Phase Partition Studies of Actinomycin–Nucleotide Complexes†

Pari Davanloo and Donald M. Crothers*

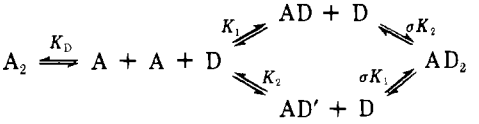
ABSTRACT: A method is reported for measuring the stoichiometry of complex formation between actinomycin and a series of deoxynucleotides. The amount of bound actinomycin is measured by distribution of the drug between two liquid phases, a buffer phase containing deoxynucleotide and an organic phase in which the nucleotide is insoluble. Using simple statistical mechanical analysis, the equilibrium equations for several models of actinomycin–deoxynucleotide complexes have been derived: actinomycin with one binding site, with two equivalent independent binding sites, and with two sites which must be occupied together. The binding of actinomycin C_3 with dpG, dpApG, dpA, and dpGpC has been examined and compared with these models. It is found that binding to dpG and dpApG involves two independent binding sites of nearly equal affinity for nucleotides, whereas binding of dpGpC to the two binding sites on actinomycin is a cooperative process. Binding of dpA to actinomycin is partially cooperative and weaker than binding of dpG. The dimerization constant of actinomycin was also determined by the phase separation technique, and found in agreement with other values, including the results of kinetic measurements reported here.

Actinomycin C_3 is an antibiotic that binds to double-helical DNA and selectively inhibits RNA synthesis (Kirk, 1960; Kersten et al., 1960; Reich et al., 1961); its interactions with nucleotides and nucleic acids have been the object of frequent study (Kahan et al., 1963; Reich and Goldberg, 1964; Cavalieri and Nemchin, 1964; Gellert et al., 1965; Cerami et al., 1967; Müller and Crothers, 1968; Wells and Larson, 1970; Sobell and Jain, 1972; Krugh, 1972; Schara and Müller, 1972; Patel, 1974a,b; Krugh and Neely, 1973a,b). In spite of all this work, some simple questions concerning actinomycin–nucleotide interactions remain in dispute. Of particular concern to us has been the stoichiometry of the complex with mono- and oligonucleotides, and the separate binding constants in cases where there are multiple binding sites for nucleotide. An illustrative example is the binding of actinomycin to deoxyguanosine, or its monophosphate d(pG). Optical spectroscopic titrations have uniformly indicated a 1:1 complex between d(pG) and actinomycin (Behme and Cordes, 1965; Gellert et al., 1965; Crothers et al., 1968; Schara and Müller, 1972), but the NMR studies of Krugh and Neely (1973a,b) indicate that as nucleotide is added spectral changes continue to occur up to a ratio of 2 nucleotides/actinomycin. This was interpreted as indicating a 2:1 complex, as also found in the crystal (Sobell and Jain, 1972).

There are reasons why the spectroscopic methods used to study the binding reaction could lead to different conclusions, and we therefore took the view that a purely thermodynamic method was required to resolve the disagreement. We found a solvent system suitable for investigation of the partition of actinomycin between an aqueous phase containing nucleotide which solubilizes actinomycin and an organic phase in which the nucleotide is insoluble. (Our choice of solvents was influenced by a comment on unpublished experiments in the paper of Gellert et al., 1965.) The phase partition method is analogous to equilibrium dialysis, and permits conclusions about total binding, not just the portion that produces a particular spectroscopic effect (Waring, 1975). We present also the necessary theory for using the measurements to test various models for the equilibrium binding reaction, and for determining actinomycin binding and dimerization constants.

Theory

We consider a general scheme for actinomycin–nucleotide interactions in which actinomycin (A) has as many as two binding sites for the nucleotide (N):



K_D allows for actinomycin dimerization, K_1 and K_2 are the binding constants for attachment of a single nucleotide to either site 1 or site 2 separately, and σ allows for cooperative or anticooperative effects when a second nucleotide is bound.

The partition function of actinomycin in the organic phase is taken proportional to its concentration C_A^{org}

$$Q_A^{org} = Q_0 C_A^{org}$$

(This equation assumes that the activity coefficient of actinomycin is unity in the organic phase solution, an assumption that must be checked by an independent experiment.) The partition function of dilute actinomycin monomer in the aqueous phase is therefore

$$Q_A^{aq} = K_P Q_0 C_A^{org}$$

where K_P is the actinomycin partition coefficient between the phases in the limit of zero concentration

$$K_P = \lim_{C_A \to 0} K_P'$$

K_P' is the apparent partition coefficient, a function of concentration,

$$K_P' = \frac{C_A^{0,aq}}{C_A^{org}}$$

and $C_A^{0,aq}$ is the total concentration of actinomycin in the aqueous phase.

