Binding Isotherms of Nucleosides and Polynucleotides Measured by Continuous Ultrafiltration at Constant Volume: A Rapid and Precise Technique†

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ABSTRACT: Equilibrium binding isotherms have been measured as a function of temperature for the interaction of 2- and 8-aminoadenosine with poly(U) by continuous, constant-volume ultrafiltration. An analytical method is described for determining the entire isotherm in a single experiment on a preequilibrated ligand–polymer solution. The method is shown to produce the same results as classical equilibrium dialysis but to be much more rapid and convenient. Values of the enthalpy, equilibrium constant, and free energy of stacking as a function of temperature are derived from the binding data. Stoichiometry of interaction is obtained from the diafiltration profiles as well as from the binding isotherms. Over the temperature range studied, the cooperativity of binding is observed to increase with rising temperature.

One of the most fundamental and important processes in biological reactions and control mechanisms is reversible binding of small ligands to macromolecules such as proteins and nucleic acids. For this reason, binding studies have long been a powerful tool in biochemical studies. Traditionally direct quantitative measurement of binding is done by equilibrium dialysis. The disadvantage of this method is that it is laborious and slow, since a whole series of experiments at different equilibrium concentrations must be performed to construct a single binding isotherm. The slowness of the process precludes use of labile material, and the batch nature of the method makes it often wasteful and costly. Various modifications of the process have been used to reduce the time and material required, but even with these changes, much of the tedium and difficulty remain. Another direct method of determining binding parameters is ultrafiltration (Blatt et al., 1968; Ryan & Hanna, 1971; Cantley & Hammes, 1973). Sophianopoulos et al. (1978) have shown that contrary to the usual belief, batch ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out. Cantley & Hammes (1973) have used an ultrafiltration technique, which they call "forced dialysis", and showed the method gives precision comparable to gel filtration or equilibrium dialysis.

A constant-volume method of continuous ultrafiltration, termed diafiltration, was first described by Blatt et al. (1968). We show here that it is possible to obtain a complete binding isotherm from a single solution by diafiltration. The apparatus used to maintain constant volume in the ultrafiltration cell and related mathematical analysis for the wash-in method were first described by Blatt et al. (1968) and have been applied to binding studies by other workers (Crawford et al., 1972). In this method, a solution of ligand is forced under pressure into a closed and magnetically stirred diafiltration cell containing a solution of the macromolecule. The bottom of the cell consists of a semipermeable membrane from which the effluent is conducted to a fraction collector. Since each unit volume of effluent is replaced by an equal volume of solution from the reservoir, the volume in the cell remains essentially constant (the effect of small deviations from constancy is discussed below).

A potential disadvantage of this method of diafiltration (termed the wash-in method) is that it may not reflect a true equilibrium process, particularly if the binding kinetics are slow compared to the filtration rate. This problem can be avoided by measuring equilibrium desorption of a preequilibrated ligand-macromolecule complex. Constant volume is maintained in this case by adding buffer or salt solution rather than ligand from the reservoir. This "wash-out" method was mentioned in the original diafiltration paper (Blatt et al., 1968), but it was not actually used, and the necessary equations were not given. We should note that the converse of the above problem would arise in cases of extremely strong binding, for which the on rate is fast and the off rate is slow. For such cases, the wash-in method would be preferable.

We have developed the procedure and necessary equations for the preequilibration or wash-out method and have recently applied them to a specific problem in polynucleotide stoichiometry (Howard et al., 1981). In this paper we describe the preequilibration method and show with chemically well-defined systems that it combines speed, simplicity, and precision in obtaining binding isotherms and the thermodynamic parameters derived from them.

The ligands used are purine nucleosides, which are known to interact with pyrimidine polynucleotides in discrete integral ratio's to form regular helices with standard complementary base pairs (Howard et al., 1964, 1966a,b; Huang & Ts'o, 1966; Davies & Davidson, 1971; Damle, 1972; Hattori et al., 1976). 2-Aminoadenosine has previously been shown to form complexes of 1:2 stoichiometry with poly(U) (Howard et al., 1966).

Since each system studied here represents a single, chemically well-defined binding equilibrium, it reveals more clearly some characteristics of diafiltration than previously reported protein binding systems involving multiple equilibria. The comparison of diafiltration with equilibrium dialysis in these simpler systems shows complete agreement rather than the significant discrepancies reported and discussed previously for the more complex protein systems.

