sphere, a is a "collision diameter," D is the dielectric constant of the medium, ϵ is the electrostatic charge, κ is the reciprocal of the radius of the ionic atmosphere, and N, R, and T have their usual meaning. The relationship between the microscopic and intrinsic constants is usually given by the approximate relationship:

$$k = k_{\text{int}} \exp 2wZ \qquad (22)$$

A distinction should be made between the quantities $(c - h)$ and Z of equations (20) and (22). The quantity h is always an integral number and equation (20), when expanded for n groups, should contain $(n + 1)$ terms. On the other hand, the quantity Z of equation (22) is

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[3] A more elaborate and perhaps more realistic theory would be that of Rice and Nagasawa (1961) or related theories. However, as elaborated under Discussion, more information than now available is necessary, and at present use of Rice and Nagasawa's or related theories is not warranted.

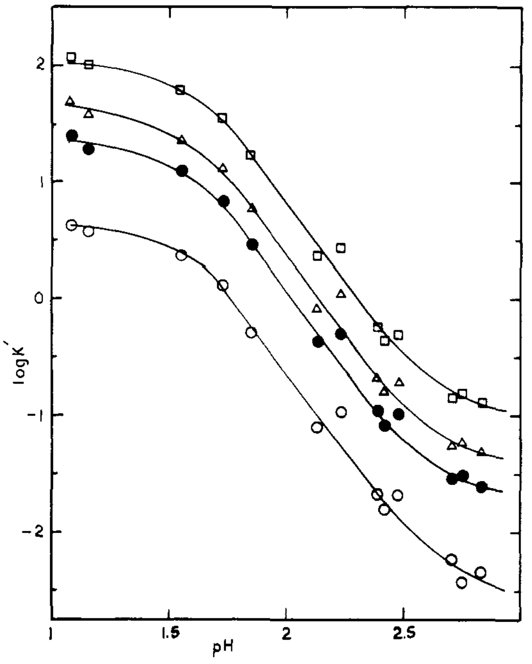

FIG. 4.—Dependence of log K' on pH at constant temperature. ○, $1/T = 3.10 \times 10^{-3}$ °K^{-1}; ●, $1/T = 3.05 \times 10^{-3}$ °K^{-1}; △, $1/T = 3.03 \times 10^{-3}$ °K^{-1}; □, $1/T = 3.00 \times 10^{-3}$ °K^{-1}.

the average net charge at a particular (H^+). In the absence of ion binding, $Z = (c - r)$, where r can be a fractional number equal to the protons removed per mole of protein at a particular pH.

If it is assumed that the pH-dependent change is entirely due to electrostatic interactions of the charged-sphere model, then the only parameter which is different in the functions $f_o(1/H)$ and ${}^df_o(1/H)$ is w. The values of k_{int}, c, n, and h are the same in both expressions. Calculations based on the foregoing model are shown in Figure 5. Here $f_o(1/H)$ and ${}^df_o(1/H)$ were calculated by assuming $pk_{\text{int}} = 3.70$, $w = 0.130$, $w_d = 0.035$, and $n = 3$, $c = 19$. $f_o(1/H)$ and $f_o{}^d(1/H)$ were obtained by equation (20) and substituted into equation (9) (in its logarithmic form).

An important relationship arises from application of the foregoing model to equation (13). It follows immediately that regardless of the value of k_{int},

$$\frac{K_{0D}}{K_{3D}} = \exp{(w - w_d)[c^2 - (c - n)^2]} \qquad (23)$$

The ratio K_{0D}/K_{3D} is again constant, in agreement with the previous assumption made. From the following it can also be seen that the ratio K_{0D}/K_{3D} is a measure of the difference between the standard free energy for the transition of the fully protonated native form to the fully protonated denatured one, and the standard free energy for the transition of the unprotonated native form to its denatured counterpart. The term "unprotonated" refers again to the complete removal of just the n protons related to denaturation. The electrostatic free energy is given by:

$$W_{el} = RTwZ^2 \qquad (24)$$

For the transition $(PH_n) \rightarrow {}^d(PH_n)$, $W_{el(PH_n)} = RT(w_d - w)c^2$; for the transition $(P) \rightarrow {}^d(P)$, $W_{el(P)} = RT(w_d - w)(c - n)^2$; and therefore,

$$W_{el(PH_n)} - W_{el(P)} = RT(w_d - w)[c^2 - (c - n)^2] \qquad (25)$$

Since K_{0D} and K_{3D} are the denaturation equilibrium constants of the fully protonated and fully unprotonated species, respectively, the difference in $\Delta F°$ of the two processes is:

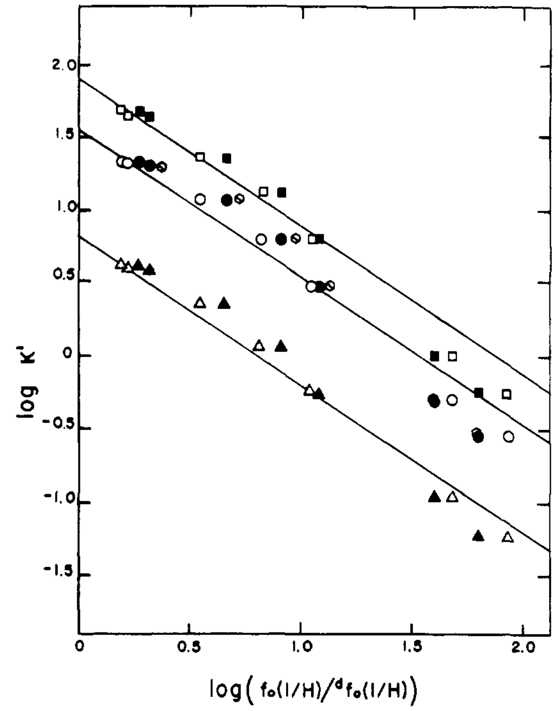

FIG. 5.—Variation of log K' with log $[f_0(1/H)/{}^df_0(1/H)]$. Filled points are calculated for model A (see text); open points are for model B. Triangles are from measurements at $1/T = 3.10 \times 10^{-3}$ °K^{-1}; circles are for measurements at $1/T = 3.05 \times 10^{-3}$ °K^{-1}; squares are for measurements at $1/T = 3.03 \times 10^{-3}$ °K^{-1}. Points ⊘ are for model C at $1/T = 3.05 \times 10^{-3}$ °K^{-1}. The straight lines are drawn with a slope −1.

$$\Delta F°_{(PH_n)\text{den}} - \Delta F°_{(P)\text{den}} = -RT \ln \frac{K_{0D}}{K_{3D}} \qquad (25a)$$

Comparison of equations (23) through (25a) shows that

$$W_{el(PH_n)} - W_{el(P)} = \Delta F°_{(PH_n)\text{den}} - \Delta F°_{(P)\text{den}} \qquad (25b)$$

Of course, equation (25a) holds true regardless of the origin of the free energy.

Analysis of Results.—Maximum reliable information can be obtained by examining the data in a number of ways. Since the values obtained depend upon the accuracy of the values of $\Delta H°$, T_{trH}, T_{trL}, and pH, experimental errors will reflect themselves in a different manner in each method used. The method which contains the least number of assumptions is that shown in Figure 4. The only significant assumption involved is that the pH, measured at 25°, remains constant with temperature. Experimental errors of $\Delta H°$ are reflected in each experimental point, since the overall average $\Delta H° = 66$ kcal was not used in obtaining these graphs.

Values from graphs such as in Figure 4 can be used to examine various models. Only models where three carboxylate groups are involved will be considered because according to equation (17) and Figure 4 the minimum number of groups is three, and titration studies of a commercial sample (Donovan *et al.*, 1960) and of the one used in this study[4] indicate only three carboxylate groups with abnormal pK_a values. The results have been analyzed by assuming one and also two groups, but the data do not fit such schemes well and will not be given further consideration. Equation (9) in logarithmic form, in conjunction with Figure 4, has been used to obtain the graphs shown in Figure 5,

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[4] Work in progress in this laboratory.

