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PERSPECTIVES REPORT

The Interaction of Aminoacridines with Nucleic Acids

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The nature of the interaction of aminoacridines and their derivatives with nucleic acids† continues to engage the attention of investigators employing many different techniques. This interest persists for various reasons. First, the aminoacridines are themselves important antibacterial and mutagenic agents. Their antibacterial action has long been the subject of intensive study[1–3] but in the last six years it is their ability to induce mutations, apparently by causing deletion or insertion of a single nucleotide in DNA, which has been to the fore.[4,5] This hypothesis has been convincingly confirmed[6] by amino acid sequence determinations on lysozyme altered by a double mutation which was induced by proflavine (3,6-diaminoacridine) in bacteriophage T4. This ability to interact with DNA must also form part, at least, of the explanation of their antibacterial activity and has been associated with their inhibition of DNA-primed DNA and RNA polymerases.[7,8] However, this inhibition of RNA polymerase now appears[9] also to involve a direct inhibition of the enzyme by the aminoacridine, and, indeed, aminoacridines are known to inhibit a number of enzymes[1] notably those involved in oxidation–reduction reactions. So the antibacterial activity of aminoacridines is likely to depend on a wider range of effects than simply their interaction with the nucleic acids.

Second, aminoacridines, on account both of their cationic charge and their three flat aromatic rings, have structural features similar to those of other compounds whose interaction with DNA is of great interest, such as carcinogens (both polycyclic hydrocarbons and benzacridines); certain antibiotics; nucleic acid derivatives (purines, nucleosides); histological dyes, e.g., those with three flat, fused rings such as pyronin, toluidine blue, and triphenylmethane dyes such as methyl green; phenanthridine trypanocides, e.g., ethidium bromide; and, of course, other acridine derivatives, many of which are noted for their antimalarial activity, e.g., atebrin.

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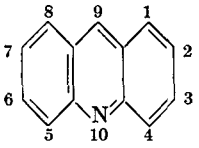
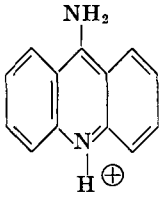
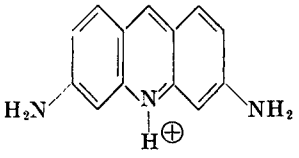
† This term is intended to include polynucleotides synthesized enzymatically *in vitro*.



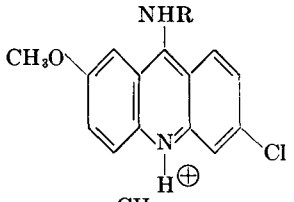
Acridine



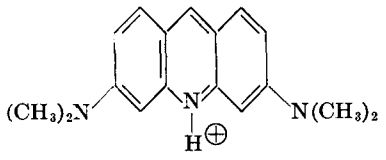
9-Aminoacridine cation



Proflavine cation

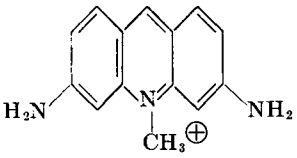


Atebrin (Mepacrine, Quinacrine) cation

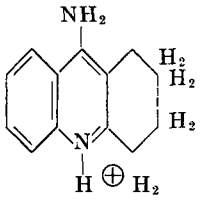


Acridine orange cation

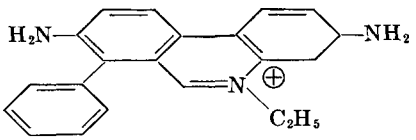
Acranil cation



Acriflavine cation



9-Amino-1, 2, 3, 4,-tetrahydroacridine cation (THA).



Ethidium cation

Third, the effect of aminoacridines and compounds, such as ethidium bromide, which have almost identical effects in untwisting and extending the DNA double helices, can be employed deliberately to modify the shape of DNA molecules. Two notable applications of this property of ethidium bromide have recently been made in order to determine the extent of supercoiling in circular DNA from mitochondria[10] and from polyoma virus.[11]

Fourth, the interaction of these small molecules with nucleic acids is representative of the wider class of interaction of small molecules with biological macromolecules: such interactions are likely to be one of the

central areas of interest in many biochemical problems, and the methods and ideas developed for this particular system should therefore be of wider import.

One of the present authors had his attention focused on this interaction[12] as a result of his earlier interests in the antibacterial action of aminoacridines[13] and his other interests in nucleic acids. Since then, with the stimulus provided by a new awareness of the biological activity of the aminoacridines, for the reasons mentioned above and because of interesting proposals concerning the structure of the complexes, which involve the idea of intercalation (see later), there have been a large number of investigations in this field.

The aim of the present survey is to provide a perspective on certain aspects of these problems as seen from the limited viewpoint of one group of workers, attempting, on the one hand, to correlate and, where necessary, correct our earlier ideas and formulations and, on the other hand, to relate our own observations with those of others. No attempt will be made to mention all the work which has contributed to our understanding of this interaction; indeed the only papers which can be mentioned are those which are particularly pertinent to points which have puzzled or continue to puzzle the authors. This approach inevitably places an emphasis on the work of one particular group which is unavoidable in the circumstances. We can only hope to stimulate others to make their own assessments. Where there seems to be general agreement concerning a particular point, no attempt will be made to describe all the evidence.

The contributions to our understanding of the nature of the interaction between aminoacridines* and nucleic acids which are afforded by a knowledge of the binding curves and of the problems involved in determining and assessing such curves will first be surveyed in some detail, since some of the difficulties in interpretation arise here. Various structural aspects of the interaction will then be summarized and the structure of the complexes will finally be discussed in the light of the various types of experimental evidence to see if a coherent picture emerges.

BINDING CURVES

These curves, whose experimental determination will be examined below, are plots of the amount (r) of aminoacridine bound per mole of nucleic acid phosphorus against the concentration (c, in moles/l) of free aminoacridine. The purpose of determining such curves is to derive the number (n_J) of each of the P classes of binding sites (of intrinsic binding constant k_J) for the aminoacridine on the nucleic acid. In general

$$r = \sum_{J=1}^{J=P} \frac{n_J k_J c}{1 + k_J c} \tag{1}$$

* Also variously called, in the following, the dye or ligand, since aminoacridines are both in the present context.

which simplifies to

$$r = \frac{n_{\mathrm{I}} k_{\mathrm{I}} c}{1 + k_{\mathrm{I}} c} + \frac{n_{\mathrm{II}} k_{\mathrm{II}} c}{1 + k_{\mathrm{II}} c} \tag{2}$$

for two classes (I, II) of binding sites; and to

$$r = nkc/(1 + kc) \tag{3}$$

or

$$r/c = kn - kr \tag{3a}$$

for one class of binding site.

In this last instance a plot of r/c against r will be linear (Fig. 1*a*) with an intercept at $r = n$ on the r axis and with a slope of $-k$, if this quantity, which is the intrinsic association constant of the group and is equal to [occupied sites]/[unoccupied sites]. [Free aminoacridine] is constant. Curvature of this plot can result from variation in one or both of two factors: (*1*) an electrostatic free energy (ΔG_r^0) dependent on r so that $k = k' e^{\Delta G_r^0/RT}$ and (*2*) the overlapping of the binding of more than one type of binding site, as expressed in eq. (1). The electrostatic effects (*1*) can be suppressed by addition of neutral salt at high concentration.