To calculate the partition function Q^{aq} for actinomycin summed over all bound states in the aqueous phase, we employ

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received March 29, 1976.* Supported by a Grant CA 15583 from the National Cancer Institute, United States Public Health Service.

a form commonly used for the binding of ligands to polymers (Crothers, 1971)

$$Q^{aq} = Q_A{}^{aq} \sum_i \exp(-\Delta G_i/RT)$$

where ΔG_i is the free energy of the actinomycin complex in state i relative to the free actinomycin monomer. The free energy change on binding a molecule at activity a' to a site with binding constant K' is

$$\Delta G' = -RT \ln K' - RT \ln a'$$

Summation over all the states allowed in the model for the binding reaction yields

$$Q^{aq} = Q_A{}^{aq} [1 + K_D a + (K_1 + K_2)m + \sigma K_1 K_2 m^2] \quad (1)$$

where a is the actinomycin monomer activity and m the activity of free nucleotide.

The total concentration $C_A{}^0$ of actinomycin in an aqueous phase containing nucleotide and in equilibrium with an organic phase containing $C_A{}^{org}$ is made up of the contribution of the monomer C_A, dimer C_D, and nucleotide complex C_C:

$$C_A{}^0 = C_A + C_D + C_C$$

The excess solubility due to addition of the nucleotide, C_C, is readily determined experimentally, as is the dimer concentration (see below). We define the quantity g as the fraction of nondimerized actinomycin which is bound to nucleotide:

$$g = \frac{C_C}{C_A{}^0 - C_D}$$

This ratio can easily be calculated from the experimental data.

The ratio g, obtained from the partition function in eq. 1 modified to exclude the dimer state, is

$$g = \frac{(K_1 + K_2)m + \sigma K_1 K_2 m^2}{1 + (K_1 + K_2)m + \sigma K_1 K_2 m^2} \quad (2)$$

The nucleotide activity m in eq 2 will be taken equal to the free concentration, assuming that complex formation is the only source of nonideality. The quantity m is not directly measured in the partition experiment, but it can be obtained by solution of eq 2:

$$m = \frac{-(K_1 + K_2)}{2K_1 K_2 \sigma} + \tfrac{1}{2}\left[\frac{(K_1 + K_2)^2}{K_1{}^2 K_2{}^2 \sigma^2} + \frac{4g}{(1-g)K_1 K_2 \sigma}\right]^{1/2} \quad (3)$$

Another expression for m is needed to eliminate it as a variable. Let the total nucleotide concentration be $C_N{}^0$, with concentration of bound nucleotide expressed by $C_N{}^b$. We define r as the ratio of the bound nucleotide concentration to the nondimerized actinomycin concentration:

$$r = \frac{C_N{}^b}{C_A{}^0 - C_D}$$

Hence $$m = C_N{}^0 - C_N{}^b = C_N{}^0 - r(C_A{}^0 - C_D) \quad (4)$$

Again excluding the dimer state, and taking the derivative $r = (\partial \ln Q/\partial \ln m)$, we obtain

$$r = \frac{(K_1 + K_2)m + 2K_1 K_2 \sigma m^2}{1 + (K_1 + K_2)m + K_1 K_2 \sigma m^2} \quad (5)$$

Equations 2 (or 3), 4, and 5 contain the variables r, m, and g, and can, in principle, be solved for those quantities if trial values of the constants K_1, K_2, and σ are assumed. The general method of data analysis is to test whether a single set of constants reproduces the experimental value of g for all $C_N{}^0$ and $C_A{}^0$. With three constants we can expect considerable latitude in adjusting them, however, and turn instead to simple limiting cases described by a single parameter to test which models can be excluded.

One Binding Site. In this case $K_2 = 0$, and we find (eq 2 and 5)

$$g = \frac{K_1 m}{1 + K_1 m} = r$$

Combining with eq 4 and rearranging,

$$g(1-g)^{-1}[C_N{}^0 - g(C_A{}^0 - C_D)]^{-1} = K \quad (6)$$

The left hand side of this equation contains only experimental quantities, and can be plotted against g. A horizontal line implies a constant value of $K = K_1$, and hence that the data can be fitted to the model of one binding site.