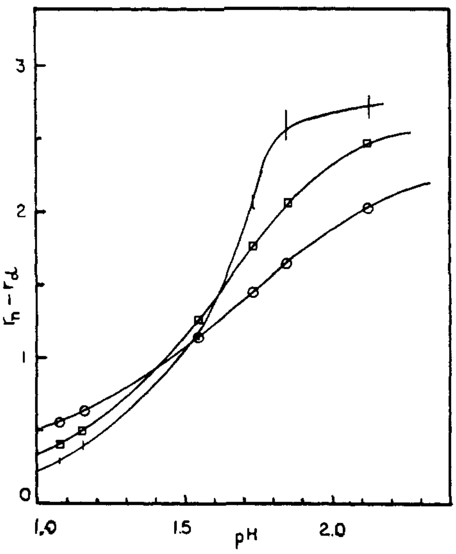

FIG. 6.—Variation of ($r_n - r_d$) with pH. Graph described by the vertical bars is experimental, and the length of the bars indicates the maximum deviation. □, Calculations for model B; ○, calculations for model A.

where results at three different temperatures are shown. In order to calculate $f_o(1/H)/{}^df_o(1/H)$, three models were considered. In models A and B electrostatic effects were neglected. For model A, the groups were considered independent and equivalent, and the closest agreement was obtained by setting $k = 1.92 \times 10^{-2}$, or $pk_a = 1.72$. Model B was calculated by assuming one independent group with $k = 3.5 \times 10^{-2}$ ($pk = 1.46$) and two groups dissociating simultaneously with $k = 2.2 \times 10^{-2}$ ($pk = 1.66$). For both models A and B the groups in the denatured form were considered equivalent and independent with $k_d = 4 \times 10^{-4}$ ($pk = 3.40$). Model C was calculated by attributing the change in pk entirely to electrostatic effects, assuming that the k_{int} in the native and denatured forms are the same, and that $w = 0.130$ whereas $w_d = 0.035$. The best fit is given by model B although models A and C can be considered acceptable. The deviations of the points above pH 2.1 could be owing mainly to the fact that no extensive attempt was made to obtain better values for k_d, because the significant regions to obtain such values would be at higher pH values and therefore high temperatures, where measurements become uncertain. The most significant deviations of the models occur between pH 2 and 1. The values used in each case were the best for the model, obtained by curve fitting to within 5% of the k values used.

Values of the ionization constants can also be obtained by using equations (17) and (18). Figure 6 shows graphs of the slopes obtained from Figure 4 versus pH and also graphs of ($r_n - r_d$) obtained from the models A and B versus pH. Again model B is the more acceptable one. It should be noted at this point that the shape of the curves at various temperatures in Figures 4 and 5 below pH 2.5 is identical. This is also borne out by the fact that the slopes obtained at three different temperatures were so close that they were averaged for use in Figure 6. This indicates that the ionization constants of the native protein do not vary appreciably with temperature and that therefore the $\Delta H°$ of ionization is small.

Perhaps the best way to obtain accurate values of $T_{\text{tr}L}$ is from Figure 5. The intercept at the ordinate gives $\log K_0$ at the temperature used, and using equation (15a), $T_{\text{tr}L}$ can be obtained. The values from Figure 5 gave for $T_{\text{tr}L}$ 43.40°, 43.20°, and 43.12°.

