Barnes, P. R., Hersh, R. T., & Kitos, P. A. (1974) *In Vitro 9*, 234.
Benson, S. C. (1977) *Biochem. Biophys. Res. Commun. 75*, 845–850.
Benson, S., Olsnes, S., Pihl, A., Skorve, J., & Abraham, A. K. (1975) *Eur. J. Biochem. 59*, 573–580.
Berridge, M., Ralph, R., & Letham, D. (1970) *Biochem. J. 119*, 75–84.
Blobel, G., & Sabatini, D. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 390–394.
Bonner, J. (1965) in *Plant Biochemistry* (Bonner, S., & Varner, J., Eds.) pp 21–37, Academic Press, New York.
Carrasco, L., Fernández-Puentes, C., & Vázquez, D. (1975) *Eur. J. Biochem. 54*, 499–503.
Cawley, D. B., Hedblom, M. L., Hoffman, E. J., & Houston, L. L. (1977) *Arch. Biochem. Biophys. 182*, 690–695.
Cawley, D. B., Hedblom, M. L., & Houston, L. L. (1978) *Arch. Biochem. Biophys. 190*, 744–755.
Fernández-Puentes, C., Benson, S., Olsnes, S., & Pihl, A. (1976) *Eur. J. Biochem. 64*, 437–443.
Grasmuk, S., Nolan, R. D., & Drews, J. (1977) *Eur. J. Biochem. 79*, 93–102.
Hamilton, M. G., Pavlovec, A., & Petermann, M. L. (1971) *Biochemistry 10*, 3424–3427.
Hedblom, M. L., Cawley, D. B., & Houston, L. L. (1976) *Arch. Biochem. Biophys. 177*, 46–55.
Hedblom, M. L., Cawley, D. B., Boguslawski, S., & Houston, L. L. (1979) *J. Supramol. Struct.* (in press).
Krystosek, A., Cawthon, M. L., & Kabat, D. (1975) *J. Biol. Chem. 250*, 6077–6084.
Marcus, A. (1972) in *Protein Biosynthesis in Nonbacterial Systems* (Last, J. A., & Laskein, A. I., Eds.) p 128, Marcel Dekker, New York.
Marcus, A., Efron, D., & Weeks, P. (1974) *Methods Enzymol. 30*, 749–754.
Montanaro, L., Sperti, S., Mattioli, A., Testoni, G., & Stirpe, F. (1975) *Biochem. J. 146*, 127–131.
Nolan, R. D., Grasmuk, H., & Drews, J. (1976) *Eur. J. Biochem. 64*, 69–75.
Olsnes, S., & Saltvedt, E. (1975) *J. Immunol. 114*, 1743–1748.
Olsnes, S., & Pihl, A. (1976) in *Receptors and Recognition Series B: The Specificity and Action of Animal, Bacterial and Plant Toxins* (Cuatrecasas, P., Ed.) pp 130–173, Chapman and Hall, London.
Olsnes, S., Fernández-Puentes, C., Carrasco, L., & Vázquez, D. (1975) *Eur. J. Biochem. 60*, 281–288.
Palmiter, R. D. (1974) *Biochemistry 13*, 3606–3615.
Roberts, B. E., & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330–2334.
Skorve, J., Abraham, A. K., Olsnes, S., & Pihl, A. (1977) *Eur. J. Biochem. 79*, 559–564.
Skup, D., & Milward, S. (1977) *Nucleic Acid Res. 4*, 3581–3587.
Sperti, S., Montanaro, L., Mattioli, A., & Stirpe, F. (1973) *Biochem. J. 136*, 813–815.
Staehelin, T., & Falvey, A. K. (1971) *Methods Enzymol. 20*, 433–446.
Wagner, A. F., Bugianesi, R. L., & Shen, T. Y. (1971) *Biochem. Biophys. Res. Commun. 45*, 184–189.

Protein–Water Interactions. Heat Capacity of the Lysozyme–Water System†

Pang-Hsiong Yang and John A. Rupley*

ABSTRACT: Calorimetric measurements of the heat capacity of the lysozyme–water system have been carried out over the full range of system composition at 25 °C. The partial specific heat capacity of the protein in dilute solution is 1.483 ± 0.009 J K^{-1} g^{-1}. The heat capacity of the dry protein is 1.26 ± 0.01 J K^{-1} g^{-1}. The system heat capacity responds linearly to change in composition from dilute solution to 0.38 g of water per g of protein (h) and is an irregular function at lower water content. The break in the heat capacity function at 0.38 h defines the amount of water needed to develop the equilibrium solution properties of lysozyme as being 300 molecules of water/protein molecule, just sufficient for monolayer coverage. The heat capacity behavior at low water content describes three hydration regions. The most tightly bound water (0–0.07 h), probably principally bound to charged groups, is characterized by a partial specific heat capacity of 2.3 J K^{-1} g^{-1}, a value close to that for ice. A heat of reaction associated with proton redistribution is reflected in the heat capacity function for the low-hydration region. Between 0.07 and 0.25 h the heat capacity increases strongly, which is understood to reflect the growth of patches of water covering polar and adjacent nonpolar portions of the protein surface. The hydration shell is completed by condensation of solvent over the weak-interacting portions of the surface, in a process displaying a transition heat.

The interactions between water and protein are generally understood to be important determinants of folding and function. Despite a large literature on protein hydration and related problems (Kuntz & Kauzmann, 1974), some important questions remain unresolved. Heat capacity measurements seem particularly well suited to probing three of these.

First, how much water is needed to fully hydrate a protein? We define the water of hydration, or the bound water, as water perturbed in its properties or that perturbs protein properties through interactions with the protein. Techniques for studying protein hydration each may see only a portion of the total bound water (Richards, 1977). Heat capacity data are likely to reflect all water important for equilibrium considerations,

† From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721. *Received December 14, 1978.* This work was supported by research funds from the American Cancer Society, the National Science Foundation, and the National Institutes of Health.