Two Identical Independent Sites. In this case $K_1 = K_2 = K$, and $\sigma = 1$. Then, combination of eq 3, 4, and 5 yields

$$\{(1-g)^{-1/2} - 1\}\{C_N{}^0 + 2(C_A{}^0 - C_D)[(1-g)^{1/2} - 1]\}^{-1} = K \quad (7)$$

Again, the left hand side contains only experimental quantities, and can be plotted against g to test for constancy of K.

Two Site, Obligatorily Cooperative Model. In this case, the only complex form considered has 2 nucleotides bound/actinomycin. Hence $\sigma \gg 1$ and we can replace $K_1 K_2 \sigma$ by K^2. Eq 2, 4, and 5 yield

$$g^{1/2}(1-g)^{-1/2}[C_N{}^0 - 2g(C_A{}^0 - C_D)]^{-1} = K \quad (8)$$

This equation is analogous to eq 6 and 7.

Two-Parameter Models. As will be seen, the data for binding actinomycin to all nucleotides except d(pGpC) fit the two independent site models, described by a single binding constant. Even though two separate binding constants cannot be determined, it is of interest to ask how different K_1 and K_2 can be without causing the model to be in disagreement with the results. We set

$$K_1 = K$$
$$K_2 = \alpha K$$

with $\sigma_1 = \sigma_2 = 1$. Routine but tedious algebra leads to the following result of combining eq 3, 4, and 5: let the function $f(\alpha, g)$ be

$$f = \frac{-(1+\alpha)}{2\alpha} + \tfrac{1}{2}\left[\left(\frac{1+\alpha}{\alpha}\right)^2 + \frac{4g}{\alpha(1-g)}\right]^{1/2} \quad (9)$$

Then

$$\left\{C_N{}^0 - (C_A{}^0 - C_D)\left[2g - \frac{(1+\alpha)f}{(1+f)(1+\alpha f)}\right]\right\}^{-1} f = K \quad (10)$$

Equations 9 and 10, which reduce to eq 7 when $\alpha = 1$ and to eq 6 when $\alpha = 0$, are employed by assuming trial values of α and plotting the quantity K as a function of g.

The other two-parameter model of interest is that with partially cooperative binding. We let $K_1 = K_2$, with

$$K^2 = K_1{}^2 \sigma$$

and denote by f the function

$$f = -\sigma^{-1/2} + [\sigma^{-1} + g/(1-g)]^{1/2} \quad (11)$$

Upon combining eq 3, 4, and 5 we find

$$[C_N{}^0 - 2f(f + \sigma^{-1/2})(1 + 2\sigma^{-1/2} f + f^2)^{-1} \times (C_A{}^0 - C_D)]^{-1} f = K \quad (12)$$

These two equations are used analogously to eq 9 and 10. When $\sigma = 1$, they reduce to equation 7, and when $\sigma \to \infty$ they are

equivalent to equation 8.

Dimerization. Dimerization is conveniently measured in the absence of nucleotides. Then

$$K_P' = Q^{aq}/Q_A{}^{org} = Q_A{}^{aq}(1 + K_D a)/Q_0 C_A{}^{org}$$

which, with $Q_A{}^{aq} = K_P Q_0 C_A{}^{org}$, is

$$K_P' = K_P(1 + K_D a)$$

Assuming that $a = K_P C_A{}^{org}$ (unit activity coefficient of the monomer in both phases), we obtain

$$K_P' = K_P(1 + K_P K_D C_A{}^{org}) \quad (13)$$

Experimental Procedure

Materials. The aqueous phase in all experiments was BPES buffer (0.008 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.18 M NaCl, 0.001 M Na_2 EDTA,[1] pH 6.8). The actinomycin C_3 was given to us by Dr. W. Müller. Hexanes and 1-chloroheptane were purchased from Fisher Scientific Co. The deoxydinucleotides were purchased from Collaborative Research, Inc., and 2′-deoxyguanosine 5′-monophosphate, d(pG), and 2′-deoxyadenosine 5′-phosphate, d(pA), were purchased from Sigma Chemical Co.