Methods

We used an Amicon Model 8MC stirred cell ultrafiltration apparatus (Figure 1) with microvolume accessory and made necessary modifications of the commercial equipment. The stirring assembly was replaced with a simple stopper. A magnetic stirring bar resting on a stainless steel screen fitted in a groove cut out at the base of the small cylindrical insert did the stirring. The membrane was on the base plate below the screen. The insert, which forms the actual ultrafiltration cell, was held in position by two O-rings, at the top and bottom,

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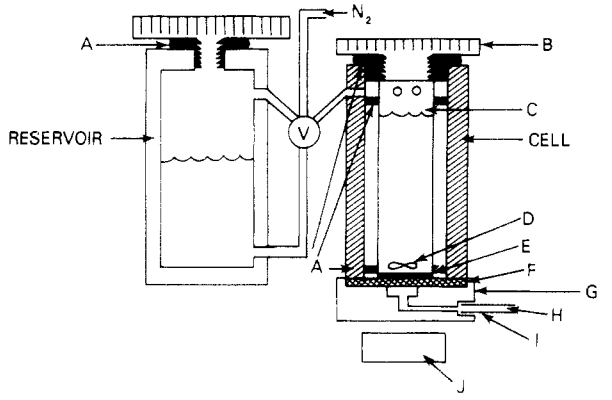

FIGURE 1: Schematic view of constant volume diafiltration apparatus (A, O-rings; B, cell cap; C, microvolume insert; D, magnetic stirring bar; E, stainless steel screen to support bar; F, membrane; G, base; H, tubing to fraction collector; I, effluent port; J, magnetic stirrer).

which prevented seepage of solution into the annular space between the body of the apparatus and the insert. The apparatus was set up in an insulated box, with necessary connections being made through snug-fit holes in the walls of the box. Three side walls were lined with copper coils, and the box had a double-glass window in the front wall and a removable insulated top. Water from a Lauda RC3 bath was circulated through the copper coils to maintain constant temperature inside the box. A thermistor was inserted in the body of the apparatus and connected to a digital thermometer. Before each run, 10–12 h were usually allowed for equilibration of temperature, which then could be maintained within ±0.1 °C. A fine-bore (0.51-mm) thick-walled plastic tube fitting snugly in the filtrate port of the apparatus served as outlet tube, the other end of which was connected to an LKB fraction collector. In this way the void volume between the membrane and the analyzer was kept small compared to the fraction volume to prevent mixing of fractions inside the collection line and to improve accuracy of mathematical analysis.

In a typical run, the ultrafiltration cell was filled with 2.9 mL of ligand–polymer complex solution, and the reservoir contained the same solvent in which the solution was made. The free end of the outlet tube (H, Figure 1) was closed with a hemostat. Pressure from a nitrogen cylinder was then applied via a valve to both the reservoir and the cell simultaneously for a few seconds. The valve was then switched so that the pressure was only on the reservoir. The pressure was then increased slightly to force the solvent from the reservoir up the connecting channel to the top of the cell. At this point, the hemostat was released, and the solvent started passing from the reservoir to the cell. For each volume of solvent entering the cell, an equal volume of solution containing unbound ligand in equilibrium with bound ligand is filtered out through the membrane and is collected in discrete fractions of equal volume for analysis. The volume of solution inside the cell thus remains constant, permitting concentrations of components to be calculated, as described below, from analysis of collected fractions. A typical run required 3–6 h.

Theory

Blatt et al. (1968) gave the equation for variation of effluent concentration with effluent volume in a wash-out experiment as

$$\ln C_0/C = (V - V')/\bar{V}_0 \qquad (1)$$

where C_0 is initial total ligand concentration in the cell, C is the concentration of ligand in the effluent, $\bar{V}_0$ is the average sample volume in the cell during the run (equal to V_0, the initial sample volume, if no change occurs), V is the cumulative filtrate volume, and V' is the apparent void volume of the system. Since in a given experiment V' and $\bar{V}_0$ are constant, a plot of $\ln C_0/C$ against V gives a straight line, the slope of which gives $\bar{V}_0$. This semilog plot, called a diafiltration profile, is, in agreement with previous reports (Blatt et al., 1968), a single straight line for diafiltration of simple solutes. When binding occurs to a macromolecule in the cell, however, we find, in contrast to previous reports, that the profile is quite different, as described later. For calculation of bound ligand in the preequilibration or wash-out method, we have formulated the following equation from a simple material balance. In the absence of binding or rejection of ligand by the membrane