0006-2960/79/0418-2654$01.00/0 © 1979 American Chemical Society

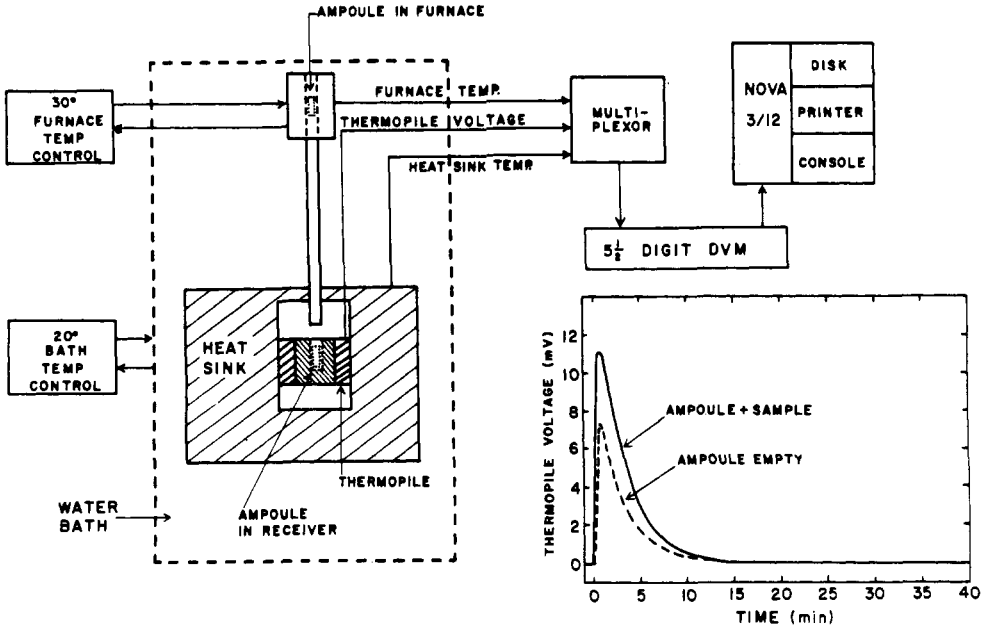

FIGURE 1: Diagram of the drop calorimeter and data acquisition assemblies. The insert at the lower right shows typical voltage–time curves.

in that variation in the heat capacity with water content comprises not only changes in the heat capacities of protein and water but also changes in all equilibria of nonzero enthalpy that depend on the extent of hydration. Heat capacity is a thermodynamic measurement, and for this reason estimates of hydration based upon it will relate most clearly to other thermodynamic properties. These include, however, many that are of general interest, e.g., folding equilibria, substrate binding, and proton ionization.

Second, what is the relationship between the properties of protein powders and the properties of proteins in dilute solution? Because the water activity and the amount of water interacting with a protein can be varied in a controlled fashion when working with protein films or powders, these have been used in a substantial number of measurements probing protein hydration. Heat capacity measurements can be made equally directly on liquid or solid samples and thus serve to link the hydrated powder and the solution state.

Finally, what is the sequence of events in the process of hydrating dry protein? The character of the change in heat capacity with system composition at low hydration is consistent with a simple picture of the hydration process.

Experimental Procedures

Protein. Lysozyme (salt free; 2× crystallized; Worthington Biochemical Corporation) was deionized by using a mixed-bed ion-exchange column, lyophilized, and stored at 5 °C. Aqueous solutions prepared from this material were of pH 8.7–9.1, owing to CO_2 absorbed subsequent to deionization.

Calorimetry. Heat capacity measurements were carried out with a drop calorimeter constructed according to Konicek et al. (1971). A diagram of the device is given in Figure 1. Furnace temperature was maintained at 30 °C, by using a modified Eurotherm temperature controller; deviations from the set temperature were within 0.001 °C over a period of 1 day, and long-term stability was only slightly worse. The calorimeter heat sink was maintained at 20 °C in equilibrium with a 100-L water bath, controlled by a Tronac Precision Temperature Controller Model PTC-40, used with a cooled heater, Model 101. The temperature of the heat sink increased slightly with each drop (0.005 ± 0.001 °C for a typical 10-cal heat flow from an ampule). Furnace and block temperatures were measured by using 2000-ohm Veco thermistors (32A47) that had been calibrated by using a Hewlett-Packard quartz digital thermometer. Thermistor resistances were converted to voltage signals by using a 0.02-mA constant-current source. The thermopiles linking the ampule receiver block and the heat sink were Cambion (800-3951-03-00-00). The voltage drops across the furnace and heat sink thermistors and the voltage signals from the thermopiles were converted to BCD, by using a 5-1/2 digit Data Precision Multimeter Model 3500. A NOVA 3/12 minicomputer was used to analyze the data, which was acquired either directly by the NOVA or with intermediate storage in an IMSAI 8080 microcomputer. Thermopile voltage was monitored at 0.5-s intervals for 40 min after the drop of the ampule from furnace to receiver. The furnace and sink temperatures were measured at 1-s intervals for 1 min before each drop, and the sink temperature was measured after each drop. The ampule was equilibrated in the furnace for about 40 minutes before a drop. An ampule was dropped repeatedly until the standard deviation of the mean of the heat flow was below 0.03%. Typical thermopile voltage signals are shown in Figure 1. The area under the thermopile voltage curve corresponds to about 10 cal for the filled ampule. The time constant of the calorimeter was 174 s. The calibration constant for the calorimeter was determined approximately weekly with water. The sample ampule was similar to the type A ampule of Suurkuusk & Wadsö (1974) and was 0.5 or 0.9 mL in volume.

Sample Preparation. Lysozyme solutions were prepared by adding water to lyophilized protein samples; 0.5 mL of a solution was added to the ampule. Hydrated solid protein samples of 0.6–0.98 weight fraction of protein were prepared by isopiestic equilibration of lyophilized protein with an H_2SO_4–H_2O mixture of appropriate water vapor pressure at 25 °C (the mean of the furnace and sink temperatures of the calorimeter). Protein samples of 0.98–1.0 weight fraction of protein were obtained by drying under vacuum over P_2O_5 at 25 °C or under diffusion pump vacuum at 60 °C. Samples of 0.2 weight fraction of protein (the solubility limit for lysozyme) to 0.6 weight fraction were prepared by adding an appropriate amount of water to a lyophilized protein sample and mixing thoroughly with a spatula. As a check on the above methods of sample preparation, hydrated solid protein samples