All concentration measurements were performed in a 1-cm path length cell on a Cary Model 14 spectrophotometer. Deoxydinucleotide concentrations were measured by their absorption at 260 nm using the extinction coefficients provided by Collaborative Research, Inc. The concentration of actinomycin in 1:1 mixtures of hexane and 1-chloroheptane was determined using the extinction coefficient: ϵ in 1:1 organic mixture = 28379 M^{-1} cm^{-1} at λ_{max} = 445 nm, T = 25 °C.

All glassware was leached in a cleaning solution containing dichromate in concentrated sulfuric acid to remove detergents, because traces of detergent cause the two phases to become turbid.

Methods. Actinomycin is soluble in a 1:1 mixture of 1-chloroheptane and hexane. To measure dimerization, a series of actinomycin solutions with different concentrations in BPES buffer was prepared, and equal volumes of a 1:1 mixture of 1-chloroheptane and hexane (organic phase, top phase) were added to each. The samples were left at a constant temperature of T = 7.1 °C for 30 min, and the two phases were then mixed by shaking the tubes vigorously for four 30-s intervals during a 5-min period. Samples were left in the bath for 5 h to allow the phases to separate. The total concentration of actinomycin in each phase was measured spectrophotometrically. Since actinomycin forms a relatively stable dimer in aqueous solution (Crothers et al., 1968), dilutions to a concentration of 0.03 mM were made before measuring the actinomycin absorbance in order to determine the concentration in the buffer phase (bottom phase). During shaking, actinomycin is distributed between the two phases according to the distribution law $K_P' = C_A{}^{0,aq}/C_A{}^{org}$, where $C_A{}^{org}$ is the concentration of actinomycin in organic phase and $C_A{}^{0,aq}$ is the total concentration of actinomycin in the buffer phase.

We used the same procedure for measuring the amount of solubilized actinomycin in the presence of deoxydinucleotide. In this experiment a series of dinucleotide solutions were prepared with different concentrations ranging from 0.1 to 4 mM. To these solutions the same total actinomycin concentration and an equal volume of organic solvents were added, followed by the phase separation procedure given above. Deoxydinucleotides, because of their charge, remain in the buffer phase, as confirmed by spectral measurements on the organic phase.

[1] Abbreviation used: EDTA, (ethylenedinitrilo)tetraacetic acid.

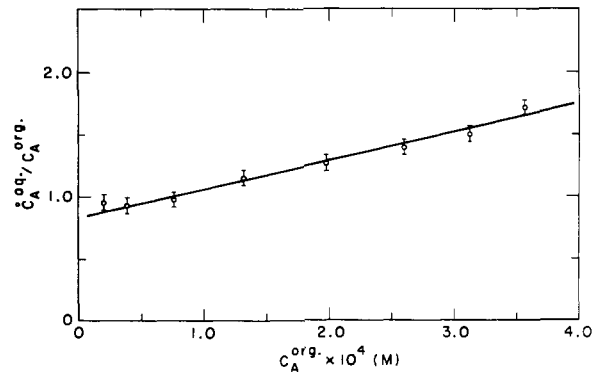


FIGURE 1: Variation of the partition ratio $K_P' = C_A{}^{0,aq}/C_A{}^{org}$ with actinomycin concentration $C_A{}^{org}$ in the organic phase. Using eq 13, we find a dimerization constant of 3.2×10^3 M^{-1} at 7.1 °C.

The total actinomycin concentration in the presence of dinucleotide was measured at the isosbestic wavelength of the actinomycin-nucleotide complex (455 nm), using the extinction coefficient ϵ_{455} = 19126 M^{-1} cm^{-1}, T = 25 °C.

Since solubilization in the presence of nucleotide is due to the formation of dimer as well as bound actinomycin in the buffer phase, the concentration of dimer must be subtracted in order to obtain the bound actinomycin concentration. The amount of aqueous phase dimer in equilibrium with a given concentration of actinomycin in the organic phase was determined in the dimerization experiments in which nucleotide was not added.

Results and Discussion

Calculation of Actinomycin Dimerization Constant from the Phase Separation Experiment. We checked the assumptions of the phase separation method by measuring the dimerization constant for actinomycin. Figure 1 shows the ratio $C_A{}^{0,aq}/C_A{}^{org}$ plotted vs. $C_A{}^{org}$. A dimerization constant $K_D = 3.2 \times 10^3$ M^{-1} was calculated from this plot, using eq 13.