When only two or three types of binding site are present, it is sometimes possible to discern distinct linear portions in the plot of r/c versus r from which the individual n_J and k_J can be determined. Even when there is a continuous overlap of a series of binding sites, the slope $d(r/c)/dr$ at a particular r is still an indication of the weighted, average association constant prevailing at that r value. For example, a case which is important in this instance (Fig. 1*b*), if there are two types of binding sites, as in eq. (2), and if, respectively, r_{I} and r_{II} are the number of aminoacridines bound per phosphorus on sites I and II, of maximum number n_{I} and n_{II}

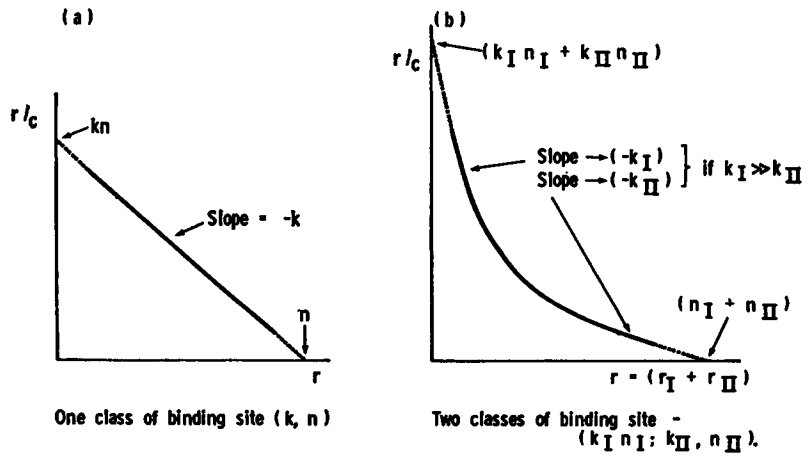


Fig. 1. Form of the r/c against r plot for: (*a*) one class of binding site (k, n); (*b*) two classes of binding sites (k_{I}, n_{I}; k_{II}, n_{II}).

per phosphorus, and k_I and k_{II} are the corresponding intrinsic association constants, then $r = r_I + r_{II}$ and equations such as (3a) can be written for each site,

$$r/c = (r_I + r_{II})/c = k_I n_I - k_I r_I + k_{II} n_{II} - k_{II} r_{II}$$
$$= k_I n_I + k_{II} n_{II} - [(k_I - k_{II}) r_I + k_{II} r] \quad (4)$$

In the limit (Fig. 1*b*), when r_I, r_{II} and r are all zero,

$$r/c \rightarrow k_I n_I + k_{II} n_{II}$$

and when

$$r/c \rightarrow 0,\ r \rightarrow (n_I + n_{II}).$$

From eq. (4), the negative slope of r/c versus r is

$$-d(r/c)/dr = k_{II} + (k_I - k_{II})\, dr_I/dr \quad (5)$$

When $k_I > k_{II}$, if this negative slope is identified with k_{II}, as might seem reasonable when r is large and r_I close to n_I, an overestimate of k_{II} will be made. However, if $k_I >> k_{II}$, a region of r is readily attainable experimentally in which dr_I/dr is virtually zero (i.e., r_I constant at the value n_I), so that as r approaches its limiting value of $(n_I + n_{II})$, the slope $d(r/c)/dr$ approaches $-k_{II}$. Correspondingly at low r, when all the binding is on sites I, $dr_I/dr = 1$, so the negative slope of the r/c versus r plot is equal to k_I. For more than two classes of binding sites the limiting slope as $r/c \rightarrow 0$, is $-k_P$, where the Pth class of sites has the smallest association constant, and the intercept is

$$\sum_{J=1}^{J=P} n_J$$

Thus inspection of the binding curves and analysis of the derived r/c versus r plots can provide information concerning the number and nature of the binding sites. However, quantitative analysis becomes difficult if there is a continuous range of binding sites with many k_J. Moreover, the treatment described is based on equations such as eq. (3) which assume a Langmuir type of binding, possibly modified by electrostatic interactions. But when molecules like the aminoacridines interact with macromolecules there is also the possibility of cooperative binding, whereby bound aminoacridine molecules facilitate the binding of further molecules. Cooperative binding curves (r versus c) are sigmoidal in character and at low c are convex to the c axis, whereas the binding curves corresponding to eqs. (1)–(3) are all concave to the c axis and two or more usually give combined curves which are also concave to it (Fig. 2*b*). A further possibility is the formation of a net binding curve (total r versus c) which is the sum of a Langmuir-type binding [eqs. (1)–(3)] and a cooperative binding curve (Fig. 2*a*). These possibilities must be borne in mind when examining the interaction between aminoacridines and nucleic acids. These curves have

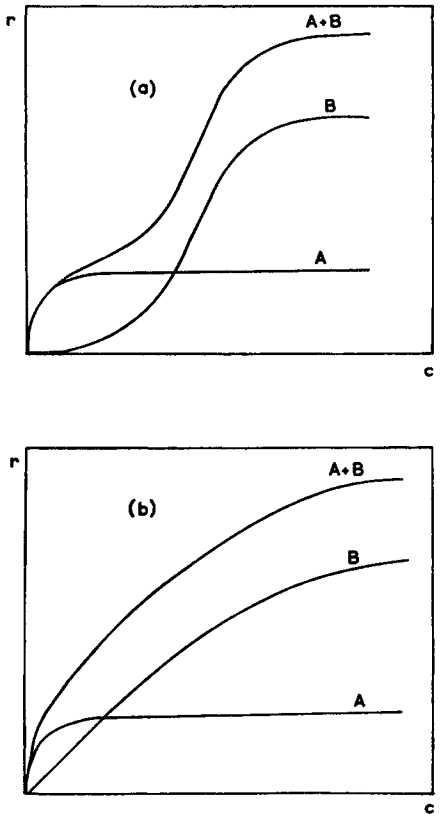


Fig. 2. Plots of r vs. c: (*a*) A = single site binding according to eq. (3), B = cooperative binding, $A + B$ = sum of A and B; (*b*) A = single-site binding according to eq. (3), B = single-site binding according to eq. (3), with larger n but lower k than in A, $A + B$ = sum of A and B.

been determined experimentally by a number of methods and the limitations and advantages of each must now be examined in order to assess the meaning of the results afforded by them. Since the majority of studies have been made by the spectrophotometric method, which is only applicable under specific conditions, this method will be examined in more detail than others.

Methods

Spectrophotometric analysis. Many dyes containing aromatic rings undergo a displacement of their electronic absorption spectrum when bound to macromolecules. The spectrum of aminoacridine molecules bound to nucleic acid is, for example, displaced to longer wavelengths when they are bound as single molecules; this bound "monomer" spectrum may only be achievable at very low values of r with aminoacridines which have a great tendency to self-aggregation. This tendency appears to be stronger in many substituted aminoacridines, most notably in acridine orange, which

is the methylated derivative of proflavine. Thus solutions of proflavine obey Beer's law over a much wider range of concentrations than do solutions of acridine orange, which in concentrated solution displays a new absorption band at 464 mμ to replace the 492 mμ monomer band.[14] Correspondingly, when acridine orange interacts with nucleic acids and other macromolecules, it displays a bound "monomer" spectrum with λ_{max} = 505 mμ (called by Michaelis[15] the α spectrum at low r, when binding sites greatly exceed in number the molecules actually bound; but as r increases,[15] with increasing ratio of acridine orange to nucleic acid phosphorus, the peak shifts to wavelengths lower than that of free monomer and a bound "dimer" spectrum, β,[15] appears with λ_{max} = 465 mμ; with some macromolecules, at still higher values of r a further bound aggregate spectrum, γ,[15] appears with λ_{max} = 450 mμ. (The terms monomer, dimer, and aggregate, when applied to the bound monoaminoacridine, will, in this context, refer to states in which the characteristic spectral shift is due to interaction between none, two, or several bound molecules, respectively. The displacement of the spectrum in the monomer is presumed to result from interaction with the macromolecules, as will be discussed below.)

Fortunately, under many conditions, the spectra of simple mono- and diaminoacridines is displaced on binding only to that of the bound monomer, the α-type spectrum. When this is so, the displacement can be used to determine the fractions of the total aminoacridine in solution which are free and bound and hence enable the binding curve to be determined. The method of this calculation has been fully described[12] and is illustrated graphically in Figure 3.

The possibility of the calculation of free and bound fractions rests on certain conditions, and the extent to which these conditions are fulfilled must be gauged for each set of experiments in order to assess the reliability of binding curves derived from spectral shifts. These conditions are (referring to Figure 3) as follows.

(*1*) The nucleic acid is nonabsorbing at all λ.

(*2*) Free and bound ligand obey Beer's law over the whole range of c_T used, i.e., ϵ_f and ϵ_b are constant.

(*3*) ϵ_b also does not vary with r.

(*4a*) There is only one species of bound ligand so, with free ligand, there are two species of ligand present in the solution; or (*4b*) if more than one species with different spectra, apart from the free ligand, are present, the relative proportion of these does not vary with r.

If conditions (*1*)–(*4*) are obeyed, then:

(*5*) there should be a clear isobestic point through which all spectra of mixtures containing a given total ligand concentration (c_T) would pass, being the λ at which $\epsilon_f = \epsilon_b$; and (*6*) the value of α_b, and so of r, calculated from ratios such as PQ/PR (Fig. 3) should be the same at all λ.