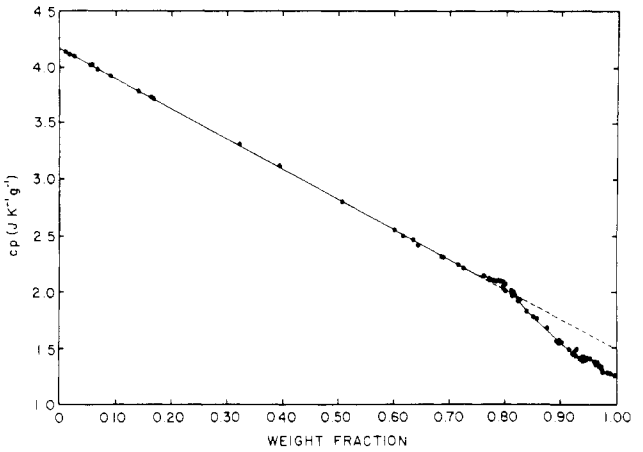

FIGURE 2: Specific heat of the lysozyme–water system from 0 to 1.0 weight fraction of protein. Least-squares analysis of the linear portion of the heat capacity function from 0 to 0.73 weight fraction of water gives $\overline{cp}_2^0 = 1.483 \pm 0.009$ J K^{-1} g^{-1}. The value of $cp_2^0 = 1.26 \pm 0.01$ J K^{-1} g^{-1}.

were prepared also by equilibrating protein solutions with appropriate H_2SO_4–H_2O mixtures, so obtaining hydrated protein films. Protein concentrations were determined, after completion of a heat capacity measurement, by drying the sample at 105 °C for 24 h.

Results

Figure 2 gives the heat capacity of the lysozyme–water system for $w_2 = 0$–1.0.[1] The median of the standard deviations of the measurements is 0.008, smaller than the symbol diameter. Thus, the linear response of the heat capacity to changes in system composition from $w_2 = 0$ to 0.73 and the irregular response between $w_2 = 0.73$ and 1.0 are significant.

It is convenient to represent the results for the low-hydration region as the apparent specific heat of the protein component, defined for a water–protein system as

$$\phi cp_2 = \frac{cp - w_1 cp_1^0}{w_2} \quad (1)$$

For an ideal binary system the specific heat is

$$cp = w_1 cp_1^0 + w_2 cp_2^0 \quad (2)$$

For a nonideal system an excess specific heat can be defined as

$$cp^{excess} = cp - (w_1 cp_1^0 + w_2 cp_2^0) \quad (3)$$

Combining eq 1 and 3,

$$\phi cp_2 - cp_2^0 = cp^{excess}[(w_1/w_2) + 1] = cp^{excess}/w_2 \quad (4)$$

Thus, the quantity $(\phi cp_2 - cp_2^0)$ is cp^{excess}, the deviation of the system from ideality, normalized to unit protein concentration.

Figure 3 gives the apparent specific heat of lysozyme, ϕcp_2, vs. the grams of water bound per gram of protein, $h = w_1/w_2$, for the range of system composition $h = 0$–0.45. In assessing the physical significance of ϕcp_2, one should keep in mind that it measures the nonideality of the system. As water is added to dry protein, the thermal properties change until the protein–water interaction reflected in cp^{excess} reaches the dilute solution limiting value, at the point of completion of the hydration process, after which cp^{excess}/w_2 remains constant. For a nonideal binary system, cp^{excess}/w_2 will be nonzero at infinite dilution, even though cp^{excess} is by definition zero for the pure component. The significance of ϕcp_2 is also brought out by the relationships between the partial specific heat capacities and the curve of Figure 3, as given by

$$\overline{cp}_2 = \phi cp_2 - h(d\phi cp_2/dh) \quad (5)$$

$$\overline{cp}_1 = cp_1^0 + d\phi cp_2/dh$$

A tangent drawn to the curve of ϕcp_2 at any value of h has a y intercept of $\overline{cp}_2$ and a slope which added to cp_1^0 gives $\overline{cp}_1$. Thus, inspection of Figure 3 shows that $\overline{cp}_1$ is constant and equal to cp_1^0, the pure water value, in region I, is less than cp_1^0 in region II, is greater in region III, and is less in region IV. The changes in cp^{excess}/w_2 parallel those in $\overline{cp}_1$. The implications of the changes in $\overline{cp}_1$ are considered in the discussion section.

Several observations relate to possible artifacts in the heat capacity measurements. First, adsorption isotherms measured for proteins show hysteresis over much of the region of hydration covered in Figure 3 [Benson & Richardson (1955); for lysozyme, see Hnojewyj & Reyerson (1959, 1961)]. Nevertheless, the lysozyme–water system is reversible in its heat capacity. The heat capacity was the same whether measured for samples of hydrated protein prepared by slowly drying a lysozyme solution to a film (data given by the square symbols of Figure 3) or by lyophilization of the solution and subsequent slow rehydration of the powder. Second, there were no systematic changes in the measured heat capacity between the first and the last drop of a sample in the set of drops constituting an experiment nor was there any difference in calorimeter response functions for solutions and hydrated protein samples (i.e., the characteristic times for the decay of the signals were the same). Thus, changes in the system reflecting the shift in temperature from that of the furnace to that of the sink (30–20 °C) must have characteristic times of less than 1 min or more than 1 h. Third, the drop in sample temperature from 30 to 20 °C can result in condensation of water vapor on the hydrated solid. This was shown not to be significant by measurements of the heat capacity made by using ampules of 0.5- and 0.9-mL volumes in the same hydration range. For example, the eight heat capacities measured between 0.60 and 0.725 h were determined half with 0.5-ml and half with 0.9-mL ampules, with no significant difference being observed. Finally, the data of Figures 2 and 3 were measured over a 1-year period by using different batches of

Table I: Modeling of Heat Capacity Results[a]

site class	water bound/protein[b]		heat capacity[c]	
region IV	0.075	60	−0.058	−830
region III	0.200	160	0.346	4950
region II	0.105	85	−0.061	−870
	0.38	305	0.227	3250
transition	**maximum and width**		**amplitude[f]**	
A	0.046[d]	37	0.045	640
	0.033[e]	26		
B	0.261[d]	207	0.053	760
	0.017[e]	13		

[a] See text for description of the function (eq 6) used in the modeling. The parameters of the function correspond to values in this table, indicated by the superscripts *b*–*f*. The two values given for each parameter correspond, in order, to a weight and a mole basis. The units of the heat capacity are J K^{-1} g^{-1} and J K^{-1} mol^{-1}, respectively. [b] w_i^{max}. [c] $\Delta\phi cp_i$. [d] h at zero of $\Delta\chi_j$. [e] σ_j. [f] $\Delta\phi cp_j$.

[1] Abbreviations used: h, grams of water per protein; w_2, weight fraction of protein; w_1, weight fraction of water; ϕcp_2, apparent specific heat of the protein solute; cp_i^0, specific heat of the pure substance i; $\overline{cp}_i$, partial specific heat of component i; $\overline{cp}_i^0$, partial specific heat of the component i at infinite dilution.