Calculation of the Dimerization Constant from Kinetic Studies. We also investigated the mechanism of dimer formation by using the temperature jump relaxation kinetic method. The relaxation experiments show one relaxation time in the range of a few tens of microseconds. Application of standard relaxation methods to the equation

$$2A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2$$

yields for the relaxation time τ,

$$1/\tau^2 = 8k_{12}k_{21}C_A{}^0 + k_{21}{}^2 \quad (14)$$

Figure 2 shows a plot of $1/\tau^2$ against $C_A{}^0$, yielding a dimerization equilibrium constant of $(2 \pm 1) \times 10^3$ M^{-1}.

Table I summarizes the actinomycin dimerization constant determined by several different methods. We conclude from comparison of the results in Table I that the phase separation technique measures dimerization accurately, and therefore that the assumptions made to derive the phase partition equations, including the assumed lack of appreciable association in the organic phase solution, are justified.

Investigation of One-Parameter Binding Models for the Interaction of d(pG), d(pApG), d(pA), and d(pGpC) with Actinomycin. Solubilization of actinomycin in the aqueous phase by nucleotides was measured as a function of nucleotide and actinomycin concentration. The quantity g, the ratio of actinomycin concentration in nucleotide complex to the total nondimerized actinomycin concentration, was calculated. The

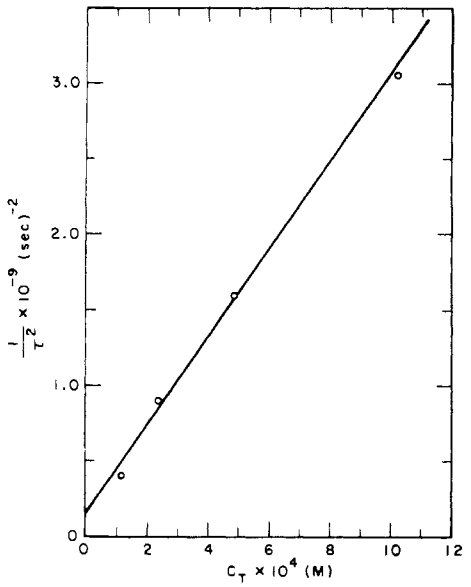


FIGURE 2: Square of the reciprocal relaxation time for actinomycin dimerization vs. concentration. Equation 14 yields $k_{21} = 1.3 \times 10^4$ s^{-1} and $k_{12} = 2.7 \times 10^7$ M^{-1} s^{-1}, with $K_D = k_{12}/k_{21} = (2 \pm 1) \times 10^3$ M^{-1}, T = 7.1 °C.

TABLE I: Equilibrium Constant for Actinomycin Dimerization.

T (°C)	K_D, M^{-1}	Method
7.1	2.0×10^3	Relaxation kinetics
7.1	3.2×10^3	Phase partition method
5	3.6×10^3	Equilibrium centrifugation
20	9.0×10^2	(Crothers et al., 1968)
4	2.7×10^3	Proton magnetic resonance
18	1.4×10^3	studies (Angerman et al., 1972)

data are shown tested against the three one-parameter models in Figure 3a–d; g vs. log K plots will be horizontal lines if the data fit the model. In each figure, curve 1 (x) refers to the model with two independent binding sites (eq 7), curve 2 (Δ) to the model with one binding site (eq 6), and curve 3 (O) to the model with cooperative occupation of both of the nucleotide binding sites on actinomycin (eq 8).

Figure 3a,b shows that binding of d(pG) and d(pApG) to actinomycin fits well with the model with two independent binding sites of equal affinity, but does not fit either of the other models. Binding of d(pGpC), on the other hand, fits only the model of cooperative occupation of the sites. This is in agreement with earlier findings (Schara and Müller, 1972; Krugh and Neely, 1973b) that were interpreted as indicating cooperative double-helix formation by two molecules of d(pGpC) around an intercalated actinomycin chromophore.

The data for d(pA) can, within the error bars for individual points, be fitted to the model with two independent equal affinity binding sites. There is, however, a clear systematic tendency for the points to slope rather than simply scatter as g changes. We show below that the best model is a two-parameter one with partially cooperative binding. Table II summarizes the best values we found for the binding constants using one-parameter models. The results for all nucleotides except d(pGpC) refer to the two independent, equal-affinity site model; the fully cooperative model was used for d(pGpC).