$$L_b = V_0C_0 - \sum_1^n V_nC_n - \bar{V}_0C_{fn} \qquad (2)$$

where L_b is the total moles of ligand bound after removal of the nth fraction, V_n is the fraction volume, C_n is the measured concentration of ligand in nth fraction, and V_0, $\bar{V}_0$, and C_0 are as defined above. C_{fn}, the concentration of free ligand inside the cell at the end of collecting the nth fraction, can be evaluated as follows. During diafiltration, if fractions collected are of infinitesimally small volume, the ligand concentration in the nth fraction (C_n) can be equated to the free ligand concentration inside the cell (C_{fn}) at that moment, i.e., $C_n = C_{fn}$. When fractions of finite volume are collected instead, the above condition is no longer valid, as the ligand concentration inside the cell decreases continuously during wash out. If all fractions have precisely the same volume, however, and are sufficiently small, C_{fn} can be related to C_n as

$$C_{fn} = (C_n + C_{n+1})/2 \qquad (3)$$

A derivation is provided in the supplementary material (see paragraph at end of paper). Application of these equations is presented in a subsequent section.

We discuss below experimental procedures and sources of error [see also Blatt et al. (1968)]:

(a) Membrane behavior: for the theory of diafiltration to be applicable, the membrane used should be completely retentive for the polymer and completely nonretentive for the ligand. One must therefore choose the membrane carefully and check for membrane retention and binding. We used PM 10 (Millipore) membrane with a cutoff value of M_r 10000. Previously dialyzed poly(U), used in this study, did not show any leakage through this membrane. Retention of ligand by the membrane can occur either because of binding of ligand to the membrane or retardation of filtration by finite solute rejection or reflection at the membrane surface. Equation 2 can reveal such binding or rejection and provide an estimate of its magnitude when a wash-out experiment of the simple ligand solution is carried out. L_b should be zero for every fraction in absence of binding or rejection, while a finite positive value would give the extent of such interaction. In the latter case, comparison of diafiltration profiles of single ligand and ligand–polymer complex solutions, under the same conditions, would permit a point by point correction to be made for these factors. In the present study, with simple ligand solutions, the calculated quantity of total ligand (sum of second and third terms in eq 2), for every fraction, did not differ more than 2% from the actual amount put in (first term in eq 2), and hence no correction was made. Another effect of significant rejection of ligand would be that $\bar{V}_0$ calculated from the diafiltration profile would be considerably larger than the initial volume V_0. In our case, these apparent increases were

within 3%, showing that rejection is not a significant problem. Retardation of ligand filtration can also occur in diafiltration of polymer–ligand complex due to polarization of polymer depositing on the membrane surface. This was avoided by working under low pressure (~20 psi) and continuous stirring of solution during the run.

(b) Fluctuation of cell volume, fraction volume, and void volume: Application of the above equations requires that the cell volume should remain constant and be precisely known. In all continuous diafiltration experiments with the apparatus described here, small fluctuations of cell volume occur. The magnitude of such fluctuation depends on the dead volume above the solution in the cell. The best way to avoid this problem is to use cells filled to capacity and with no gap between solvent meniscus and solution (cf. Ryan & Hanna, 1971). Design of the Amicon apparatus, however, did not permit us to fill the cell completely. Modification of the apparatus as described above helped to minimize fluctuation to a negligible value, if not eliminate it. Since fluctuations are random, the final essential result contains a random error in evaluation of the amount of bound ligand with eq 2. If fluctuations are small, random errors are also small, permitting a good fit to the data points. In any event, the diafiltration profile permits evaluation of the average volume $\bar{V}_0$ even if small fluctuations occur. The problem of the void volume is that V' in Blatt's equation is an apparent void volume and is different in different runs. Ideally, as eq 1 predicts, the best way to avoid this problem is to devise an inline analysis (making V' almost equal to zero) so that no correction for contributions of void volume to fraction volume is necessary. Equation 1 also shows that this contribution is insignificant when cummulative fraction volume V is very large compared to V', i.e., toward later fractions. In application of eq 2, however, the void volume does not pose any problem if there is no mixing of solute in the collection system. Otherwise a small correction term is necessary, which again will be important only in the first few fractions. We tried to avoid this correction by using a small void volume (<100 μL) compared to the fraction volume (583 μL or more). The fraction volume was precisely determined by weighing.