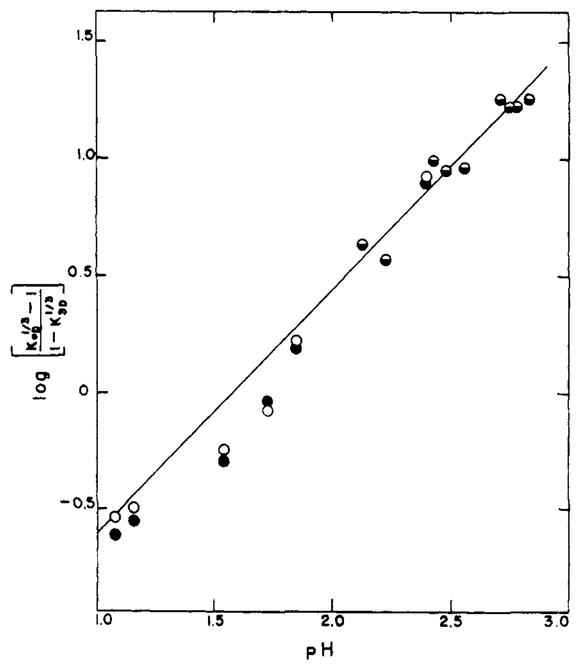

FIG. 7.—Variation of $\log\,[(K_{0D}{}^{1/3} - 1)/(1 - K_{3D}{}^{1/3})]$ versus pH, according to equation (16b). ○, $T_{\text{trL}} = 43°$; ●, $T_{\text{trL}} = 43.24°$; ◒, superposition of ○ and ●. For both graphs it was assumed that $T_{\text{trH}} = 77°$, $\Delta H° = 66$ kcal.

If $T_{\text{tr}L}$ and $T_{\text{tr}H}$ are known accurately, equation (16b) can be used to determine the ionization constants of the native form. Equation (16b) is limited to model A. A similar equation where all the protons dissociate simultaneously can be derived:

$$\log \frac{(K_{0D}{}^* - 1)}{(1 - K_{3D}{}^*)} = -pK + npH \qquad \text{(16c)}$$

where n is the number of protons dissociating simultaneously. Equation (16c), with acceptable values of $T_{\text{tr}L}$, $T_{\text{tr}H}$, and $\Delta H°$, did not give a particularly good fit. In Figure 7 are shown two graphs which differ in the values of $T_{\text{tr}L}$ used, $T_{\text{tr}L} = 43°$ and $T_{\text{tr}L} = 43.24°$. It is obvious that such graphs are extremely sensitive to the values of $T_{\text{tr}L}$ used. However, the significant deviations are shown again in the region pH 2 to 1 and are of the same nature as in Figure 5. Such deviations could be due to experimental error, but the values used at low pH are perhaps the most accurate ones, and there should be appreciable errors in measuring pH for such deviations to occur. One reasonable conclusion is that none of the models used describes completely the ionization scheme.

DISCUSSION

Any of the three models described can be acceptable, although model B agrees best. Reasonable values of pK_a for the three groups are obtained; such values could not be obtained previously from titration studies at room temperature (Donovan *et al.*, 1960). Refining the models further has not been attempted because lack of reliable information regarding parameters such as w, and anion binding at various temperatures does not warrant such effort. A study (Beychok and Warner, 1959) at room temperature and to pH 2 indicates extensive anion binding, but no data are available below pH 2. The value of w below neutral pH is not definitely established (Beychok and Warner, 1959; Tanford and Wagner, 1954). Titrations of the

TABLE 1
THERMODYNAMIC PARAMETERS FOR THE TRANSITION AT 25°

Overall Process	$-RT \ln K$ (kcal/mole)	$\Delta F°$ (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$(eu)
$PH_3 \rightarrow {}^dPH_3$	3.8	3.7	66	209
$P \rightarrow {}^dP$	9.8	9.7	66	189
$PH \rightarrow P + H^+$	7.0–6.5		0[a]	−23.5
${}^dPH \rightarrow {}^dP + H^+$	13.9		0[a]	−46.7

[a] It has been assumed that $\Delta H°$ is zero; see Discussion for reasons. The column "$-RT \ln K$" gives $\Delta F°$ calculated from the appropriate equilibrium constant, whereas the column "$\Delta F°$" has been calculated from the relation $\Delta F° = \Delta H° - T\Delta S°$.

samples used in this study[4] agree closer with that of Armour lot 381-187 (Donovan *et al.*, 1960) than any of the others reported. In one case, the apparent discrepancy of Armour lot D638-040, which was reported to have five "extra" carboxyls (Donovan *et al.*, 1961), has been resolved since it has been shown[4] that the "extra" carboxyls originated in unidentified but dialyzable contaminants.

Model B may be somewhat unusual because it assumes the simultaneous dissociation of two protons. However, by reference to scheme (M6) it can be shown that if the denaturation constant K_{2D} is much larger than K_{3D}, the result would be a model similar to model B.