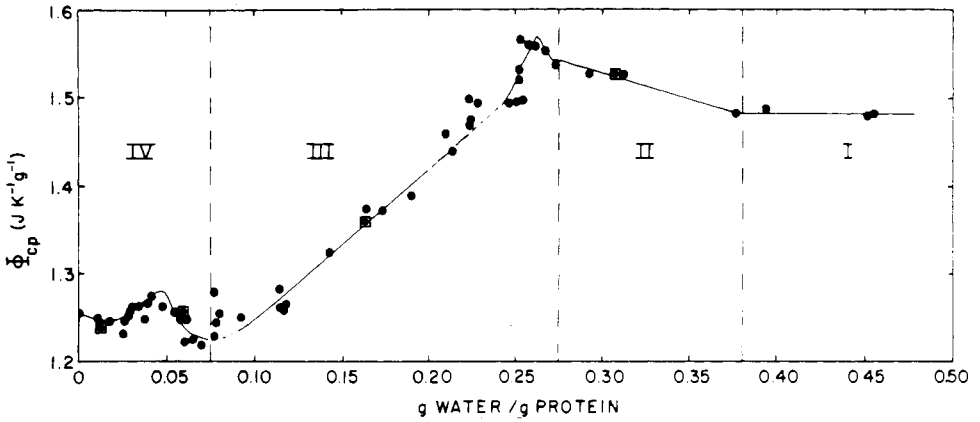

FIGURE 3: The apparent specific heat capacity of lysozyme from 0 to 0.45 g of water per g of protein. The curve is calculated by using the parameters given in Table I for a heat capacity function with four regions of linear response to ligand composition (breaks at 0.07, 0.27, and 0.38 *h*) and two transitions (or reactions) centered at 0.05 and 0.26 g of water per g of protein. The heat capacity measurements were made with lyophilized powders of lysozyme, appropriately hydrated, except for the four measurements indicated by the square symbols, for which the sample was a film formed by slowly drying a concentrated solution of lysozyme.

protein. To summarize, we feel that there are no systematic errors or artifacts affecting the data given in Figures 2 and 3.

The heat capacity function shown in Figure 3 can be viewed as a relatively smooth response to change in *h* with superimposed heat effects at near 0.25 and 0.05 *h*. Effects of this kind, i.e., a rise and a fall in the heat capacity function, almost certainly reflect heats of reaction and are analogous to those observed in scanning calorimetric measurements, in which the reaction would be, for example, denaturation of a protein or disordering of a membrane.[2] Heats of reaction make a positive contribution to the heat capacity, because this contribution originates in the temperature dependence of equilibria relating states or components of the system. A thermochemical measurement of heat capacity necessarily involves a change in temperature. If any equilibrium process of the system responds to the change in temperature, there will be absorption of heat in addition to the heat absorbed as required by the heat capacities of the system components. The following reactions might contribute to the measurements of Figure 3 for the hydrated solid protein: (a) changes in interaction at the protein–water interface, for example, transfer of water from an adsorption site of one class to one of another class that has a different enthalpy of sorption; (b) rearrangements of the structure of the hydrated solid protein, comprising changes in internal contacts, contacts between neighboring molecules, or both; (c) transfer of protons from one ionizable group to another; and (d) rearrangements entirely within the water shell surrounding the protein. Possibility (d) is unlikely, as at the compositions at which transitions were observed (Figure 3) the amount of water is insufficient for even monolayer coverage.

With the comments of the preceding paragraph in mind, we can describe the heat capacity function in terms of four regions: *region I*, dilute protein solution to 0.38 *h*, within which $\overline{cp_2}$ for the protein is constant and the heat capacity of solvent is that of bulk water; *region II*, 0.38–0.27 *h*, which includes a substantial heat of reaction at the juncture with region III and within which $\phi\, cp_2$ shows a small increase from the dilute solution value; *region III*, 0.27–0.07 *h*, within which there is a nearly linear drop in $\phi\, cp_2$ to a value slightly below cp_2^0; and *region IV*, 0.07 *h* to dry protein, which also includes a substantial heat of reaction and in which the heat capacity rises slightly to the dry protein value. A minimal model can be based on this description by assuming that regions II–IV each represent one class of water-binding sites of the protein and that there are two reactions or phase changes that contribute heat effects centered at system compositions 0.05 and 0.26 *h*. The curve of Figure 3 was calculated by using the function

$$\phi\, cp_2 - cp_2^0 = \left(\sum_i \Delta\phi\, cp_i\right)\frac{w_i}{w_i^{max}} + \sum_j \Delta\phi\, cp_j \exp[-(\Delta\chi_j\sigma_j)^2] \quad (6)$$

The first summation on the right-hand side of eq 5 comprises three terms, one for each of the regions II–IV. The quantity w_i^{max} is the amount of water required to fill the *i*th class of sorption sites, w_i is the amount of water bound to the *i*th class of sites at a given extent of hydration, *h*, and $\Delta\phi\, cp_i$ is the change in apparent heat capacity associated with filling all sites of the *i*th class. The second summation on the right-hand side of eq 6 represents the two transitions that appear in the heat capacity measurements. These heat effects are assumed to be Gaussian, $\Delta\phi\, cp_j$ being the amplitude, $\Delta\chi_j$ being the deviation in extent of hydration *h* from the midpoint, and σ_j being the breadth of the transition. The values of w_i were estimated by numerical integration. A principal assumption of this model is that the affinity constant for sites of one class does not vary with *h* and differs greatly from those for other classes. There is no assumption as to the shape of the sorption isotherm. Table I gives the parameters used in calculating the curve of Figure 3. The standard deviation of the fit was 0.012, to be compared with the standard deviation of the data, 0.013. The model is almost certainly incomplete. The dashed portion of the curve of Figure 3 at 0.1 *h* indicates that the scatter in the data is greater than experimental error, and it signals perhaps some additional event in the hydration process.

[2] The correspondence between the response of the heat capacity to change in temperature, as in a scanning calorimetric study, and to change in composition, as in this work, can be understood by using a simple example. Consider a protein denaturation process that shows a decrease in transition temperature with decrease in pH. A scanning calorimetric measurement on a sample of high pH will show a rise and fall in the heat capacity as the sample temperature passes through the transition region. A drop calorimetric experiment carried out for a sample of the same pH and at an average calorimeter temperature fixed at well below the transition region will give a heat capacity value within the base line region preceding the transition seen in the scanning experiment. A series of drop calorimetric measurements are now made over a range of pH values, at the lower end of which the fixed calorimeter temperature is well above the transition temperature for the denaturation process. In effect, the change in system composition (pH) has moved the transition region of the heat capacity vs. temperature curve by steps through the set temperature of the drop calorimeter. A rise and fall in the drop calorimetric heat capacity as a function of system composition would have been observed and can be seen to mirror the rise and fall in the scanning calorimetric experiment. The relationship between the two experiments is determined by the dependence of *K* (and ΔH) for the denaturation process on temperature and pH.