(c) Leakage at the filtrate port: Though the outlet tube (H, Figure 1) is snugly fitted into the outlet port (I, Figure 1), minor leakage can occur at this junction under certain conditions of operation. If the tube H is kept closed with a hemostat for a considerable period of time after the system has been pressurized, visible leakage occurs at I. When there is no clamp on H or when the clamp is removed promptly after pressurizing the system, no leakage is observed. Proper manipulation can thus prevent leakage or keep it to a negligible value. The effect of an initial leak is to cause an apparent upward shift of the isotherm. Even if such a leak does occur, however, values of the calculated parameters (stoichiometry, midpoint, and slope of binding isotherm) are not affected, as shown in the supplementary material.

We see in Figure 3 that an isotherm obtained from a run in which no leakage occurred is completely coincident with a second isotherm in which the lower plateau of a shifted curve (caused by the initial leakage discussed above) is brought into register with the base line of the first curve. All parameters referred to above are the same for both runs.

Results and Discussion

In Figure 2 we show representative diafiltration profiles for the 8-aminoadenosine–poly(U) system. The graph is not a single straight line as Blatt et al. (1968) postulated but instead has three distinct linear portions. We interpret the profile as

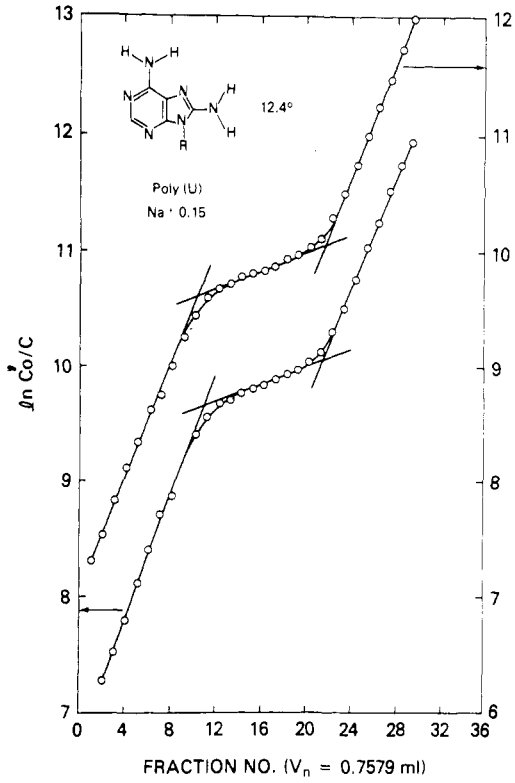

FIGURE 2: Diafiltration profiles for the 8-aminoadenosine–poly(U) interaction of replicate runs. Excess ligand is washed out along the left limb of the curves, the complex dissociates along the middle limb, and dissociated ligand is eluted along the right limb. The average cell volume $\bar{V}_0$ is determined from the slopes of the left and right limbs, and the stoichiometry is determined by the distance between the intersections of the line extensions (cf. text). For convenience of scaling, C_0 in eq 1 has been multiplied by 1000 and expressed as C_0'.

follows. The first linear segment describes wash out of excess ligand present. As soon as the ligand concentration passes below a critical concentration, the complex starts dissociating. The middle segment describes this progressive dissociation and elution. When all the complex has dissociated, the sample has only free ligand in solution, which washes out along the third linear segment. We consider such a diafiltration profile to be characteristic of cooperative binding. For weakly cooperative processes, the breaks should be less sharp and the middle part less pronounced. It is unlikely that resolution into discrete steps can be observed in cases of multiple binding equilibria, particularly if the binding is weak. We assume that the data of Blatt et al. (1968; binding of anions to serum albumin, Figure 7), for which a single straight line was drawn through somewhat scattered points, represents such a case of overlapping multiple equilibria. With a single, discrete equilibrium, as in Figure 2, however, the characteristic nature of the profile immediately gives us a clear indication of the cooperative process and a simple procedure for determining stoichiometry of binding. Since every fraction is analyzed for its ligand content, the difference between the total amounts of ligand filtered out at the two inflection points gives the total amount of bound ligand. The inflections are best determined by the intersections of extensions of the linear portions. In this way we obtain a value very close to 0.5 for ligand per residue of poly(U). Parallel slopes of the first and third linear segments of the graphs in Figure 1 indicates negligible variation of sample volume.

Binding isotherms calculated by using eq 2 are shown in Figures 3 and 4. In evaluating $\bar{V}_0$ from eq 2, we used the third linear segment of the diafiltration profile. $\bar{V}_0$ values thus calculated were within 3% of the initial volume V_0.