Two-Parameter Models. The data in Figure 3a indicate that binding of d(pG) by actinomycin is much better described by a model with two independent equal-affinity sites than by a single site model. However, the results presented in Figure 3a do not rule out the possibility of intermediate models in which there are two sites, with one binding constant greater than the other. We therefore tested the data against eq 9 and 10 which allow for two binding constants whose ratio is α. Figure 4 shows the results. The best fit is with $\alpha = 1$, which is equivalent to the

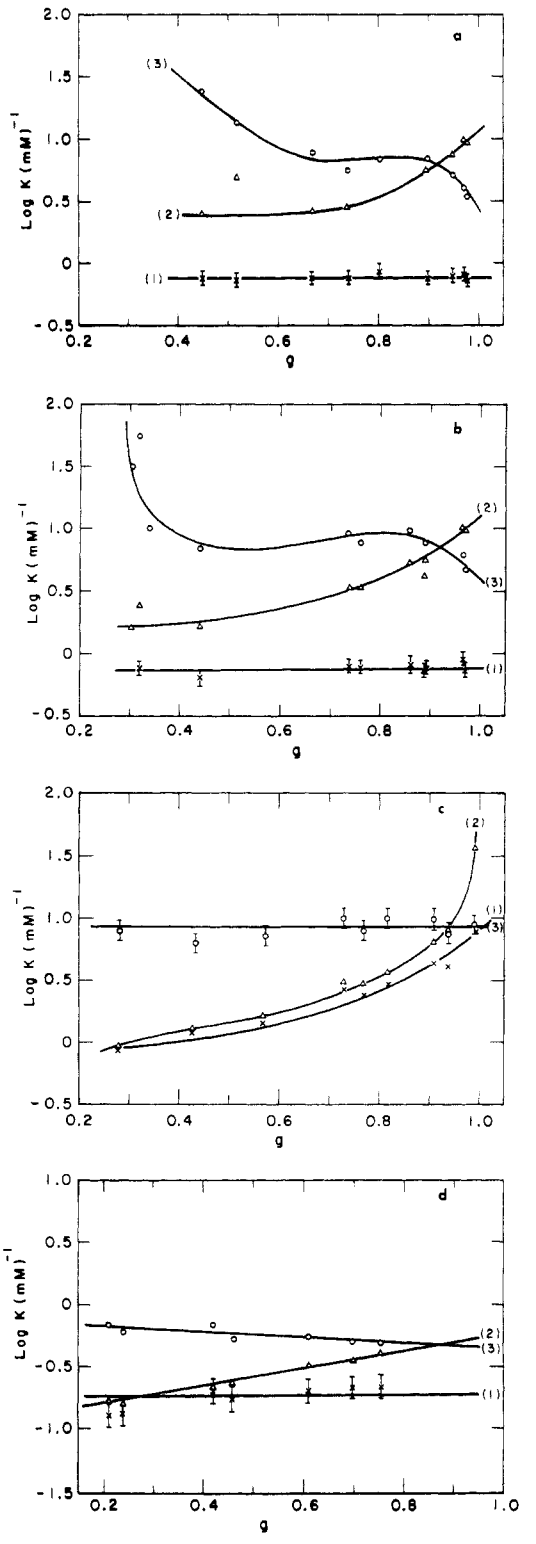


FIGURE 3: Test of three one-parameter binding models for actinomycin–nucleotide interactions. In each case the curves are (1) two identical independent sites, eq 7; (2) one binding site, eq 6; (3) two site, obligatorily cooperative model, eq 8. The nucleotides used are (a) d(pG), (b) d(pApG), (c) d(pA), and (d) d(pGpC). T = 7.1 °C.

TABLE II: Equilibrium Constants for Mono- and Dinucleotide Binding to Actinomycin C_3 (BPES Buffer, 7.1 °C).

Nucleotide	K
d(pG)[a]	0.75×10^3 M^{-1}
d(pApG)[a]	0.76×10^3 M^{-1}
d(pA)[a]	0.16×10^3 M^{-1}
d(pGpC)[b]	8.2×10^3 M^{-1}

[a] K expressed as the binding constant for one of the two independent equal affinity sites, $K = K_1 = K_2$. [b] K expressed as the square root of the equilibrium constant for binding two dinucleotides in a cooperative process. $K = (K_1 K_2 \sigma)^{1/2}$.

model with equal affinity sites. As α becomes smaller than 1 the fit is less good; by $\alpha = 0.3$ there is a clear systematic deviation of the quantity $K(g)$ from constancy. Hence, we conclude that the two binding sites have affinities that differ by less than a factor 3, or by less than 700 cal/mol in the standard free energy of binding.