Table II: Partial Specific Heat Capacities (J K^{-1} g^{-1}) of Water and Lysozyme, in Regions I-IV[a]

I		II		III		IV		
cp_1^0	$\overline{cp}_2{}^0$	$\overline{cp}_1$	$\overline{cp}_2$	$\overline{cp}_1$	$\overline{cp}_2$	$\overline{cp}_1{}^0$	$cp_2{}^0$	ref
4.183 ± 0.003	1.483 ± 0.009	3.35	1.75	5.8	1.09	2.3	1.26 ± 0.01	present work
4.1792 ± 0.0004	1.494 ± 0.0007			5.42 ± 0.03	1.192 ± 0.005			Suurkuusk (1974)
	1.34 ± 0.09							Privalov & Khechinashvili (1974)
4.1791 ± 0.0001	1.760 ± 0.001							Millero et al. (1976)

[a] The regions are defined in the text. The cp_i values in regions II-IV were determined by use of eq 5 and the parameters of Table I. The cp_i values for region I were determined by use of least-squares analysis of data for $w_2 \leq 0.73$.

The dashed region of the curve near 0.2 *h* similarly indicates possible incompleteness of the model. The transition at 0.26 *h* is almost certainly not Gaussian, as assumed in eq 6. We emphasize that the intent of the above simple modeling is, first, to show that the apparently complex behavior of the heat capacity function in the region of low hydration can be represented by a relatively simple response with superimposed heats of reaction and, second, to serve as a framework for discussion.

In view of the importance attached to the rise and fall in the heat capacity at 0.05 and 0.26 *h*, the reliability with which the data distinguish these effects requires comment. First, the amplitude of the change in signal is 4× the standard deviation of the measurements in the dry protein range (0.013, calculated from the variation in the voltage responses during data collection). Second, data points were accumulated at high density for the ranges 0–0.07 and 0.24–0.27 *h*. The results show internal agreement to better than the above standard deviation and clearly follow a rise-and-fall pattern. Thus, we believe that the two heat effects are real features of the hydration process. An understanding of the magnitude of the reaction or transition associated with these effects can be obtained as follows. Careri et al. (1979) have concluded from IR spectroscopic studies that drying of lysozyme from a solution of neutral pH results in the reversal of the p*K* order for three carboxylate and three basic groups, so that these groups, in the carboxylate and protonated base form in solution, are carboxylic acid and free base in the dry protein. Addition of water to the dry protein in the range 0–0.1 *h* leads to normalization of the p*K* order. We believe that this proton redistribution accounts principally or perhaps entirely for the reaction heat centered at 0.05 *h* in the heat capacity measurements and that this effect therefore reflects changes in the chemistry and environment of six ionizable protein groups interacting with more than 10 water molecules. The heat effect centered at 0.26 *h* is of comparable size, but it would involve an even greater proportion of the protein surface, because of the 2–3 times smaller enthalpy of sorption of water vapor at high coverage of the surface. The transition heat contribution varies as the square of the enthalpy; thus, as many as 100 water molecules could be involved in the 0.26 *h* transition.

Table II summarizes the values of $\overline{cp}_1$ and $\overline{cp}_2$ in the four regions of the heat capacity function and compares these results with data reported previously. The values for region I (cp_1^0 and $\overline{cp}_2{}^0$) agree closely with those of Suurkuusk (1974). The difference between the higher value of $\overline{cp}_2{}^0$ obtained by Millero et al. (1976) and Suurkuusk's and our values is outside experimental error and is unexplained. The lower value of $\overline{cp}_2{}^0$ reported by Privalov & Khechinashvili (1974) was determined by using a scanning calorimeter, which precisely measures changes in heat capacity but less accurately determines the absolute value of the heat capacity. The value of cp_2^0 obtained in the present work is higher by 0.07 J K^{-1} g^{-1} than the value obtained by Suurkuusk (1974). This difference is outside the limit of experimental error. The present data are in excellent agreement with five measurements made by Suurkuusk at 0.05–0.20 weight fraction of water, but his single point at 0.02 weight fraction of water falls substantially below our data, accounting for the different extrapolated value for the pure protein component. In view of the range of composition used by Suurkuusk in determining cp_2^0, his value should be compared to our value estimated for region III. The value of Suurkuusk (1974) for $\overline{cp}_1{}^0$ was determined similarly by using data over the range 0.02–0.2 *h* and corresponds to the value obtained in the present work for region III.

Discussion

There are two principal conclusions from the heat capacity measurements of Figures 2 and 3. First, the linear heat capacity function between $w_2 = 0$ and 0.73 requires that $\overline{cp}_1$ and $\overline{cp}_2$ be invariant in this region and thus that the change in heat capacity of the system reflect simply the addition of bulk water to a fully hydrated protein. As viewed by heat capacity, therefore, the amount of water required to hydrate lysozyme is 0.38 g of water per g of protein. It is important that there is no discontinuity in the heat capacity curve at the start of the transition from aqueous solution of lysozyme to the hydrated solid at about $w_2 = 0.2$. We emphasize that the heat capacity is a particularly sensitive measure of protein and water time-average properties. Apparently, most of the bulk water about the protein can be removed without altering the heat capacity.