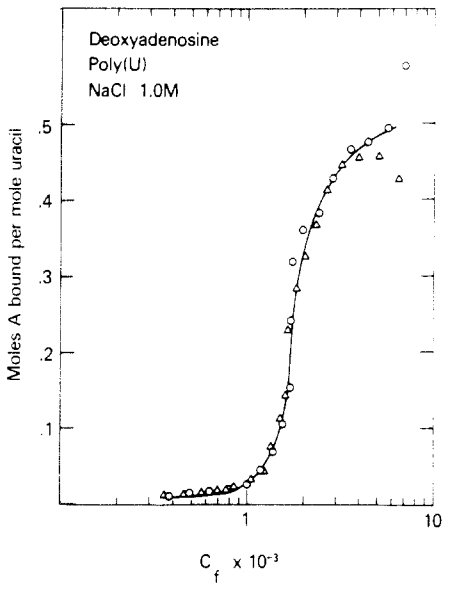

FIGURE 3: Binding isotherm for the deoxyadenosine–poly(U) system, obtained by diafiltration using eq 2 and 3. The symbols ○ and △ represent replicate runs, but in the first of these, the lower plateau had an ordinate value of 0.18. It has been shifted to the plateau of the second curve to show that the isotherms are coincident and give the same values of C_m, slope, and derived parameters. These values from the runs shown here agree well with those of Davies & Davidson (1971) measured by equilibrium dialysis.

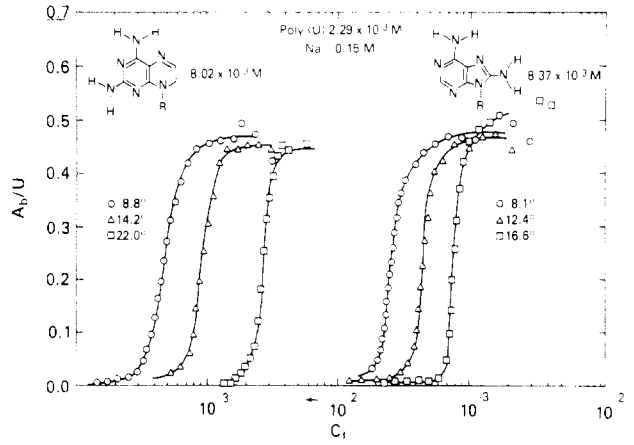

FIGURE 4: Binding isotherms of 2-aminoadenosine and 8-aminoadenosine with poly(U), measured by constant volume diafiltration at the indicated temperatures. The change of slope with temperature is discussed in the text.

At the outset of diafiltration, there is an initial decrease in cell volume, presumably due to a holdup volume of 85 μL below the membrane. This initial decrease combined with the problem of void volume, as discussed above, caused a scattering of the first few points in the binding isotherm (upper plateaus, Figure 3). Using sufficient excess of ligand, nevertheless, permitted us to reproduce well-defined top and bottom plateaus of the isotherm, which clearly define the stoichiometry.

Validity and precision of our method was examined by comparing our results with those of the detailed equilibrium dialysis study of the deoxyadenosine–poly(U) system, reported by Davies & Davidson (1971). These authors found that for the deoxyadenosine–poly(U) reaction (5 °C, pH 7.1, 0.15 M Na^+), the midpoint of the isotherm was well characterized and reproducible [C_f at $\theta = 0.5$ is $(1.83 \pm 0.1) \times 10^{-3}$], but the slope ($d\theta/d \ln C_f$ at $\theta = 0.5$) showed much greater variation in replicate determinations (standard deviation of six determinations 25%; highest and lowest values are 1.81 and 0.9). Our results in Figure 2 show that at the midpoint of the isotherm in two different runs, $C_f = 1.77$ and 1.8×10^{-3}, in excellent agreement with the value from equilibrium dialysis. The isotherm exhibited saturation at 0.5 mol of dA/mol of U, demonstrating 1:2 stoichiometry. The slope of the curve we estimate to be 2.1, somewhat larger than the reported mean value of 1.43 ± 0.3. In Figure 4 we show the binding isotherms for 2-aminoadenosine and 8-aminoadenosine with poly(U) at different temperatures. All the curves are sigmoid, indicating a cooperative process. They exhibit saturation at 0.5 mol/mol of U, demonstrating 1:2 stoichiometry, as has been found by infrared spectroscopy for 2-aminoadenosine (Howard et al., 1966a,b).