Although model C shows the poorest fit, its apparent agreement with the results indicates that even such sharply *p*H-dependent transitions could be explained purely on electrostatic grounds. The main argument for the existence of side-chain hydrogen bonds has been, so far, that electrostatic interactions cannot be used to describe transitions occurring in a narrow *p*H range. It should be remembered that the W_{el} of the charged sphere model favors the transition to random coil at any *p*H away from the isoelectric point. At low *p*H for example, the calculated W_{el} in this study, neglecting anion binding, is approximately −20 kcal. The experimental $\Delta F°$ for the transition at low *p*H is 4 kcal. Therefore other intramolecular interactions must contribute approximately a $\Delta F° = -24$ kcal to stabilize the compact configuration so that the T_{trL} is 43° rather than 25°. Thus the effect of increasing electrostatic interactions with decreasing *p*H is in essence to increase the electrostatic free energy beyond the contribution of other stabilizing forces. Such increase could be sharp, especially for such basic proteins as muramidase and ribonuclease at low *p*H, as can easily be seen from equations (20) to (25b).

In spite of the apparent agreement of model C with the results, a number of reasons argue against it, the main one being the apparent lack of ionic strength dependence. Values of $w = 0.230$ at 0.03 ionic strength and $w = 0.175$ at 0.15 ionic strength have been reported (Tanford and Wagner, 1954). The values of $w = 0.080$ above neutral *p*H has been accepted (Tanford and Wagner, 1954; Beychok and Warner, 1959), and this value can be used in acid *p*H if anion binding is taken into account. For the calculations in this study, the values $w = 0.130$ and $w_d = 0.035$ were employed. Since the increase of w with lowering ionic strength for the denatured form is much less than that for the native form, ionic strength effects should be obvious within the ionic strength ranges used here. Another reason is that chloride binding has been neglected; by neglecting chloride binding, $Z_{max} = 19$ and at *p*H 2, $Z = 17$. However, a net charge of approximately 8 is reported when anion binding is taken into account (Beychok and Warner, 1959). Meaningful calculations such as for model C would be impossible if chloride binding is taken into account. Another reason is that the titration of carboxyl groups is not as artificially abrupt as it was taken for model C, since from titration studies the value of r changes by about 2 between *p*H 3 and 2 after subtracting the contribution to r of the three abnormal carboxyls.

Thermodynamic values for the transition can be obtained if it is assumed that the $\Delta H°$ for the transition is independent of *p*H. Although the experimental value was constant, 66 ± 1.5 kcal, a larger deviation of ±3 kcal could be accepted. However, no trend with *p*H was observed; and, because of lack of evidence to the contrary, $\Delta H°$ for the transition will be assumed to be independent of *p*H. If $\Delta S°$ is assumed to be independent of temperature at constant *p*H, the values shown in Table I are obtained. The values of $\Delta S°$ can be obtained at the two limits of *p*H since $\ln K' = \ln K_0$ at low *p*H, $\ln K' = \ln K_3$ at high *p*H, and $\Delta F°_{(PH_n)den} = -RT \ln K_0$, $\Delta F°_{(P)den} = RT \ln K_3$ independent of *p*H, and $\Delta F° = 0$ at the transition temperature. It is seen that there is a difference of approximately 20 eu between the entropies for the transition of the fully protonated species and the unprotonated one. This difference cannot be attributed to experimental error in measuring $\Delta H°$, because even if it is assumed that the value of $\Delta H°$ varies with *p*H and is reduced from 71 kcal at high *p*H to 61 kcal at low *p*H, the value of $\Delta S°$ is reduced to only approximately 11 eu. It has already been pointed out that if the $\Delta H°$ for the transition is independent of *p*H, the sum of the enthalpies of ionization of the three groups in the native form must equal the sum of the heats of ionization of the same groups in the denatured form. Since the groups are presumably "normalized" in the denatured form and since the $\Delta H°$ of ionization of carboxyl groups is nearly zero, it follows that the heat of ionization of the groups in the native molecule is also nearly zero. In any event, no significant enthalpy changes seem to be associated with the abnormal lowering of the pK_a values in the native molecule. Rather, such changes seem to be largely entropic in nature. The average change in $\Delta S°$ of ionization per group between the native and denatured forms is of the order of 7 eu. This is of the same order of magnitude as the difference in the $\Delta S°$ of ionization of the carboxyl groups of α-aminobutyric acid and γ-aminobutyric acid, two acids in which the amino group is close to and far from the carboxyl group. It has been postulated previously (Donovan *et al.*, 1961) that lysyl or arginyl residues may be in close proximity to the abnormal carboxyls of muramidase in the native state. Localized electrostatic interactions should not, in general, be so ionic-strength dependent as is the charged sphere model.