Occasionally nucleic acid solutions have a slight optical density at 400–500 mμ, the range in which proflavine and acridine orange absorb: correction is easily made for this. The need to meet condition (*1*) renders

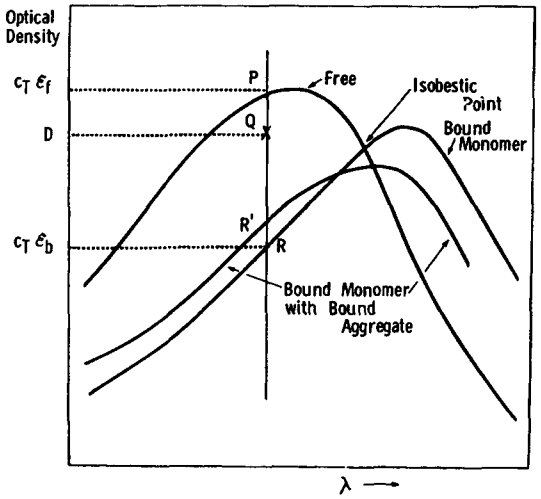


Fig. 3. Spectra of proflavine, free and bound as monomer (experimental[12]). The spectrum of proflavine bound both as monomer and as aggregate is schematic only, though based on experimental observations.[16] Optical density (absorbance) D at wavelength λ is given by

$$D = c\epsilon_f + c_b\epsilon_b$$

where c and ϵ are concentrations and extinction coefficients, respectively; subscripts f and b refer to free and bound ligand, respectively; c is concentration of free ligand. Then:

$$D = (1 - \alpha_b)\, c_T\epsilon_f + \alpha_b c_T\epsilon_b$$
$$= (1 - \alpha_b)\, D_f + \alpha_b D_b$$

where α_b is the fraction of the total ligand concentration (c_T) which is bound. D_f ($= c_T\epsilon_f$) and D_b($= c_T\epsilon_b$) are the optical densities when all the ligand (at concentration c_T) is completely free and completely bound, respectively. Hence $\alpha_b = (D_f - D)/(D_f - D_b) = PQ/PR$, and $c = (1 - \alpha_b)c_T$ and $r = \alpha_b c_T/P$, where P is nucleotide phosphorus concentration (in mole/l). If bound aggregate contributes to the optical density D at point Q, the correct reference spectrum is then given by the curve passing through the corresponding reference point R', and the true $\alpha = PQ/PR' > PQ/PR$.

the ultraviolet region unsuitable for determining the binding curves on nucleic acids.

Condition (*2*) is usually met by restricting the total ligand concentrations (c_T) to the range within which Beer's law is obeyed.

Condition (*3*) needs more examination and is logically linked with (*4*), since if there are two or more types of bound species, each with different spectra (e.g., the α, β, and γ spectra), and ϵ_b inevitably varies with r. Condition (*4b*), which would allow α_b and r to be determined, is not likely to be fulfilled, since different bound species will usually be in dynamic equilibrium with each other and, since one is likely to be an aggregate of the other, their relative proportion will change with r and c. This condition might be fulfilled when more than one type of binding occurs, if there were two forms, with different spectral characteristics, of the bound

monomer or aggregate in an equilibrium governed by a constant independent of r and c. Such a situation is conceivable for, say, tautomeric forms of a given dye, but there is no evidence that any such equilibrium occurs between different forms of bound monomer aminoacridines (or bound dimer or bound aggregate).

One consequence of a lack of fulfilment of conditions (*3*) and (*4*), in either of its forms, is illustrated in Figure 3, where the line passing through R' represents the spectrum of the fully bound ligand as it would be if the ratio of monomer to aggregate binding were the same as in the mixture corresponding to Q. This line has been drawn with a greater short wavelength component than the limiting spectrum of bound monomer, because point Q is now taken to correspond to a value of r at which conditions (*3*) and (*4*) are not fulfilled and some of the bound aminoacridine is in the aggregate form (i.e., β or γ spectrum is beginning to contribute). It should be noted that the series of spectra between those of free and bound monomer is usually obtained by adding increasing amounts of nucleic acid to a given aminoacridine solution of concentration c_T, so that the first spectra obtained correspond to high r values (because c is high) and includes any contribution of aggregates. The limiting spectrum is recorded when a large excess of nucleic acid is present so that $c_b = c_T$ and r is extremely low. In this procedure, therefore, the limiting spectrum always corresponds to the bound monomer (α) spectrum. Hence it is the point R' which corresponds to the optical density at the chosen λ of ligand fully bound in the form prevailing at Q, i.e., with the same proportion of aggregates and monomers. So the true value of $\alpha_b = PQ/PR' > PQ/PR =$ observed value of α_b. So if aggregates are present and the experimentally observed limiting spectrum is used to calculate α_b, this quantity, and r, will be underestimated.

If conditions (*3*) and (*4*) are not fulfilled, (*5*) does not follow, as there is no clear-cut isobestic point, nor does (*6*). The practical problem is usually to decide if deviations from (*5*) and (*6*) are sufficiently small to allow a determination of α_b, and so of r, within the degree of accuracy required. The type of obscuration of the isobestic point which is frequently observed is well illustrated by the series of spectra recorded with proflavine in DNA at pH 2.8[16] and proflavine on poly (A + U).[16] Acridine derivatives, such as acridine orange, which aggregate more readily display no semblance of an isobestic point.[15,17]

In the original spectrophotometric study[12] of the interaction of proflavine with DNA, it was noted that although α_b and hence r values determined by observing changes of extinction were consistent when the wavelength of observation was in the range 430–444 mμ, the values so obtained did not agree with α_b and r, derived from measurements at 460 mμ, even when the isobestic points were satisfactory. This was, no doubt, partly due to the much lower accuracy of the calculations of α at λ = 460 mμ, since the difference ($\epsilon_b - \epsilon_f$) was so much smaller (about half that at λ = 440 mμ). This aspect of the method has been re-examined

more closely[18] in this laboratory, and it is concluded that when the isobestic point is sharp (as with native DNA at all ionic strengths μ and denatured DNA at $\mu \geqslant 0.1$), the values of α_b, and so of r, derived from extinction measurements at λ in the range 410–440 mμ with proflavine are in agreement within the extent to be expected from the actual accuracy of the extinction measurement. Agreement is least when only relatively small amounts of DNA have been added to a given proflavine solution, so that r has the maximum value for the series, and there is the possibility that aggregate binding is beginning to occur. Determinations of α_b and of r by measurement on the long wavelength side of the isobestic point of proflavine and DNA (i.e., $\lambda > 454$ mμ) were not all consistent with each other or with the determinations at $\lambda = 410$–440 mμ, and the reason given above still seems an adequate explanation for this. When the system gave poor isobestic points (proflavine on denatured DNA at $\mu < 0.1$), not surprisingly the α_b and r values derived at $\lambda = 410$, 420, 430, and 440 mμ agreed less well, although the values from measurements at $\lambda = 430$ and 440 mμ were often close enough to justify confidence in the form of the r versus c curve derived from them, if not in the precision of the actual r values.

The spectrum of free proflavine resolves[18,19] into a weaker Gaussian peak with a maximum at 410–415 mμ and a stronger one at 445 mμ. On becoming bound as monomer, the spectrum is displaced, to give the α spectrum, and the weaker contributory peak at 410–415 mμ then appears at 433–440 mμ, and the stronger 445 mμ contributory peak at about 460 mμ.[18,19] So each peak is displaced by $+20 \pm 5$ mμ on binding.

The existence of a spectrum of bound proflavine of the β type, with a maximum at shorter wavelengths than free proflavine, has only recently been detected, although well known with acridine orange. Chambron[20] has observed a peak at 420–425 mμ at high r values for proflavine bound to DNA at $\mu = 0.01$, 0.1, and at relatively high concentrations c of free ligand, and similar observations have been made in this laboratory.[18] This β spectrum of bound aggregate (or dimer) proflavine is so close to the weaker 433–440 mμ component of the α spectrum of bound monomer that aggregation could be occurring but on increasing r would not be as obvious and as easily detectable as it is with acridine orange.

This would explain why the binding curves of proflavine which are deduced by spectrophotometry often agree well with those derived from equilibrium dialysis studies, even when both curves show, by the presence of an inflection, a transition from strong monomer to weaker aggregate binding. The upward inflections in spectrophotometric binding curves of proflavine on DNA, which have been observed under many conditions, therefore represent a genuine transition to a region of c in which another, weaker, mode of binding is dominant; the spectrophotometric method will be tending to underestimate α and r to an increasing extent as c increases, so any corrections will only serve to increase r above the spectrophotometric value and so to accentuate the inflection.

The spectrophotometric method will therefore not mislead one as to the value of c at which weaker, aggregate binding begins to dominate over stronger, monomer binding, but will tend to underestimate r in this range. Spectrophotometric values of r up to 0.3 should be as accurate as in the true monomer range because of the relatively small shift of spectrum when aggregation first occurs.