Table I

material	temp (°C)	$C_{\theta=0.5}$ ($\times 10^3$ M)	$\frac{d\theta}{d \ln c}$ at $\theta = 0.5$ [a]	Q (M^{-1})	q (M^{-1})	W (kcal M^{-1})
2-NH_2A	8.8	0.48	1.44 ± 0.03	2083	63.1	−2.0
	14.2	0.895	1.73 ± 0.09	1117	23.2	−2.2
	21.0	2.13	2.36 ± 0.2	469	5.2	−2.6
	22.0	2.70	2.48 ± 0.3	370	3.8	−2.7
8-NH_2A	8.1	0.265	1.87 ± 0.09	3773	67.7	−2.2
	12.4	0.457	2.33 ± 0.08	2186	25.2	−2.5
	16.0	0.710	2.82 ± 0.08	1408	11.1	−2.8

[a] Values from Davies & Davidson (1971).

Pertinent equations for analysis of binding isotherms, which have been applied to monomer–polymer interaction (Davies & Davidson, 1971; cf. Damle, 1972; Hill, 1973; Gukovskaya et al., 1980), are

$$C_m = (1/q) \exp[w/(kT)] \text{ at } \theta = 0.5 \quad (4)$$

$$Q = 1/C_m \text{ at } \theta = 0.5 \quad (5)$$

$$\left[\frac{d\theta}{d \ln c}\right]_{\theta=0.5} = \frac{\exp[-w/(2kT)]}{4} \quad (6)$$

where C_m is the concentration of free ligand, θ is the degree of saturation, q and Q are the equilibrium constants for binding of a monomer onto an isolated site and onto a site adjacent to an occupied one, respectively, and w is the corresponding difference in free energies. The results thus obtained are given in Table I. It is clear that these compounds bind much more strongly than adenosine (for adenosine, $Q = 550$ M^{-1} at 5 °C and 1.0 M NaCl). It is expected that the ability to form an extra hydrogen bond and hence a favorable enthalpy contribution should make the binding of the 2- and 8-aminoadenosines stronger. From the data it appears, however, that extra free energy for binding also comes from a stronger stacking tendency of these amino derivatives compared to adenosine. In spite of the same number of H bonds involved in both cases, interestingly, 8-aminoadenosine shows a stronger and more cooperative binding than 2-aminoadenosine. This could be due to either stronger hydrogen bonding of 8-NH_2 hydrogens or greater free energy of stacking of 8-NH_2 derivatives. The difference for these two nucleosides is probably not one of hydrogen bonding, since both these compounds have similar q values, while w, which is a measure of stacking free energy, is greater for 8-aminoadenosine. It must be pointed out here that in this treatment of binding data, as stated above, C_m and Q are the most accurately determined parameters, while q and w, which are estimated from the slope of the isotherm, are subject to larger error.

From eq 4 it follows (Davies & Davidson, 1971) that

$$\frac{d \ln C_m}{dT} = -\frac{d \ln q}{dT} + \frac{d}{dT}[w/(kT)] \quad (7)$$

$$= -(\Delta H_0 + \Delta H_w)/(RT^2) = -\Delta H_t/(RT^2) \quad (8)$$

Table II: Thermodynamic Data for Monomer-Polymer Complexes

complex		ΔH_t (kcal/M)	ΔS_t (eu/M)	ΔH (kcal/M)
poly(U):2-NH_2A [b]	pH 7.0, 0.15 M Na^+	−21.4 ± 0.8	−60.6	−15.8 [a]
poly(U):8-NH_2A	pH 7.0, 0.15 M Na^+	−19.9 ± 0.5	−51	
poly(U):2-NH_2-adenine	pH 7.0, 0.1 M Na^+	−19.3 ± 0.6 [c]	−53 [c]	−15.9 [a]

[a] Values from Scruggs & Ross (1970). [b] A, adenosine. [c] Values from Davies & Davidson (1971).