Figure 5 shows an analogous treatment of the data on d(pA) using a model with partially cooperative binding, eq 11 and 12. The best fit is obtained with $\sigma = 20$ and $K = K_1\sigma^{1/2} = 0.36 \times 10^3$ M^{-1}. In this case, the two-parameter model fits the data better than either of the limiting one-parameter models.

In summary, our results confirm the conclusion of Krugh and Neely (1973a,b) that actinomycin has two nucleotide binding sites. Our method also provides a considerably more accurate view of the thermodynamics of association than do the NMR measurements, which are complicated by the actinomycin dimerization reaction at the necessarily high concentrations employed. Optical titration methods can, in general, distinguish 1:1 and 1:2 complex stoichiometries, but since most measurements were made at large excess of nucleotide, the ability to discriminate the two mechanisms is limited. In addition, it is likely that the two actinomycin binding sites have different spectral changes when nucleotide is bound, so that quantitative analysis of the complex binding equilibrium by optical methods is virtually impossible.

Finally, our results provide an explanation of an apparent conflict between optical titrations and the NMR results of Krugh and Neely (1973a). The latter authors found that d(pA) occupied an actinomycin binding site nearly as strongly as did d(pG), in contrast to optical measurements which showed a much weaker binding for d(pA) than d(pG). Our results demonstrate the partially cooperative nature of the binding, so that at lower concentrations of actinomycin and nucleotide the apparent binding constant would be approximately $K_1 = 0.08 \times 10^3$ M^{-1}, while when both the sites are occupied at higher concentration the apparent binding constant would be $K_1\sigma^{1/2} = 0.36 \times 10^3$ M^{-1}. The latter is only a factor two smaller than the binding constant for d(pG), thus explaining the NMR-based observations, which were made at high concentrations. Interaction, for example by hydrogen bonding, between bound d(pA) residues could account for the cooperativity of binding. An alternative explanation is that the first d(pA) residue bound alters the actinomycin conformation in a way that favors binding of the second nucleotide. As usual, it is impossible to distinguish structural models on the basis of thermodynamic measurements.

Specificity of binding to particular nucleotides, on the other hand, involves thermodynamic parameters, so its origin is not always clear from a structural study. Sobel and Jain (1972) used their structural results to suggest that hydrogen bonding interactions between actinomycin and adjacent G·C base pairs

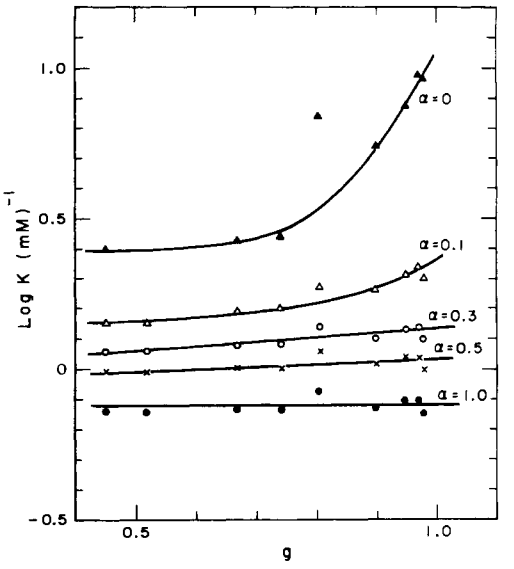


FIGURE 4: Test of a two-parameter model for binding actinomycin to d(pG). α is the ratio of the binding constants, $\alpha = K_2/K_1$. The quantity K ($=K_1$) is plotted using eq 9 and 10. We conclude that the best model is $\alpha = 1.0$.

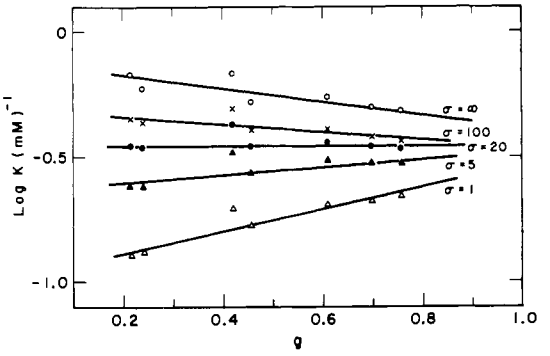


FIGURE 5: Test of a two-parameter partially cooperative binding model for interaction of actinomycin with d(pA). The model has $K_1 = K_2$, and σ is the ratio of the binding constant for attaching the second nucleotide to the constant for the first binding step. The data are plotted using eq 11 and 12. The best fit is obtained with $\sigma = 20$.