In order to understand this process, it is helpful to consider separately the phase change and the thermodynamic properties of the protein, the heat capacity being that of interest here. The situation is like that for protein crystals, which behave as a typical small-molecule solid phase in solubility but generally show other thermodynamic properties to be the same as found in solution, except for those properties that reflect surface regions part of lattice contacts (Rupley, 1969). Thus, the free energy of binding of a ligand can be unaffected by transfer of the protein from solution to solid phase. Analogously, the data of Figure 2 show that the heat capacity of the protein is unaffected by transfer from solution to the amorphous solid.[3] It is important that for this to be observed, the intermolecular contacts in the solid must form with $\Delta cp = 0$. This is expected for two reasons: (a) judging from protein crystals, lattice contacts are dominated by polar and charged groups, and Δcp for the formation of such interactions is small and (b) because the hydrated solid is amorphous and the lysozyme molecule has irregular shape, the extent of intermolecular contacts is expected to be less than in the crystal. In this regard, if a set of irregular parts that fit closely together are separated and then mixed randomly, the volume occupied is greater and the extent of contact between pieces

[3] Professor F. R. Salemme of this department has measured the X-ray scattering from a wet sample of hydrated lysozyme, of $w_2 \ll 0.7$, during dehydration to the limit of air drying. No specular reflections were observed at any stage of the drying nor were any observed for an air-dried film of lysozyme. Measurements were with a 1600-W copper fine-focus source.

is smaller than for the original ordering. Lysozyme in the crystal has 25–30% of the surface in 10 lattice contacts (Shrake & Rupley, 1973); if there are two to three contacts in the gel, one would estimate 5–10% occlusion of the surface. The nature of the solid phase is not important for the present arguments. Specifically, it is immaterial whether essentially all the protein in the sample for compositions $w_2 > 0.2$ is part of the amorphous solid phase, whether the solid phase entraps the solution phase, the amount of which diminishes as w_2 increases, or whether there are a sequence of solid phases between $w_2 = 0.2$ and 0.73. However, the simplest picture of the hydrated amorphous solid is a loose aggregate of protein molecules, an open gel, that, as solvent is removed, shrinks without significant change in protein conformation or intermolecular contacts.

The second principal conclusion from the heat capacity results is that the complex response of the function in the region below 0.38 h (a) reflects the interactions between several classes of protein sites and water and (b) contains contributions from reactions or changes in state that occur within narrow ranges of the low-hydration region (centered at 0.05 or 0.26 h). The fact that regions of the heat capacity function can be distinguished means that the hydration process must be viewed as a sequence of at least three events; i.e., within each of the regions I–III, essentially all protein molecules of the solid sample must be undergoing the same changes in thermodynamic properties as water is added. If this were not the case, i.e., if the hydration process were all or none, if in the hydration process water molecules were bound randomly at sites about the protein surface, or if the partially hydrated solid protein sample were heterogeneous in structure, then a monotonic response of the heat capacity to water added below 0.38 h would be expected, which is contrary to observation. For example, an all-or-none hydration process, in which protein molecules would be either essentially fully hydrated or unhydrated, would show a linear change of ϕc_{p_2} from the value for the dry protein to that for the solution. The change in slope of the function ϕc_{p_2} vs. h and the rise and fall of the function at 0.05 and 0.26 h indicate that the water bound at the different steps of the hydration process is different either in environment or in effect on protein properties.

The heat capacity behavior observed for lysozyme may be obtained generally for proteins. Bull & Breese (1968) have carried out a similar study of the heat capacity of ovalbumin over the full range of system composition. They observed a linear response of the heat capacity from dilute solution to $w_2 = 0.75$. The data of Bull & Breese (1968) were not sufficiently accurate to define nonlinearity in the low-hydration region. Suurkuusk (1974) measured the heat capacity of three proteins (lysozyme, ovalbumin, and chymotrypsinogen) in solution, $w_2 = 0$–0.25, and in the region of low hydration, $w_2 = 0.8$–0.98. In each case he saw some deviation from linearity in the region of low hydration and linear or nearly linear response for the solution range. Horn & Mennie (1935) determined the heat capacity of gelatin gels from 0.4 to 4.4 h. They observed a reaction heat at 0.5 h like the effect seen with lysozyme at 0.26 h. The response of the heat capacity function to composition was linear above 1.0 h. In this regard, gelatin and collagen are more heavily hydrated than most proteins, as determined by a variety of methods (Kuntz & Kauzmann, 1974). Haly & Snaith (1968) measured the heat capacity of wool from 0 to 0.7 h. The low-hydration region was nonlinear and a break was observed at 0.4 h. The range of $c_{p_2}^0$ values is 1.23–1.29 J K^{-1} g^{-1} for the following proteins: lysozyme [this work & Suurkuusk (1974)], insulin (Hutchens et al., 1969), chymotrypsinogen (Suurkuusk, 1974), ovalbumin (Bull & Breese, 1968; Suurkuusk, 1974), gelatin (Horn & Mennie, 1935), collagen (Haly & Snaith, 1971), and wool (Haly & Snaith, 1968). Values of $\overline{c_{p_1}}$, corresponding to that for region III as defined in this work, range from 4.6 to 5.8 for the above proteins. To summarize, the results obtained for lysozyme fit well with heat capacity results for other protein–water systems.

The estimate of full hydration from the heat capacity measurements, 0.38 h, can be compared with estimates for lysozyme made by using other approaches. Only hydrodynamic methods give a higher estimate of the hydration, 0.34–0.52 h (Kuntz & Kauzmann, 1974). The amount of nonfreezing water is 0.34–0.35 g of water per g of protein determined by NMR (Kuntz & Kauzmann, 1974; Hilton et al., 1977). About 0.25 g of water per g of protein has been located at the surface of the lysozyme molecule by X-ray diffraction (Imoto et al., 1972), and a similar value, 0.24 g of water per g of protein was obtained by analysis of preferential hydration (Cox & Schumaker, 1961).

The amount of water bound at full hydration as indicated by heat capacity can be compared with minimum and maximum estimates of the amount of water in a monolayer covering the protein surface (Richards, 1977). The minimum estimate is 320 molecules, calculated for covering a sphere of the mass and density of lysozyme with water molecules of radius 1.6 Å. The maximum estimate can be based upon the surface exposed to solvent, calculated according to the methods of Lee & Richards (1971) or Shrake & Rupley (1973), which give a surface approximately twice that of the equivalent sphere, i.e., one requiring ca. 600 water molecules for monolayer coverage. The latter estimate is a maximum because it does not take into account invaginations of the protein surface for which one water molecule covers a region greater than its projection upon a surface of lower curvature. Contacts between molecules of the lyophilized protein would reduce only slightly the amount of surface available for interaction with water.

There are several points to be brought forward, considering the comments of the preceding paragraphs and the fact that the heat capacity reflects essentially all equilibrium chemistry. First, the estimate from the heat capacity of 0.38 g of water per g of protein is likely to be as true an estimate as can be made of the hydration level required to obtain the dilute solution thermodynamic properties of the protein. In this regard, the heat capacity is sensitive to interaction of water with nonpolar elements of the surface, which should be the most weakly interacting portions. Kinetic properties of the protein may not exhibit dilute solution behavior at 0.38 h, as indicated by the generally larger amounts of bound solvent estimated from hydrodynamic measurements (Kuntz & Kauzmann, 1974) and by the increases in enzymatic activity of lysozyme observed above 1 g of water per g of protein (Yang and Rupley, unpublished experiments). Second, the 300 molecules of water per protein molecule required to bring ϕc_{p_2} to the dilute solution value are at most just sufficient for monolayer coverage. Thus, multilayer phenomena need not be considered, and equally importantly, the monolayer water shell about the protein must mesh well with the bulk solvent. The small number of water molecules required to cover the surface perhaps reflects local ordering of the hydration shell through interaction with polar surface atoms of the protein. The polar atoms represent about half of the protein surface and are distributed relatively randomly. The average distance between polar sites is 4–5 Å, which is that between nonhydrogen-bonded neighbors in water. Such locally ordered arrangements should mesh well with the bulk solvent.