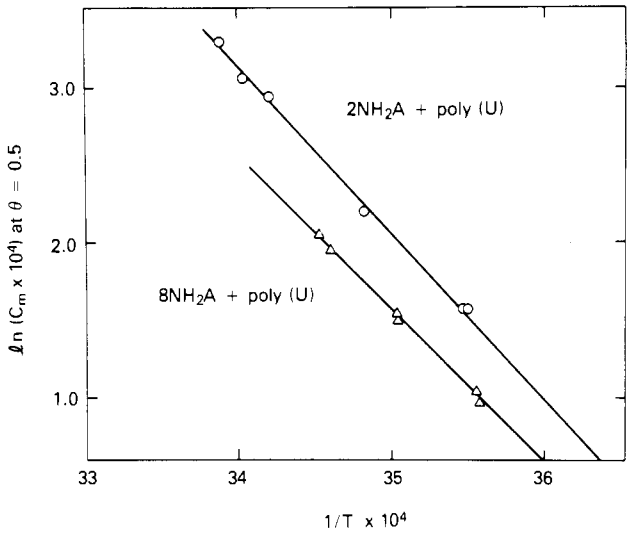

FIGURE 5: van't Hoff plots of data obtained from Figure 4. Enthalpies calculated for the overall reactions are −21.4 and −19.9 kcal/mol for the 2-amino- and 8-aminoadenosine systems, respectively.

where ΔH_0 is the enthalpy of binding a monomer to an isolated site and ΔH_w is the enthalpy of transferring a monomer from an isolated site to a site adjacent to an occupied one, so that ΔH_t is the total enthalpy of cooperative binding of a monomer (Table II).

The value of ΔH_t for the overall reaction can be obtained from a van't Hoff plot (Figure 5) of $-\ln Q$ (or $\ln C_m$) vs. $1/T$ using the data of Figure 4. These data represent the first measurements by a direct equilibrium binding method of monomer–polymer complex formation at different temperatures, though derived binding isotherms have been calculated from spectroscopically observed melting curves. The values are −21.4 and −19.9 kcal/mol for the 2- and 8-aminoadenosine reactions, respectively. The 2-aminoadenosine value, like the temperature coefficient value of Davies & Davidson (1971), is larger than the calorimetric value of Scruggs & Ross (1970). ΔH_0 can be estimated from a plot of $-\ln q$ (Table I) vs. $1/T$, though these are less reliable because of the uncertainty in q noted earlier. The values are −35.7 and −38.8 kcal/mol for 2- and 8-aminoadenosine, respectively. The corresponding values of ΔH_w are then 14.4 and 18.8 kcal/mol. These positive values for ΔH_w are a consequence of the experimentally observed fact that the slope of the isotherm (or cooperativity of binding) increases significantly and progressively with temperature for both the aminoadenosines. The values of the slopes at $\theta = 0.5$ are 1.4, 1.7, 2.4, and 2.5 for the 2-aminoadenosine interaction at 8.8, 14.2, 21, and 22 °C, respectively. A similar trend is observed with 8-aminoadenosine (Table I). The effect of self-association of the monomers, with its inverse temperature dependence, would be in the observed direction, though probably of much smaller magnitude. Thus, for example, if we assume a dimerization constant for 2-aminoadenosine at 9 °C of 100 M^{-1}, the extent of dimerization at $\theta = 0.5$ would be only 8%, and when corrected for this reduction in C_f, the slope becomes 1.72, compared to the observed 1.4. Departure from the observed value because of dimerization would become progressively smaller with increasing temperature as K_D diminished with T. Since reported self-association constants (Ts'o & Chan, 1964; Broom et al., 1967; Solie & Schellman, 1968) for nucleotides and purines fall in the range 6–15 M^{-1}, the above calculation is probably conservative and suggests that self-association is not the major cause of the observed temperature dependence of the slope (we estimate, in fact, that the self-association constant would need to have an improbably large value, such as $\sim 10^4$, at 8 °C to produce the observed effect).

We may note that a micelle formation tendency, dependent largely upon the strong self-association of water molecules, is associated with a positive enthalpy and entropy. While the bases, with many polar atoms, are not hydrophobic in the sense of hydrocarbon residues (Scruggs et al., 1972), differences in solvation of bases in the stacked and unstacked states on the polymer lattice may contribute to the observed results.

We have shown in the foregoing sections that constant-volume diafiltration of preequilibrated ligand–polymer complexes possesses major advantages over classical equilibrium dialysis in obtaining equilibrium binding isotherms but that the same information is obtained. We have also discussed experimental problems and sources of error in both methods.

Supplementary Material Available

Derivation of eq 2 and 3 and calculated parameter values (5 pages). Ordering information is given on any current masthead page.