Elucidation of the specific conformational changes which occur upon denaturation, as well as information concerning the forces responsible for maintaining the compact structure of muramidase, should be obtained from further studies, now in progress, on the thermodynamics of the proton and anion binding of muramidase, and the effect of the solvent medium on denaturation.

Acknowledgments

We wish to thank Dr. K. E. Van Holde for valuable discussions and suggestions on the subject; some exploratory experiments pertaining to this work were carried out by one of us (A.J.S.) in Dr. Van Holde's laboratory in 1960–61.

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On the Similarity of Dihydrofolate Reductases from Amethopterin-sensitive and Amethopterin-Resistant Mouse Leukemia (L1210) Cells[*]

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The dihydrofolate reductase activity of amethopterin-resistant mouse lymphoid-leukemia (L1210) cells increases manyfold over that of drug-sensitive cells. This has been attributed to the selection of resistant cells that produce the same kind of dihydrofolate reductase molecules as their sensitive ancestors but in greater numbers. This conclusion now appears to be justified in view of the identical behavior of the two enzyme proteins (one from amethopterin-sensitive cells, the other from the amethopterin-resistant subline) on ion-exchange columns, as well as the striking similarity in kinetic behavior with a variety of folate analogs used either as inhibitors or as substrates. Identical enzyme activations were observed with sodium chloride or *p*-mercuribenzoate.

Amethopterin treatment of mice given implants of lymphoid-leukemia (L1210) cells results in sublines of tumor cells that are resistant to the inhibitory action of the antifolate (Law, 1956). This resistance may be owing in part to a marked rise in dihydrofolate reductase, an enzyme known to be exquisitely sensitive to the inhibitory action of amethopterin. The enhanced enzymatic activity allows folic acid compounds to be reduced to the coenzymatically active tetrahydrofolates in quantities sufficient for cellular multiplication in the presence of the drug (Misra *et al.*, 1961). Selection of amethopterin-resistant mutant leukemic cells with elevated enzyme activity rather than induction is suggested by the persistence of high enzyme levels for at least ten transfers of the leukemia cells in drug-free animals (Friedkin *et al.*, 1962a). Mathematical models consistent with the foregoing concept have been developed which relate dihydrofolate reductase activities in resistant and sensitive cells, mutation rates, and the life spans of leukemic mice treated with amethopterin (Friedkin and Goldin, 1962).

The dihydrofolate reductases from amethopterin-sensitive and -resistant leukemia cells have now been compared in order to determine whether there has been a selection of mutant cells in which a new molecular species of a more active reductase is produced, or whether the selection pressure is toward a cell producing a greater number of the original enzyme molecules. The striking similarity of the reductases of amethopterin-resistant and -sensitive cells reported in this communication strongly supports the latter possibility. A preliminary report has been presented elsewhere (Kashket *et al.*, 1964).

Experimental Procedures

Materials.—Folic acid as well as the various analogs were reduced to the dihydro form with dithionite in 1 M mercaptoethanol (Friedkin *et al.*, 1962b). The folic acid analogs 10-nitrosofolic acid, 3′-iodo-10-nitrosofolic acid, 3′-bromofolic acid, and 3′,5′-dibromofolic acid were prepared by Dr. L. Plante at Tufts University. Homofolic acid was prepared by Drs. L. Goodman and J. DeGraw, Stanford Research Institute (Goodman *et al.*, 1964). Amethopterin (Lederle Laboratories) was purified by the method of Noble (1961). Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956). NADPH was purchased from Pabst Laboratories.

Preparation of Dihydrofolate Reductase.—Acetone powders were prepared from tumors formed by the implantation of male mice (CDBA, (National Institutes of Health animal farm)) with amethopter-in-sensi-

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