Features of the heat capacity function in the region of low

hydration correlate well with features of the sorption isotherm. Many proteins, including lysozyme (Hnojewyj & Reyerson, 1961), exhibit a "knee" in the isotherm at 0.05 h, which presumably reflects the hydration process within region IV and the heat of reaction contribution to the heat capacity at 0.05 h. Maxima in the heat of sorption as a function of extent of hydration have been observed at low hydration for several proteins [e.g., for lysozyme, Hnojewyj & Reyerson (1961)].[4] The heat of sorption in the region of low hydration (region IV) is about twice the heat for sorption at higher coverages and decreases in region III to equal the heat of vaporization of water. The sharp upturn in the sorption isotherm at 0.8–0.9 relative humidity corresponds to the transition heat at the juncture of regions II and III of the heat capacity function.

Hill (1949) has given a statistical mechanical treatment of the localized adsorption of interacting molecules onto a heterogeneous surface. Calculations were carried out for an adsorbent with a linear distribution of site energies, the lower limit being zero, and for adsorbate–adsorbate interaction energy of an intermediate value. This model exhibited the following characteristics: at low coverage the bound adsorbate is dispersed about the surface; as coverage increases, the bound adsorbate undergoes a two-dimensional phase change, from a state of dispersal about the surface to a condensed phase of clusters of adsorbate molecules; as coverage increases further, the clusters grow until the surface is nearly covered and only the weakest sites remain open; and condensation of adsorbate over these regions completes the adsorption process.

The heat capacity results obtained in this work, like other thermodynamic measurements, do not give information on structure. It therefore is of interest that a protein–water system resembles the Hill model described above, in that (a) adsorption energies range from ca. 20 kcal for strongly interacting polar or charged sites to near zero for covering nonpolar sites and (b) the average adsorbate–adsorbate interaction energy would be several kilocalories. The Hill model provides a satisfactory framework for understanding the heat capacity changes with hydration. The first water molecules to interact with dry protein are bound at charged sites (Leeder & Watt, 1974) and thus would be dispersed about the protein surface. The value of $\overline{cp}_1$ in region IV is approximately that for ice, an observation consistent with solvent arrangements lacking configurational freedom. Infrared measurements (Careri and Gratton, personal communication) show that the transition heat observed at 0.05 h in the heat capacity exactly parallels a proton redistribution process, in which protons are transferred from carboxylic acid to basic groups of the protein. The change in sign of the derivative of the heat capacity function at 0.07 h can be understood, in the context of the Hill model, to reflect the surface water concentration becoming sufficiently high for clusters to form. In this regard, at the transition between regions IV and III, there is scatter in the heat capacity data, perhaps reflecting a two-dimensional condensation process. The clusters can be viewed as mobile arrangements centered on polar or charged protein atoms and increasing in size or number with added water. The strong increase in the heat capacity from 0.07 to 0.27 h thus would mark a configurational contribution to the heat capacity originating in temperature-dependent cluster rearrangements, analogous to what one obtains in bulk water. Above 0.2 h, with polar sites being largely saturated, the condensation of water over the remaining unfilled portions of the surface, presumed to be principally weakly interacting nonpolar regions, is reflected in the transition heat observed in the heat capacity at 0.26 h and corresponds to the high-coverage condensation in the Hill model.

The heat capacity results bear upon the relationship of the partially hydrated protein to the protein in dilute solution. This relationship is important because there is a substantial literature on the hydration of films and powders and because it is desirable to vary water activity when studying protein–water interactions. Three points follow from the heat capacity results. (a) The invariance of ϕcp_2 above 0.38 h, i.e., after completion of the hydration process as seen by the heat capacity, means that the protein at 0.38 h does not differ significantly in thermodynamic properties from the protein in dilute solution. Thus, studies on solid samples of hydration level greater than 0.38 bear directly on the dilute solution state. Also, the heat capacity results show that, with regard to understanding the thermodynamics of water–protein interactions, attention should be focused on the chemistry within the monolayer of solvent contacting the protein.

(b) It is possible that there are changes in protein structure during the process of hydrating dry protein to 0.38 h. One picture (Kuntz & Kauzmann, 1974) is dehydration below about 0.3 h results in substantial conformational changes that allow the protein molecules to interact and to pack more closely; the driving force for these changes would be the need to replace the lost solvent–protein interactions, so as to avoid the creation of "voids", unsatisfied hydrogen-bonding sites, etc. If this view is correct, powder studies are not interpretable without a detailed understanding of the changes in structure. It is therefore significant that the display in the heat capacity results of stages in the hydration process indicates that, if the conformation changes with hydration, the conformation states are distinguishable and thus should be describable.

(c) Several measurements bear on the protein conformation above 0.1 h, i.e., in regions II and III of Figure 3. The IR spectroscopic properties of lysozyme (Careri et al., 1979) change continuously above 0.1 h, and equally importantly, the amide I′ band shows the changes expected for hydration of carbonyl groups and does not show the changes expected for alteration of secondary structure; the enzymatic activity of lysozyme is detectable at 0.2 h (Yang and Rupley, unpublished experiments); the partial specific volume of β-lactoglobulin in the crystal remains constant at the dilute solution value with dehydration to between 0.1 and 0.2 h (Richards, 1977); the melting temperatures for β-lactoglobulin (Rüegg et al., 1975) and for lysozyme (Fujita & Noda, 1978) increase above the dilute solution value with decreasing hydration below about 0.5 h, suggesting that the protein becomes locked into the dilute solution conformation, perhaps because of the kinetics of denaturation, as the solvent is removed. The above facts seem sufficient to exclude changes in conformation involving more than perhaps 20% of the backbone during hydration from 0.1 to 0.38 h, and in our view the changes are likely smaller, comparable to the restrictions on surface side-chain movement associated with crystallization. In any event, if the changes in structure associated with the hydration process are limited in extent, then measurements of this process, by using IR spectroscopy, heat capacity, etc., define aspects of the water–protein interaction that can be expected to persist through hydration to the dilute solution, such as the strength of the interaction of surface elements with the water, the organization of water at the surface, and the mobility of water

[4] A reaction heat contribution to the heat capacity centered at ca. 0.05 h from a process such as proton transfer would be paralleled by a contribution to the heat of sorption. The heat of proton transfer from carboxylic acid to amino groups is expected to be negative and thus would result in a more negative heat of sorption. Also, the function describing the dependence of the heat of sorption on h changes with temperature according to the dependence of the heat capacity on h (Berezin et al., 1973).

within regions of the surface layer.