are responsible for the observed deoxyguanosine specificity, whereas Müller and Crothers (1975) used comparative thermodynamic measurements to suggest that electronic interactions between an intercalated chromophore and an adjacent base pair provide an alternate basis for specificity. Our present results confirm that deoxyguanosine specificity is much less pronounced at the nucleotide level than for DNA, but since either the hydrogen bonding or the electronic interaction models could explain this result on the basis of a more precisely defined geometry in the double helix, the origin of specificity must be considered still to be an open question.

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Circular Dichroism Studies on Glycogen Phosphorylase from Rabbit Muscle. Interaction with the Allosteric Activator Adenosine 5′-Monophosphate†

Shoji Shimomura and Toshio Fukui*

ABSTRACT: Circular dichroism (CD) spectra of glycogen phosphorylase from rabbit muscle have been measured in the presence of various ligands, particularly in the near-ultraviolet wavelength region. Phosphorylases *a* and *b* gave similar positive CD spectra as each other, in the 250–310-nm region. The differences in CD between the *a* and *b* forms, as well as the CD changes induced by binding of substrate and other ligands except nucleotides to the enzyme, are all relatively small. Binding of AMP and other nucleotides to phosphorylases *a* and *b*, and $NaBH_4$-reduced phosphorylase *b*, however, induces much larger CD spectral changes than the above. The difference CD curve obtained by subtracting the phosphorylase *b* curve from that of the enzyme–AMP complex is smooth, with a positive maximum at 266 nm and a negative at 289 nm. The results with various other nucleotides show that the induced Cotton effects are dependent on the base chromophore of the nucleotides. The rotational strength of the induced Cotton effect in phosphorylase *b* by AMP increases under various conditions, under which the affinity of the enzyme for AMP is enhanced, e.g., the addition of glucose 1-phosphate, inorganic phosphate, fluoride ion, divalent metal cations, and spermine, low temperatures, and conversion of the enzyme to the *a* form. On the contrary, these factors little affect the induced Cotton effects by IMP, GMP, and dAMP. Amylodextrin gave no effect on the extrinsic Cotton effect by binding of AMP plus Mn^{2+} to phosphorylase *b*, while it did retard the AMP-induced tetramerization of the enzyme. It is suggested that the interaction of nucleotides with phosphorylase involves tacking between the base ring of the bound nucleotides and an a ɔmatic amino acid residue at the allosteric site of the enzyme, ar. ¹ that, in the high affinity form of the enzyme for AMP, particular bondings are newly formed between the enzyme and the nucleotide allowing the heterotropic cooperativity.

Glycogen phosphorylase *b* from rabbit muscle shows an absolute requirement for the allosteric activator AMP[1] for catalytic activity (Cori et al., 1938). The interaction between AMP and phosphorylase *b* has been studied under a variety of conditions by the kinetic, gel filtration, equilibrium dialysis, and calorimetric methods. The affinity of phosphorylase *b* for AMP is enhanced by various ligands, e.g., glucose 1-phosphate, P_i, divalent metal ions, fluoride and sulfate ions, protamine, polylysine, and polyamines (Krebs, 1954; Helmreich and Cori, 1964; Sealock and Graves, 1967; Kastenschmidt et al., 1968; Wang et al., 1968; Mott and Bieber, 1970). It is also affected by a variety of environmental conditions, e.g., kinds of buffer, temperature, and pH (Kastenschmidt et al., 1968), and is enhanced through the enzymatic conversion to the *a* form which is active without AMP (Helmreich et al., 1967). On the other hand, activation of phosphorylase *b* by AMP is known to result from alterations in both V_{max} and K_m for each substrate (see Graves and Wang, 1972).

In addition to AMP, many other nucleotides also activate phosphorylase *b* to different extents (Cori et al., 1938; Okazaki et al., 1968; Black and Wang, 1968, 1970; Mott and Bieber,

† From the Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka 565, Japan. *Received March 30, 1976.*

[1] Abbreviations used: CD, circular dichroism; glucose 1-phosphate or glucose-1-P, α-D-glucopyranose 1-phosphate; P_i, inorganic phosphate; AMP, adenosine 5′-monophosphate; NMR, nuclear magnetic resonance.