The foregoing has been written with proflavine chiefly in mind, since this has been much more fully studied, but should also apply to those other aminoacridines, e.g., 9-aminoacridine and its derivatives, which have low tendencies to aggregate. In practice, the spectrophotometric method is limited to ranges of c where Beer's law is obeyed ($<2.5 \times 10^{-5}M$ in proflavine) but has the advantage that it yields values for r at lower c than the equilibrium dialysis method.

Equilibrium Dialysis. In this method, the unbound ligand is allowed to equilibrate across a membrane impermeable to the macromolecule. The macromolecule is usually contained either in dialysis tubing, which constitutes the membrane, when the concentration of free ligand c in the outer solution is determined; or special cells are built, often in Perspex, in which two chambers are separated by a circular membrane made from dialysis tubing, when c is determined in the chamber free of macromolecules. The method has been much applied to binding studies on proteins,[21,22] and an analysis of the best conditions for reducing fractional errors in r has been made.[12] Errors in r are proportionately greater at low r. Its practical disadvantages are that a minimum concentration of neutral salt must be present to eliminate Donnan equilibrium effects; that equilibrium can only be established in 1-3 days; and that, either the uptake of ligand by the membrane must be reproducible and separately determined, or that the total ligand concentration must be determined not only in the "outer" solution but also in the "inner" solution containing macromolecule, and this can sometimes be difficult.

The virtues of this method are that it can cover a wider range of c and so of r than other methods, notably the spectrophotometric; and that the binding curves so determined rest on no special theoretical assumptions.

Sedimentation Dialysis. If a solution containing a small ligand molecule and a macromolecule is subjected to a high centrifugal field, the concentration of free ligand remains constant throughout the cell, since it is far too small to sediment even in these high fields. But the macromolecule sediments and its concentration, after a slight correction for the radial dilution effect, is known in the plateau region. The sedimentation process may be followed at a wavelength at which the ligand absorbs but the macromolecule does not. The absorption of light in the region of the cell above the boundary is due only to free ligand whereas the absorption below it is due to both bound and free ligand. From the absorption of the incident light by these regions, as measured photographically or photoelectrically, it is possible to determine a binding curve for the ligand–

macromolecule system. The method corresponds to the equilibrium dialysis method in that the sedimenting boundary replaces the dialysis membrane. If the wavelength used in the "sedimentation dialysis" method is the isobestic wavelength of the ligand–macromolecule system, the ligand molecules have the same extinction coefficients whether bound or free and total ligand concentrations can be determined in the presence or absence of the macromolecule, provided it too does not absorb at this wavelength.

This method has been used by Steinberg and Schachman[23] to measure the binding of small molecules to proteins and has recently been applied to the proflavine–DNA system.[24] When the two molecules in such an interacting and sedimenting system have sedimentation coefficients of comparable magnitude, complicated reaction boundary phenomena make the simple interpretation above impossible, but application[24] of an equation given by Schachman[25] shows that in a system such as proflavine–DNA, in which the proflavine has virtually a zero sedimentation coefficient, the c above and below the boundary of sedimenting DNA differ by less than 0.1%. So all the difference in c_T can be attributed to binding to the DNA and the proflavine concentration above the boundary is the same as the concentration of free proflavine c in the presence of the DNA below the boundary.

This method is applicable only to proflavine solutions of c_T and c within the Beer's Law range, but otherwise a wide range of solution conditions (μ, pH, temperature) can be used. It also has the added advantage that the sedimentation coefficient of the complex can be determined.[24]

Partition Analysis. In this method,[26] the free ligand is partitioned between the aqueous solution containing the nucleic acid and a nonmiscible organic solvent whose partition coefficient for the ligand with respect to water is known. Hence c, and so c_b and r are determined. It has the advantage over equilibrium dialysis that, like sedimentation dialysis, there is no need for a correction for absorption at the surface separating the two phases. However the presence of an organic solvent, with some low solubility in water, introduces unknown effects on the binding process itself. The concentration range covered is similar to that of equilibirum dialysis, but the method is quicker. Theoretical considerations show that a minimum quantity of neutral salt must again be present, though this requirement seems frequently to have been ignored. The method has not been widely used but has been applied to determining the binding of rosaniline and triphenylmethane dyes[27] and of acriflavine[28] to nucleic acids.

Fluorescence Quenching. The fluorescence of aminoacridines is quenched when bound to nucleic acids, and this has been used to determine the fraction of ligand molecules which are bound,[28–30] since

$$c = c_T(F/F_0 - p)/(1 - p) \tag{9}$$

and, as before,

$$r = c_b/P = (c_T - c)/P \tag{10}$$

where F_0, F are the observed intensities of fluorescence of the aminoacridine in the absence and presence of nucleic acid and p is the ratio of the observed quantum yield of fluorescence of the bound dye to that observed with the free dye. The fluorescence intensity is observed with increasing amounts of nucleic acid, at a given c_T, which is much lower than that used in any other method. Then $(F/F_0) = p$ as $P \rightarrow \infty$ and $c \rightarrow 0$, and, if there is only one binding site (with parameters n, k),

$$[F_0/F - 1]_{r\to 0} = (1 - p)P_{nk} \tag{11}$$

If there is more than one binding site, it is more useful to calculate r and c and to plot r/c versus r as with the other methods.

This method has the advantage of being able to determine the relation of r to c at $r < 0.02$ and, in the case of acriflavine (3,6-diamino-10-methylacridinium chloride, i.e., N-methylated proflavine) at $c_b \backsim 10^{-7}M$. If the plots of r/c versus r display curvature at μ at which electrostatic effects can be ignored, then it can be inferred that the binding sites are heterogeneous with respect to p within the low range of r covered by the method. But if the different sites have different p values, then the basis of the calculation of r itself [eq. (10)] is undermined. So, although heterogeneity of the sites can be detected, the estimates of r and so of k and n of each type of site which this method affords are less certain.

Comparison of Methods. Most investigations of binding of aminoacridines to nucleic acids have relied mainly on one of the foregoing methods. However some comparisons have been made.

Several investigators[12,18,31–33] agree that the spectrophotometric analysis and equilibrium dialysis give the same binding curves on a given DNA, at $\mu = 0.1$, and 20°C. This agreement, which extends[18] to low r and up to 70°C is not surprising in view of the excellent isobestic points observed with native DNA at $\mu = 0.1$ (e.g., Fig. 1, ref. 12). Under these conditions, there is also reasonable agreement between the binding curves on DNA from various sources, as studied by the different authors. With DNA from a given source, Chambron et al.[20,33] in an extensive study have obtained good agreement between the values of $k = [r/c(n - r)]$ obtained by the two methods, when $\mu = 0.01$ and 0.1 over the temperature range 20–70°C, and when $\mu = 1.0$ at 20 and 35°C, but when $\mu = 1.0$ at 50 and 70°C k obtained by spectrophotometry was less than that obtained by equilibrium dialysis. This last difference can be understood if binding on sites (probably at base pairs), whose structure is more sensitive to temperature, occurs preferentially at low r and at lower temperatures, since the range of r in the equilibrium dialysis experiments at $\mu = 1.0$ was 10 times greater than in the spectrophotometric studies.[33] A discrepancy between the binding curves obtained by the two methods was also observed[31] with some benzacridines on DNA at $\mu = 0.1$ and 25°C, but not at 20°C. Such discrepancies probably arise from the different types of binding having different temperature coefficients and different spectral contributions.

The binding curves at $\mu = 0.1$ and 20°C of proflavine on native and heat-denatured DNA have been determined[24] spectrophotometrically and by sedimentation dialysis. The binding curves determined by the two methods agreed well up to $r = 0.13$ beyond which the r values from sedimentation dialysis were significantly greater than those determined spectrophotometrically. When $r > 0.13$, binding of aggregates by a weaker process becomes prominent so, for the reasons previously given, it is to be expected that the spectrophotometric method would underestimate r; even so, the observed discrepancy was not more than about 10%.

Partition analysis and equilibrium dialysis also gave[28] concordant values for the binding of acriflavine on DNA at $\mu = 0.1$ and 4°C, which implies that the *n*-hexanol used in the partition analysis did not interfere with the interaction. The values of the strongest binding constants which dominate as c tends to zero and which were determined by appropriate extrapolation of the results of fluorescence quenching and partition analysis studies were also in satisfactory agreement.[28]

Results

The binding curves of proflavine and other aminoacridines on nucleic acids which have been obtained by the foregoing methods exhibit, in summary, the following features.