Acknowledgments

We are grateful to Louis Dall'Aglio for machining the calorimeter assembly, to Dr. Bonner Denton for his advice on the calorimeter–computer interface, and to Dr. Giorgio Careri, of the University of Rome, Dr. Enrico Gratton, of the University of Illinois, and Dr. F. R. Salemme, of the University of Arizona, for numerous stimulating discussions. A North Atlantic Treaty Organization research grant (1336) made possible a collaboration between Drs. Careri, Gratton, Salemme, and ourselves that led to the picture of the hydration process used above in interpreting the heat capacity results. Dr. Walter Kauzmann has made thoughtful and much appreciated comments, which have produced a more intelligible discussion of these results.

References

Benson, S. W., & Richardson, R. L. (1955) *J. Am. Chem. Soc. 77*, 2585–2590.
Berezin, G. I., Kiselev, A. V., & Sinitzyn, V. A. (1973) *J. Chem. Soc., Faraday Trans. 1 69*, 614–619.
Bull, H. B., & Breese, K. (1968) *Arch. Biochem. Biophys. 128*, 497–502.
Careri, G., Giansanti, A., & Gratton, E. (1979) *Biopolymers* (in press).
Cox, D. J., & Schumaker, V. N. (1961) *J. Am. Chem. Soc. 83*, 2439–2445.
Fujita, Y., & Noda, Y. (1978) *Bull. Chem. Soc. Jpn. 51*, 1567–1568.
Haly, A. R., & Snaith, J. W. (1968) *Biopolymers 6*, 1355–1377.
Haly, A. R., & Snaith, J. W. (1971) *Biopolymers 10*, 1681–1699.
Hill, T. L. (1949) *J. Chem. Phys. 17*, 762–771.
Hilton, B. D., Hsi, E., & Bryant, R. G. (1977) *J. Am. Chem. Soc. 99*, 8483–8490.
Hnojewyj, W. S., & Reyerson, L. H. (1959) *J. Phys. Chem. 63*, 1653–1654.
Hnojewyj, W. S., & Reyerson, L. H. (1961) *J. Phys. Chem. 65*, 1694–1698.
Horn, W. R., & Mennie, J. H. (1935) *Can. J. Res. 12*, 702–706.
Hutchens, J. O., Cole, A. G., & Stout, J. W. (1969) *J. Biol. Chem. 244*, 26–32.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665–868.
Konicek, J., Suurkuusk, J., & Wadsö, I. (1971) *Chem. Scr. 1*, 217–220.
Kuntz, I. D., & Kauzmann, W. (1974) *Adv. Protein Chem. 28*, 239–345.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.
Leeder, J. D., & Watt, I. C. (1974) *J. Colloid Interface Sci. 48*, 339–344.
Millero, F. J., Ward, G. K., & Chetirkin, P. (1976) *J. Biol. Chem. 251*, 4001–4004.
Privalov, P. D., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.
Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151–176.
Rüegg, M., Moor, U., & Blanc, B. (1975) *Biochim. Biophys. Acta 400*, 334–342.
Rupley, J. A. (1969) *Biol. Macromol. 2*, 291–352.
Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351–371.
Suurkuusk, J. (1974) *Acta Chem. Scand., Ser. B 28*, 409–417.
Suurkuusk, J., & Wadsö, I. (1974) *J. Chem. Thermodyn. 6*, 667–679.

Cooperative Binding of a Platinum Metallointercalation Reagent to Poly(A)·Poly(U)†

Jacqueline K. Barton and Stephen J. Lippard*

ABSTRACT: The cationic complex (2-hydroxyethanethiolato)(2,2′,2″-terpyridine)platinum(II), [(terpy)Pt(HET)]$^+$, binds cooperatively to poly(A)·poly(U) by intercalation. The melting temperature of poly(A)·poly(U) in low-salt buffer is increased by 6 °C in the presence of [(terpy)Pt(HET)]$^+$, indicating stabilization of the duplex structure by the bound platinum reagent. Viscosity measurements provide evidence for comparable lengthening of the polynucleotide in the presence of [(terpy)Pt(HET)]$^+$ and the intercalating dye, ethidium bromide. Scatchard plots of the binding of [(terpy)Pt(HET)]$^+$ to poly(A)·poly(U) and poly(I)·poly(C), determined through ultracentrifugation pelleting methods, show large positive curvature, reflecting the strong cooperativity associated with the platinum complex–RNA interaction. The characteristics of the binding isotherms are interpreted in terms of a model where cooperative pair units of [(terpy)Pt(HET)]$^+$ intercalate into the double-stranded polymer. At saturation, two platinum molecules are bound for every three base pairs. This stoichiometry may be compared with the nearest-neighbor-exclusion binding observed previously in the interaction of [(terpy)Pt(HET)]$^+$ and the ethidium cation with DNA, in which one intercalator occupies every other interbase-pair site at saturation. The striking differences observed in the interaction of [(terpy)Pt(HET)]$^+$ with DNA and RNA suggest that drug recognition is sensitive to the constraints imposed by nucleic acid secondary structure.

There have been extensive studies of the binding of aromatic heterocyclic dyes (Figure 1) to duplex DNA. The primary mode of interaction, first proposed by Lerman (1961), involves intercalation, where the cationic dye inserts between adjacent base pairs of the polynucleotide. The changes in physical properties that accompany the binding of dyes to double-stranded RNAs have also been interpreted in terms of an intercalation model (LePecq & Paoletti, 1967; Waring, 1961; Aktipis & Martz, 1974; Schmechel & Crothers, 1971; Zama

† From the Department of Chemistry, Columbia University, New York, New York 10027. *Received January 10, 1979.* This work was supported by National Institutes of Health Research Grant CA-15826 from the National Cancer Institute and by a National Science Foundation Predoctoral Fellowship to J.K.B.

0006-2960/79/0418-2661$01.00/0 © 1979 American Chemical Society