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Binding of Mercury(II) to Poly(dA-dT) Studied by Proton Nuclear Magnetic Resonance†

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ABSTRACT: The binding of Hg(II) to poly(dA-dT) has been examined with proton NMR spectroscopy. Addition of $HgCl_2$ between r (Hg^{2+}/nucleotide) = 0 and 0.25 results in loss of the exchangeable imino N3H resonance of thymine, indicating preferential binding at this site. The nonexchangeable base resonances AH8, AH2, and TH6 shift their intensity downfield in a cooperative manner, indicating complexation which is slow on the NMR time scale and changes in the polymer conformation upon binding. At r = 0.25, the polymer is cross-linked, and an increase in temperature does not result in denaturation of the polymer, as evidenced by the thymine proton resonance chemical shifts. The chemical shifts of the AH2 and T(CH_3)5 base resonances allow some general conclusions to be made about the stereochemistry of this complex.

Metal ions exert a wide range of effects on the structure and biological properties of nucleic acids. These effects are seen to be closely tied to the manner in which the particular ion concerned binds to nucleic acids. [For reviews, see Barton & Lippard (1980) and Marzilli et al. (1980) inter alia.] Of particular interest has been Hg(II) because of its specific binding to the DNA bases as opposed to the more common phosphate backbone binding (Thomas, 1954). This has led to its use as a probe of DNA structure both kinetically (Williams & Crothers, 1975) and statically, as in viral (Katz & Santilli, 1962; Dorne & Hirth, 1970) and chromatin complexes (Simpsom & Sober, 1970; Bryan et al., 1976; Ding & Allen, 1980a). Furthermore, Hg^{2+} has been used to separate DNAs of differing base composition (Nandi et al., 1965; Wang et al., 1965; Davidson et al., 1965), while the related CH_3Hg^+ ion is used as a standard method for denaturing both RNA and DNA (Gruenwedel & Davidson, 1966).

The binding of Hg(II) to DNA is poorly understood at a detailed molecular level. It has been determined that the preferred mononucleoside binding sites are N3 of thymidine and N1 of guanosine, with several weaker sites available in adenosine and cytosine (Simpson, 1964; Eichorn & Clark, 1963). However, these preferences may not pertain in the polymeric situation where stereochemical factors may also be important. Furthermore, there has been little progress in understanding the effects of Hg(II) binding on the local conformation of DNA though it is known to result in a large change in its viscosity (Katz, 1952).

A suitable method for monitoring the effects of metal binding in nucleic acids would appear to be proton NMR spectroscopy, particularly when the sequence is repetitive and individual resonances can be clearly resolved and assigned. This is the case for poly(dA-dT) (Patel & Canuel, 1976), which has also been examined for its Hg(II) binding spectrophotometrically (Yamane & Davidson, 1961; Katz, 1963; Nandi et al., 1965) and so is a good candidate for critical study. This paper reports the analysis of Hg(II) binding to poly(dA-dT) as monitored by proton NMR. Binding at the N3 site of thymidine is confirmed, and we are led to suggest a variant of the model of Katz (1963) to explain the data.

Materials and Methods

Poly(dA-dT) was purchased from Sigma and dialyzed extensively against 10 mM Tris-HCl (pH 7) and water and then concentrated by lyophilization. Thermal denaturation of this polymer monitored by proton NMR gave results essentially identical with those obtained previously (Patel & Canuel, 1976), except that the T_m was 57 °C in the present conditions. Poly(dA)·poly(dT) was obtained from Collaborative Research, Waltham, MA, and was similarly dialyzed and lyophilized.

Proton NMR titrations were performed in 0.01 M NaCl-O_4-0.1 M sodium phosphate buffer (pH 8.0) and spectrophotometric titrations were performed in this and in 0.01 M $NaClO_4$-0.01 M sodium cacodylate buffer (pH 7.1). The nonexchangeable proton spectra were taken in D_2O solutions. The D_2O was purchased from Wilmad (99.8%) and vacuum distilled to remove divalent ions before use.

Proton NMR spectra were taken on a Bruker WH-360 spectrometer operating at 360 MHz in the Fourier-transform mode (nonexchangeable resonances) or in the correlation mode (exchangeable resonances). Temperature control was effected by the Bruker regulator operating on a continuous flow of air. Areas under resonances were determined gravimetrically by using a linearly extrapolated base line. Chemical shifts were measured relative to internal DSS (sodium 4,4-dimethyl-4-silapentanesulfonate).

Spectrophotometric changes were monitored on a Perkin Elmer Model 552 recording spectrophotometer at room temperature.

Results

Spectrophotometry of the Binding of $HgCl_2$ to A-T Containing Polymers. In Figure 1 is shown the binding of Hg^{2+}

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