Heterogeneity of Binding Site. The form of the binding curve is that of $(A + B)$ of Figure 2*a* and the r/c versus r plots (Fig. 1*b*) are curved even at very high ionic strengths,[12] which means that the heterogeneity is present even when electrostatic effects have been suppressed. There is general agreement that the binding process can be divided into:[12] a process (I) by which up to 0.1–0.2 molecules of aminoacridine per nucleotide are bound strongly, with a ΔG of -6 to -9 kcal/mole aminoacridine (cf. curve A of Fig. 2*a*); and a weak process (II) by which further aminoacridines are bound up to the electroneutrality limit of $r = 1.0$ (cf. curve B of Fig. 2*a*). This conclusion is based on the observation that the r/c versus r plots fall into two distinct portions: one of high slope corresponding to I up to $r \simeq 0.2$ and the other of low slope at r from 0.2 to 1.0. Process I can be characterized by a maximum value of r_I denoted as n_I, but not usually by a single value of the association constant, k_I, since the weaker binding invariably overlaps the lower c range in which I is dominant. The binding curves of ethidium bromide afford linear r/c versus r plots, and so a unique value of k_I for given conditions, but the corresponding plots for aminoacridines are usually curved[12]. This inherent heterogeneity of the strong binding I can be attributed to either a continuous change in the binding constant as a result of a continuous modification of the nucleic structure on binding or to a structural heterogeneity in the binding sites dependent on their base composition.[30,34] The form of the binding curves (compared with Figs. 2*a* and 2*b*) suggests that the weaker binding process II is co-operative, whereby the binding of one aminoacridine cation facilitates the binding of the next, especially as it only occurs at values of c at which self-aggregation occurs in proflavine solutions alone.

Ionic Strength. Increase in ionic strength diminishes the extent of binding, but binding by process II is more sensitive to such changes than binding by process I.[12,32,33,35] Hence it is concluded that electrostatic forces contribute greatly to both binding processes but are relatively more important in II than in I.

A decrease in ionic strength increases r_I of proflavine on DNA at a given c by, for example, a factor of 4 in going from $\mu = 2.0$ to $\mu = 0.01$ but the binding by II is changed even more dramatically: for r_{II} can reach its maximum value at $c = 2 \times 10^{-5}$ at $\mu = 0.005$, while at this same value of c at $\mu = 0.1$, no weak binding II is observable at all.[35]

Temperature. The effect of temperature is complex. Binding of proflavine on DNA decreases with rise in temperature[32,34,36] and this can be explained partly by a decrease in the number n of binding sites. However the ΔH of binding is not constant and also decreases with temperature.[32] The effects are therefore complex even when electrostatic effects are suppressed at $\mu = 1.0$, and involve* not only the different temperature coefficients of binding by processes I and II but also the effects on binding (both n and k) of some change of state in the DNA which occurs at a much lower temperature than its usual melting point.[32] In addition, a cooperative decrease in binding occurs at a temperature which is higher than the actual melting temperature of DNA when binding proflavine at $\mu = 0.01$ and 0.1.[33]

Denaturation. The effect of disorganization of the double-helical structure of DNA, by heating and cooling rapidly to 0°C, on its ability to bind aminoacridines is clearly of interest with reference to the problem of whether or not the intact double helix is necessary for binding. The investigations made in this laboratory[35] on the binding of proflavine, 9-aminoacridine, and the quinoline derivative, 9-amino-1,2,3,4-tetrahydroacridine (THA) by native and heat-denatured DNA were made by means of the spectrophotometric method and need to be re-examined in the light of the critique of this method made above. In particular, the results obtained with proflavine[35] will be discussed.

Isobestic points are satisfactory with native DNA and proflavine at μ from 0.005 to 0.1 or higher. They are not sharp with heat-denatured DNA and proflavine at $\mu = 0.01$ or lower, so the values of r for this system are probably underestimated. The increase of binding of proflavine by DNA after denaturation at $\mu < 0.01$[35] is therefore, if anything, underestimated. The form of these curves, with a marked inflection and rapid increase to $r = 1.0$, shows that binding by process II is strongly enhanced relative to binding by I as a result of the denaturation of DNA, as well, as usual, by lowering μ. The cooperative binding by II therefore outweighs the strong binding I even at quite low c, when $\mu < 0.01$ and the DNA is denatured; this fully explains the higher values of r ($= r_I + r_{II}$) observed at a given c with denatured DNA. However, these observations at $\mu < 0.01$ cannot

* It is therefore not surprising, in view of such differences, that binding curves obtained spectrophotometrically and by dialysis equilibrium can agree at one temperature and disagree at another.

settle the question of how strong binding I is affected by heat denaturation. This can only be judged by examining the binding curves at a sufficiently high μ (preferably > 0.1) when the isobestic points are sharp with both native and denatured DNA and over a range of c ($0\text{–}2 \times 10^{-5}M$ for proflavine) when binding by II is virtually absent.

Our earlier studies on this system under these conditions[35] suggested that the binding of proflavine by heat-denatured DNA was somewhat greater than by native DNA. However a systematic error is now known to have occurred in the calculation of r for the denatured DNA, as a result of the tendency of the complex of proflavine and denatured DNA to come out of solution when DNA is in excess and of a consequent error in the value of ϵ_b (at the usual reference $\lambda = 440$ mμ). When precipitation is avoided and a correct value of ϵ_b is obtainable, the gap between the binding curves of proflavine on native and denatured DNA is reduced to the limit of experimental error, almost up to the point of inflection where process II becomes prominent and this has been confirmed by subsequent redeterminations[37] of these binding curves in this laboratory. The previously published binding curves of proflavine on denatured DNA at $\mu = 0.06$ and 0.1[35] represent, in fact, the upper limit of error of r, and the lower limit of error extends to the r versus c curve of the native DNA. In view of this, we would not now place the emphasis on the ratio of $r_{\text{denatured}}/r_{\text{native}}$ which was made earlier,[35] or on any deductions from it. The basic conclusions of this work, that destruction of the long runs of double helices in the DNA by heating did not reduce the strong binding of proflavine by process I, still stands with its implications that such helical structures are not a necessary condition of the strong binding I. Indeed, strong binding of proflavine by process I by native and heat-denatured now appear to be identical and this is the experience of other investigators who have found this identity not only with proflavine,[38] but also with actinomycine[38] and ethidium bromide[39] at high ionic strengths.

However, Chambron et al.[20,33] report a marked reduction in binding of proflavine which is coincident, at $\mu = 1.0$, with the breakdown of the double helical DNA structure. This implies that denatured DNA does not bind proflavine at these high temperatures (>90°C) but this does not settle the question of whether or not it binds at lower temperatures, around 20°C.

This re-assessment of the effect of denaturation on the relative binding of proflavine by native and heat denatured DNA has been extended to the binding of 9-aminoacridine and, in this instance, the evidence at present is that denaturation still appears to cause some increase in strong binding by process I.

The effects on the interaction caused by denaturation with agents other than heat has not been much studied. At pH 2.8, DNA still binds proflavine, as has been demonstrated[37] by observing, by means of absorption optics at $\lambda = 454$ mμ (the usual isobestic point), the sedimentation boundary at pH 2.8 of proflavine bound to DNA.

The series of spectra of proflavine[16] obtained when increasing amounts of DNA are added at pH 2.8, are like those of heat-denatured DNA at low ionic strength. The limiting spectrum, at high concentrations of DNA and very low r values, is identical with the usual spectrum of strongly bound (process I) monomeric proflavine (isobestic point at $\lambda = 454$ mμ). At a given c_T, as the DNA concentration decreases, and r increases, the spectrum includes an increasing contribution from aggregates, and this is also indicated by a rough r/c versus r plot.[16] Thus acid-denatured DNA at acid pH (2.8) tends to bind proflavine more by II than by I. This observation has been related to the loss on lowering the pH of the optical activity which is induced in proflavine when it is bound to DNA.[16]

Weak Binding. Binding by weak process II may be identified with binding as aggregates, to judge from the spectral shifts observed, which are analogous to those more clearly identified in the metachromatic shifts which occur when an aggregating dye like acridine orange is bound to a macromolecule. The tendency to bind by process II is apparently enhanced at low ionic strength and by disorganization of the double-helical DNA structure either by heating and shock-cooling or by lowering the pH.

Bradley and his colleagues[17] have studied in detail the aggregation of dyes like acridine orange on nucleic acids and other polyelectrolytes and have introduced the concept of "stacking" which may be loosely described as the enhanced tendency of a ligand to bind next to a ligand already bound, i.e., a cooperative phenomenon. In their model of the stacking situation, these investigators conceive the aminoacridines, or other dye, as bound externally to the polyelectrolyte mainly by electrostatic forces. If the polyelectrolyte is DNA, this suggests a model in which the planes of the aminoacridine are parallel to the base rings of the DNA and are stacked on top of each other in the same direction as the DNA axis, with their positive ring nitrogens close to the external phosphate groups of the DNA chains. This model is very feasible for acridine orange bound to DNA, since the forces between the ligand molecules are then relatively strong and are comparable with the electrostatic forces between ligand and the DNA. However, the forces between bound proflavine molecules are much weaker (cf. its lower tendency to aggregate in solution) so, although the picture of proflavine being bound externally by predominantly electrostatic forces in process II is probably substantially correct, this ought not always to be described as stacking. For the tendency of proflavine to interact with proflavine in a direction parallel to the DNA axis will be markedly less than with acridine orange. That this tendency is not absent is shown by the evidence that proflavine on nucleic acids can indeed exhibit an aggregate-type spectrum, with the accompanying loss of a good isobestic point. But this tendency is markedly less than with acridine orange. For these reasons it is unwise to assume that all binding by process II, which is "weak" in the energetic sense, can be described as due to stacking, in Bradley's sense, though it is usually true that all stacked acridines are weakly bound, energetically speaking. We also think it is confusing to

describe either weak binding by process II or stacking, as defined by Bradley et al., simply as electrostatic binding. For, although electrostatic forces play a greater (probably dominant) role in process II compared with process I, and in stacking, yet attractive forces between bound aminoacridine molecules are also clearly involved—just as in binding by process I, attractive forces between aminoacridine and nucleotide rings must be involved in addition to electrostatic forces. So binding by process I, by process II, and stacking can none of them be accurately described simply as electrostatic binding as if such forces were alone operative.

The other main categories of evidence on the nature of the interaction of aminoacridines with nucleic acids are summarized in the following section.

STRUCTURAL ASPECTS OF THE INTERACTION

The Effect of Aminoacridine Structure on Binding

The greater tendency of aminoacridine derivatives, such as acridine orange, which self-aggregates readily, to bind as aggregates on nucleic acids (stacking or process II binding) has already been mentioned.

As with their ability to act as bacteriostatic agents,[1] only those aminoacridines interact strongly which are in the fully cationic form at the pH (normally 6–8) of the binding experiments, which usually means[40] that an amino group which may be substituted is present at the 3-, 6-, or 9-positions of the acridine ring. It was also shown that a minimum planar area of 38 Å^2 (corresponding to the three rings of acridine) was required in the acridine ring for bacteriostatic activity. Hydrogenation of one ring of 9-aminoacridine to form the quinoline derivative, 9-amino-1,2,3,4-tetrahydroacridine (THA) practically eliminated its bacteriostatic activity[1] and reduced its maximum capacity to bind to DNA by process I by more than a half,[35] presumably on account of its possession of a bulky, buckled ring in place of one of the aromatic rings of 9-aminoacridine. However, THA could still bind to DNA, presumably through possession of its two planar rings, and in this respect it is analogous to the quinoline derivative chloroquine[70] which probably, like the acridines, intercalates (see "Binding by Process I"), at least partially, into DNA. When long and bulky side chains are attached to the 9-amino group of 9-aminoacridine (as in acranil and atebrin) the binding is not reduced but somewhat enhanced.[35] The structure of the complex must be such that the 9-position is free to attach long side chains without detriment to the interaction. The dependence of ability to bind to nucleic acids on the basicity of the acridine has recently been confirmed by Löber,[41] who also showed that alkylation of the ring nitrogen (e.g., *N*-methylation of proflavine to acriflavine) enhances the binding ability of an aminoacridine to an extent not very dependent on the length of the alkyl chain.

Effect of Nucleic Acid Structure on Binding

It has long been known[42,43] that changes in secondary structure of the nucleic acid can have considerable influence on the binding process as revealed by the observed metachromasy, the displacement of the absorption spectrum of the bound dye. Denaturation of DNA caused an increase in the proportion of acridine orange which was bound in the 'stacked' form, as indicated by the need to increase the ratio of DNA to dye to bring about complete unstacking.[17] The evidence[35] has been described above and it indicates that denaturation by heating, followed by shock cooling, causes no decrease in the tendency of DNA to bind proflavine by process I but enhances the binding by process II, which corresponds to the stacking of acridine orange. Binding by 9-aminoacridine and of THA was not decreased and probably enhanced by denaturation of the DNA.[35] As already mentioned, this can only mean that destruction of long runs of intact helices does not reduce the extent of binding by process I. However, DNA denatured by heat, and shock-cooled, still contains entangled interacting chains and this result by itself still leaves as an open question the exact nature of the structural requirements in nucleic acids for the binding of acridines by process I.

Ribosomal RNA exhibits the same type of binding curve as does DNA[12,16] although it binds less proflavine and the inflection point, at $\mu = 0.1$, is at about $r = 0.09$ instead of at $r \simeq 0.2$, as with DNA. This suggests that the maximum binding (n_1) of proflavine by process I is, for RNA, about half of its value with DNA. Since such RNA contains both single-stranded and helical regions, through the chain doubling back on itself, it is not clear from the binding curve alone, to which parts of the chain the acridine is bound. The relation of the extrinsic Cotton effect (see "Effect of Binding on the Aminoacridine") to r suggests, however, that only one mode of binding is in fact operative at $r < 0.2$ when strong binding by process I prevails, as judged from the binding curves (ref. 16, Figs. 3 and 6).

The spectral displacements and the induction of Cotton effects indicate a similarity between the interaction of proflavine with double-helical poly(A + U)[16] and with double-helical poly A at acid pH[71] on the one hand, and, on the other hand, DNA under conditions where both monomers and aggregates are binding and the isobestic point is not exact (e.g., DNA at pH 2.8, heat-denatured DNA at $\mu < 0.1$). Determination of binding curves by equilibirum dialysis has recently[71] confirmed that proflavine binds not only to poly A in its helical form at acid pH but also extensively to neutral poly A in its single stranded, though somewhat stacked, form at neutral pH; the visible spectrum of the proflavine is then markedly depressed,[16] with only a small displacement of its maximum. Addition of poly U to solutions of proflavine[16] had only very slight depressive effects on the spectra which suggests that little or no interaction was occurring.

The various evidence is thus somewhat confusing. It seems possible to rationalize it by asserting that, in order for aminoacridines to be bound strongly by process I to a system of polynucleotide chains, the conditions must be such that there is definite interaction, by either hydrogen bonding or by stacking forces, between the bases, which may be on the same or on different polynucleotide chains. These conditions are fulfilled not only by intact double-helical DNA but also by other, though not all, polynucleotide systems. Whether or not this generalization can be upheld will depend on further investigations but it appears to represent the situation better than the apparently simpler assertion that the existence of strong binding is dependent on long runs of an intact double-helical structure. This generalization also seems to be consistent with the observations[39b] on the interaction of nucleic acids and of polynucleotide homopolymers and their mutual complexes with ethidium bromide, which behaves like proflavine in so many respects.[39a]

There is some evidence that the base compostion of nucleic acids is also important to the interaction. Binding of acridine orange to polynucleotides, and probably RNA fractions, is dependent on the base composition,[44] and fluorescence quenching studies[50] suggest that binding is greatest to those sites with least quenching efficiency, which appear to be the adenine-thymine base pairs. This agrees with the observation[45] that the T_m of complexes of DNA with acridine orange increases with the adenine–thymine content of the DNA, with the observed ability of adenine and thymine mono- and polynucleotides to suppress the fluorescence of proflavine solutions[46,47] and with theoretical calculations.[34]

Relation of the Plane of the Aminoacridine to the Helix Axis and to the Planes of the Nucleotide Bases

Studies[48] on the polarized fluorescence of atebrin bound to flowing DNA (at $r \simeq 0.015$) showed that the plane of the atebrin ring was nearer to that of the bases than that of the helical axis of DNA. Since the oreintation of the DNA molecules by flow could not be complete under the conditions employed this evidence cannot go further than affirming that in the complex the planes of the acridine rings are within $\pm 30°$ of those of the DNA bases.

Flow dichroism studies[48,49] on the complexes of acridine derivatives with DNA show that the DNA itself it slightly more dichroic[48] when the acridine is bound, thus the DNA bases are not tilted from their usual relation to their helical axis, and the dichroism of the acridine and of the DNA bases are similar so that the planes of the bases appear to be approximately parallel to the planes of the bound acridine rings. But again, the actual experimental evidence and the incompleteness of orientation of the DNA by flow only allow the assertion that the plane of the acridine ring is within $\pm 36°$ of that of the DNA bases. Unfortunately the values of r at which observations have been made are not always clear so that the relative extents of binding by I and II are uncertain. This is an important factor in the inter-

pretation of such optical evidence: for example, the change of circular dichroism on flowing complexes of acridine orange and DNA indicated[50] that the acridine orange cations were roughly halfway between being perpendicular and parallel to the DNA helix axis; however, the value of r was 0.5 so that at least half the acridine orange was bound by process II and this evidence could therefore not give an unequivocal indication of the orientation of acridine orange with respect to DNA when bound by process I. Subsequent more detailed studies[51] of flow circular dichroism, however, still indicate that, when acridine orange is bound as monomer (process I) to DNA, it is more perpendicular than parallel to the DNA axis, but deviates sufficiently from the perpendicular to require some tilting of the bases to accommodate the acridine orange. That the transition from process I to process II binding of proflavine involves a progressive change from an orientation of proflavine approximately perpendicular to the DNA axis to a more disordered state is nicely demonstrated by the observation of Houssier and Fredericq[52] that, when the DNA in nucleohistone is oriented in an electric field, there is a progressive loss in the negative dichroism of the bound proflavine as the value of r increases.

Effect of Binding on the Nucleic Acid

Combination of proflavine and acridine orange with DNA causes a marked increase in intrinsic viscosity and a decrease in sedimentation rate. Both of these properties have now been determined[24,53] as a function of r, and the best available hydrodynamic model for DNA, that of the worm-like chain, has been applied in order to interpret the results in terms of the change in the configuration of the DNA. The change in viscosity corresponds closely, up to r values of about 0.2, to an increase in contour length for each bound aminoacridine (proflavine or 9-aminoacridine) roughly equal to the normal spacing (3.4 Å) between DNA bases.[53] This process of extension reaches a limit when r attains a value corresponding to the completion of binding by process I (i.e., $r_{\mathrm{I}} = n_{\mathrm{I}}$), after which no further increase in viscosity occurs with increase in r ($= n_{\mathrm{I}} + r_{\mathrm{II}}$, in this range). The decrease in sedimentation coefficient with r also indicates an increase in contour length of the DNA double helix[24,54] but when the two sets of observations, on viscosity and sedimentation coefficients, are combined it is found[24] that the value of the Scheraga-Mandelkern coefficient,

$$\beta = Ns^{\circ}_{20}[\eta]^{1/3}[\eta]_0/M^{2/3}(1 - \bar{v}\rho)$$

decreases by about 20% from $r = 0$ to $r = 0.16$. Normally this parameter is very insensitive to shape changes, and an examination[24] of this change in the light of the available hydrodynamic theories leads to the conclusion that, although the contour length is indeed increased as a result of binding of the aminoacridine, the persistence length, which is a measure of the stiffness of the DNA, is in fact decreased from 700 to 300 Å. Study of a wider range of compounds also showed that basic dyes with three or four fused rings,

benzacridines, and a triphenylmethane dye all enhanced the viscosity of DNA.[55]

It is interesting to note[53] that the viscosity of heat-denatured DNA, which is, of course, much less that of native DNA, either does not change at all on complexing with proflavine and 9-aminoacridine at $\mu = 0.1$, or is decreased along a flat curve at $\mu = 0.001$. The last effect is indistinguishable from the effect of adding extra salt to a solution of $\mu = 0.001$ and is an example of ion-shielding on the viscosity of a polyelectrolyte. The absence of a specific increase in the viscosity of denatured DNA on interacting with aminoacridines suggests that the increase in contour length on strong binding of aminoacridines by process I is a specific feature of the interaction with the intact double-helical structure. Conversely, it is clearly possible for such strong interaction by process I to occur, without there being an increase in the contour length, in polynucleotide structures other than the intact double helix.

The increase in contour length has been confirmed by autoradiography,[56] and light scattering[57] reveals a corresponding increase in the radius of gyration. This expansion is accompanied by a decrease in the mass per unit length, along the helical direction, according to low-angle x-ray measurement[58] and light-scattering measurements,[57] and by a loss of the hypochromicity of the DNA at 260 mμ.[36,46] X-Ray diffraction patterns obtained from fibers of the DNA-proflavine complex were[54] qualitatively consistent with a model in which the two DNA strands both untwist and extend on interacting with DNA, and similar conclusions have been drawn from such studies[59] on the complexes of ethium bromide and DNA. A more detailed study[60] has, in general, confirmed this view, although the phenomena are much more complex than first appeared. For example, the x-ray evidence is consistent with an unwinding of between 12° and 45° from the original relative orientation of +36° between successive base pairs, so that the final relative orientation may be between +24° and −9°.

The interaction of aminoacridines with native DNA produces not only the configurational changes mentioned above, but it also stabilizes the macromolecule towards thermal denaturation[20,34,54,61,62] mainly through its electrostatic effect.[33]

Effect of Binding on the Aminoacridine

The most characteristic effect of interaction with nucleic acids on acridine derivatives is the displacement of the electronic absorption spectrum to longer wavelengths, when bound as single cations (α spectrum), and the reversal of this trend when the acridine is bound as dimers and aggregates (β and γ spectrum). Quenching of the fluorescence of the acridine on binding is also a notable change. Comparison[46] of the spectra obtained in the case of acridine–acridine interaction with a related study of the change in the fluorescence spectra of aminoacridines bound to nucleic acids, and the observation[20] that the oscillator strength of proflavine bound to DNA is constant up to the point where process II supersedes I lead to

the conclusion that in strong binding by I the absorption characteristics of the bound acridine are altered, at least partly, as a result of coupling with the nucleotide bases; whereas in weak binding by II or stacking, the alterations are the result of coupling between bound acridines, as in concentrated acridine solutions.[63,64] This interpretation is important in deciding the structure of complexes I and II. A full theoretical treatment of the electronic and vibrational energy levels is not yet available, although this does not preclude the practical use of the changes in absorption spectra and in fluorescence in determinations of binding curves. It should be noted that the spectral displacements are not confined to the visible regions but also occur in the ultraviolet absorption bands.

When aminoacridines are bound to nucleic acids they become optically active as a result of the mutual asymmetric coupling of electric dipole transitions of aminoacridines bound adjacently to each other, in groups of 2 or 3.[51,65,66] This coupling can arise when the bound aminoacridines are separated by 6 or 7 Å, sufficiently distant for the binding energies not to be affected. The induction of such an extrinsic Cotton effect can therefore be associated with binding by nucleic acids of proflavine by process I[65] as single molecules, which then interact in pairs (or threes). However when dimers of acridine orange (not usually optically active) are bound to nucleic acids, optical activity can also be induced by coupling between the acridines in adjacently bound dimers, and the resulting optical rotation can show complex variations with wavelength and with the ratio of dye to nucleic acid.[51] These phenomena have been studied in some detail and the possibility of optical activity being induced in the bound acridine has been found to depend in subtle ways on the structure, rigidity and helicity of the polynucleotide chains.[65]

The chemical reactivity of the amino groups of the aminoacridines is reduced when they are bound to DNA: thus there is a fiftyfold reduction in the rate of diazotization of proflavine and other aminoacridines on binding to DNA.[67] This reduction is much greater than when they interact with synthetic polymers, and it must be concluded that in the complex with DNA the amino groups are much less accessible to these nitrosating reagents.

THE STRUCTURE OF THE COMPLEX

The binding curves clearly indicate the existence of a stronger (I) and weaker (II) mode of binding of the aminoacridines on nucleic acids and the structure of each type of complex must be considered separatively.

Binding by Process I

Binding by process I has the following experimental characteristics.

(*a*) It is strong, i.e., the binding energy is of the order of 6–10 kcal/mole of aminoacridine bound.

(*b*) It is favored by the possession by the acridines of three flat aromatic rings (ca. 38 Å^2) which can interact with the nucleotide bases of the nucleic

acid, although two such rings also appear to allow such binding.[35,70] These interactions can be broadly described as van der Waals forces and are dependent on the nature of the bases.

(*c*) It is, at least in part, electrostatic for the interaction increases with decreasing μ. Only the cations bind strongly.

(*d*) The contour length of double-helical DNA is increased and its mass per unit length is decreased on binding.

(*e*) The contour length of denatured DNA is not increased on binding, if μ is large enough to suppress polyelectrolyte effects.

(*f*) The planes of the bound acridine cations are approximately (to within 30°), parallel to the nucleotide bases and perpendicular to the axis of double helical DNA.

(*g*) The reactivity of the amino groups of the aminoacridine is diminished on binding.

(*h*) Binding by process I to denatured DNA occurs to the same extent and with approximately the same range of binding constants as on intact double-helical DNA.

(*i*) Binding by process I occurs with approximately the same range of binding constants though with fewer sites (lower n) on single-stranded RNA as on DNA.

(*j*) Long side chains attached to the 9-amino position do not reduce binding in the 9-aminoacridine series.

(*k*) There is an upper limit at $r \simeq 0.2$ to 0.25 to binding by I.

Features (*b*) and (*c*) have been known since the first investigation of metachromasy of acridine dyes in cell staining reactions, and in 1947 Michaelis[15] affirmed that the flat dye molecules might lie between different bases with their positive groups close to the negative phosphates of the DNA. As additional evidence accured this view was reaffirmed by Oster,[29] and, after the discovery of the double-helical structure of native DNA, it was suggested in 1955 and 1956 that the acridine rings could slip into a plane parallel to and between successive base pairs of this structure.[12,28]

After the two types of binding had been distinguished,[12] and the strength of binding by I clearly noted, Lerman[54] made the initial observations on which (*d*) was based. This, and subsequent evidence for (*f*) and (*g*), led to his proposal[48,54,55] of an intercalation model, in which the aminoacridine cation is inserted between and parallel to, successive base pairs of double-helical DNA, which has to untwist and extend in order to accommodate them. The aminoacridine cation, in this model, lies centrally over a base-pair so that the positive nitrogen atom is near to the central axis of the molecule and equidistant from the two polynucleotide chains. Since there must be a limit to which the DNA can unwind in this way to accommodate acridine cations, the existence of a limit (n_I) in binding by I would be expected.

This model accounts adequately for the observations summarized in (*a*), (*b*), (*c*), (*d*), (*f*), (*g*), (*k*), but in our view, needs modifying to account also for observations (*e*), (*h*), (*i*), (*j*), which have emerged subsequently.

The modification (Fig. 4) which we have proposed[68] is that, when acridine cations are bound by process I, the acridine lies between successive nucleotide bases on the same polynucleotide chain, in a plane approximately parallel (*f*) to the base planes, but at an angle (looking down the polynucleotide chain) such that the positive ring nitrogen is close to the polynucleotide phosphate group. This condition is met equally well by native double-helical DNA, denatured disordered DNA, and single-stranded RNA [(*h*) and (*i*)]. Construction of this model shows that the 9-amino position is so placed that long chains attached at this point do not interfere with the structure (*j*), which is not true for the previous intercalation model[48] in which the acridines (and so the 9-position) are inside the center of the double helix. Moreover, this modified intercalation model does not make the presence of intact double helices the condition of strong binding by process I, for such strong binding occurs when the nucleic acid structure is more mixed [(*h*) and (*i*)]; and it accords better with those structural requirements for strong binding of aminoacridines by polynucleotide structures which appear to be emerging from the various evidence (Section "Effect of Nucleic Acid Structure on Binding"). It also explains why THA, which is 9-aminoacridine with one ring buckled by hydrogenation, can bind at all, though to an extent less than 9-aminoacridine. For if intercalation between and exactly over the base pairs was necessary for strong binding it is difficult to see how the THA structure, and that of chloroquine,[70] could be inserted at all.

In order to bind acridines through this modified intercalation, double helical DNA would have to untwist and extend, as observed (*d*), but denatured DNA and single stranded RNA would already have open extended chains, like that in Figure 4, into which the acridine cation could be inserted without any further modification of the contour length of the polynucleotide. This accounts satisfactorily for observations (*d*) and (*e*), therefore. If acridines bind strongly *only* by interclating into double helices, it is difficult to see why the viscosity of denatured DNA does not increase when they bind. Moreover the "hair-pin" double helices of RNA would have to untwist, which would be sterically difficult.

Finally, it might be added that this modified intercalation model has the advantage of making it clearer how proflavine could maintain the spurious extension of the polynucleotide chains of DNA during transcription to RNA, when the double helix must open out, so that misreadings of base sequences can occur with their consequent genetic effects.[6]

At least it is clear that "strong binding" must not be too readily identified with and taken as semantically equivalent to "intercalation" with all the features originally proposed.[54]

Binding by Process II

Binding by process II has the following experimental characteristics.

(*a*) It is weak, of the order of, at most, a few kilocalories per mole of bound aminoacridine.

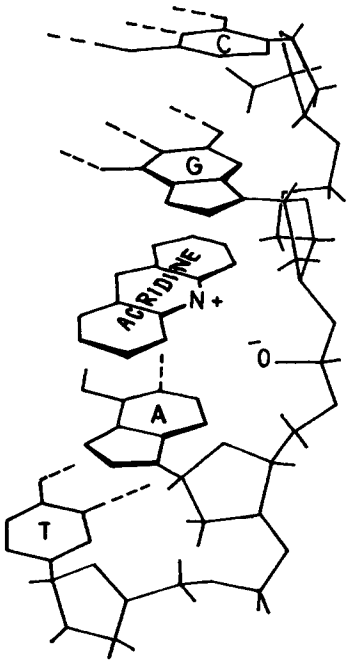


Fig. 4. Modified intercalation model for the complex of proflavine with polynucleotide chains.[68]

(*b*) It involves interaction between bound aminoacridine molecules.

(*c*) It is electrostatic and enhanced relatively more by decreases in ionic strength than in binding by I.

(*d*) Since it follows binding by I, which is more internal and covers the available base rings, it is probably an external binding process.

(*e*) The planes of the acridine rings are more disordered with respect to the planes of the base rings than in strong binding I, though this does not exclude some degree of ordered stacking.

(*f*) Denaturation of DNA enhances the tendency to bind by II.

(*g*) Binding by II can proceed up to the limit prescribed by the condition for electroneutrality, i.e., to $r = 1.0$.

The general picture of process II binding which emerges is that of acridine cations attached approximately edgewise and externally to the double helix of DNA, with their positive ring nitrogen atoms close to the phosphate groups. When r is large enough, the acridine rings can stack upon each other in a direction parallel to the helix axis. However, when r is as large as this, the helix will be much disordered by strong binding I with intercalation, so this direction of the helical axis may itself be very ill-defined and no effect on the viscosity of DNA would be anticipated. The more open structures of denatured DNA would be expected to be more accommodating to this stacking and self-aggregation of acridine cations attached to phosphate groups so that the observation (*f*) is not surprising.

The mutual interactions between bound acridines can be sufficiently strong and directed in some cases, e.g., with acridine orange, that this type of binding may be described as binding of dimers or of aggregates, which are similar to those which exist in free concentrated solutions and give rise to similar spectral shifts.[63,64]

CONCLUSION

In spite of the intricate and wide-ranging character of the experimental evidence, a reliable picture is emerging of the nature of the complex between nucleic acids and the aminoacridines, an important group of bacteriostatic and mutagenic agents. It would be tempting to attribute the entire biological effects of these aminoacridines to this interaction. Indeed, a recent study[69] has shown that the inhibition of protein synthesis by proflavine may be connected with its ability to interact with transfer RNA in particular. However, other causes are sometimes possible for the biological effects of the aminoacridines. For example, it appears[9] that the inhibition of *E. coli* RNA-polymerase by proflavine can occur not only by virtue of its interaction with the DNA primer, which is necessary for the reaction, but also by direct interaction between the proflavine and the binding sites on the RNA polymerase which interact with the nucleotide bases of the primer DNA and with the substrate nucleotide triphosphates. Other instances may prove equally mixed, and each case must be examined on its merits.

Finally, it is worth noting that most of the studies on the interaction of acridines with nucleic acids have been made on systems at thermodynamic equilibrium (but see ref. 72). Under the dynamic conditions of the living cell it is possible that it is the actual *rate* of formation and dissociation of these complexes which is relevant to their biological action. If this is so, one would expect forms of nucleic acids, in which the chains have been opened out, to bind and dissociate acridines more rapidly than long, intact double helices which have to untwist to accommodate them. Moreover, various structural features of the acridines and nucleic acids and various environmental conditions may well differ in their effects on the kinetic processes and on the equilibrium position.

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