THE INTERACTION OF AMINOACRIDINES WITH DNA.

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SUMMARY

Complete understanding of the interaction of amino-acridines with DNA requires a knowledge of the influence of DNA structure on the binding process. To this end, the interaction of 9-aminoacridine with native and denatured DNA and with several synthetic polynucleotides has been investigated.

Influence of temperature on the binding of 9-amino-acridine and of proflavine to DNA in 10^{-3}M NaCl solution has been determined by a spectrophotometric technique. The inadequacy of the expression normally used for the determination of the extent of binding is discussed with reference to measurements at temperatures above which dissociation of the double helix occurs. A method of determining the relative extents of binding to native and denatured DNA at elevated temperatures is discussed.

The binding of 9-aminoacridine to native and denatured DNA at 25^{o}C has been studied and the greater extent of binding observed to denatured DNA is attributed to an increase in the number of available intercalation sites when DNA is denatured.

The thermal stability of denatured DNA-9-aminoacridine complexes is discussed in some detail. It is shown that dye release occurs from these complexes at much lower temperatures than from complexes with native DNA. This difference in thermal stability is explained in terms of the lifetime of conformations in which sufficient overlap occurs between the intercalated dye cation and the adjacent bases. Base oscillations are postulated

to be responsible for this phenomenon.

A decrease is observed in the sedimentation coefficient of native DNA when complexed with 9-aminoacridine but no such change is noted for denatured DNA-9-aminoacridine complexes. It is concluded that marked changes do not occur in the conformation of denatured DNA when interacted with 9-aminoacridine.

Binding of 9-aminoacridine to several synthetic polynucleotides has been investigated. It is shown that these molecules have peculiar binding properties and their use as model systems for the study of aminoacridine-DNA interactions is not valid.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, nor any material previously submitted for a degree or diploma in any University, except when due reference is made in the text.

L. N. SANSOM

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ABBREVIATIONS

The following abbreviations have been used in this thesis:

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Poly A	polyriboadenylic acid
Poly dAT	Copolymer containing adenine and thymine deoxyribomononucleotides in alternating sequence.
Poly U	Polyuridylic acid
Poly (A).Poly (U)	Double-stranded complex of associated poly A and poly U.
Poly (dA).Poly (dT)	Double-stranded complex containing one strand of an adenine deoxyribopolynucleotide and one strand of a thymine deoxyribo-polynucleotide.
Poly (dG).Poly (dC)	Double-stranded complex containing one strand of a guanine deoxyribopolynucleotide and one strand of a cytosine deoxyribopolynucleotide.
O.R.D.	Optical rotatory dispersion.
C.D.	Circular dichroism.
N.M.R.	Nuclear magnetic resonance.

CHAPTER I

INTRODUCTION

The fundamental role of nucleic acids in the maintenance of life in all forms of living matter is now well established. These compounds are responsible for gene duplication, by which genetic information is transferred from parent to progeny, and also for gene expression which involves the translation of gene structure into polypeptide structure. Two types of nucleic acids are involved in these processes. Deoxyribonucleic acid (DNA) acts as the template for both gene replication and expression, while ribonucleic acid (RNA) is the means by which the information stored in the DNA is translated into proteins. In some viruses lacking DNA, (for example F2 and Qβ phages), RNA can fulfil both functions.

Although nucleic acids were first discovered by Miescher in 1869[1], their structure and function has only been elucidated in detail during the past 20 years. Both DNA and RNA consist of linear chains of alternating sugar and phosphate moieties joined together by C_3 - C_5 phospho-diester linkages, with purine and pyrimidine bases attached to the C_1 atom of the sugar group[2]. The unit of a phosphate group, sugar residue, and an attached base is referred to as a nucleotide. The major differences between RNA and DNA are the type of sugar involved (D - Ribose for RNA and 2' - Deoxyribose for DNA) and the nature of the heterocyclic

bases attached. The bases usually found are adenine, guanine, cytosine and either uracil (RNA) or thymine (DNA), although other bases are known to occur especially in some viral nucleic acids[3]. The genetic information is contained in the sequence in which these bases are arranged in the DNA (or RNA for certain viruses).

The most significant single contribution made to nucleic acid chemistry has been the characterization of the secondary structure of DNA by Watson and Crick[4,5]. These workers proposed that the DNA molecule is composed of two polynucleotide chains coiled around a common axis in the form of right hand helices with the planes of the bases arranged perpendicular to the major helix axis, the chains being held together by hydrogen bonds between specific pairs of bases. The base pairing arrangement is adenine with thymine and guanine with cytosine. This double helical structure of DNA has since been confirmed by many other workers[6] and is now generally accepted as the basic structure of DNA both in vivo and in vitro, although single stranded DNA molecules have been isolated from some viruses (e.g. ϕ x 174).

In view of the dominant role of DNA in biological systems, study of its interaction with other chemical species has developed into a major field of research especially in the areas of biochemistry and medicinal chemistry. Changes

in the composition or structure of DNA, induced by interacting compounds, can be expected to have a profound effect on the normal functioning of DNA in living cells. The biological activity of a wide range of compounds possessing mutagenic[7-9], carcinogenic[10-15], antibacterial[16-18] and antiprotozoal[19-21] activity, has been explained by their ability to interact with DNA. Studies on the interaction of compounds with DNA have also contributed to a further understanding of DNA structure and its behaviour in solution.

The binding of aminoacridines to nucleic acids has been the subject of extensive investigation for many years. The interest in these compounds comes from their antibacterial and mutagenic activity[8,22] and their use in histological staining techniques[23,24].

The interaction between DNA and aminoacridines has been described in terms of two distinct binding processes[25-27]. Cavalieri et al[25,26] proposed that this heterogeneity arose from the binding to the primary and secondary phosphate groups of the DNA. This proposal has since been rejected because of the insufficient number of secondary groups involved to account for the extent of binding. Further, because the pKa of the secondary phosphate group is of the order of 6-7, the observation that the binding is independent of pH in the range 3.5 - 7.4 indicates that these groups are unimportant

in the interaction[27]. Peacocke and Skerrett[27] assigned the two processes to a strong interaction of monomeric dye cations, predominant up to a ratio of bound dye per atom of DNA phosphorus (r) of approximately 0.2, and to a weaker interaction of dye aggregates up to a value of r = 1.0. Although the importance of the purine and pyrimidine bases in the interaction was recognised[27-30], the Watson and Crick model, in which the bases are in close Van de Waals contact, seemed to preclude the direct interaction between the bases and the bound dye cations. However, in 1961 Lerman[31] proposed that if the DNA molecule extended, then dye cations could be intercalated between adjacent base pairs thus allowing interaction between the dye and the bases. Evidence of such a change in the configuration of the DNA when aminoacridines are bound has been provided by many workers employing a variety of experimental techniques[31-41]. Although modifications to the Lerman model have been proposed[42-45], the basic concept of a structural change of the DNA allowing the insertion of the dye cation between the bases has been generally accepted as the mode of the strong, primary binding of aminoacridines to DNA. A detailed discussion of the current intercalation models is presented in Chapter II.

The intercalation hypothesis provides an explanation of the mutagenic activity of aminoacridines.

On intercalation the distance between base pairs is doubled as a result of the extension of the DNA molecule[31-33]. This modification in structure allows for the insertion or deletion of a base pair during DNA replication[8,16,37]. The ability of aminoacridines to induce mutants of this type has been utilized in the elucidation of the triplet nature of the genetic code[52]. The intercalation model has also been used to describe the interaction of DNA with many other biologically active compounds[46-51].

The weaker secondary binding is believed to involve the attachment of dye cations to the exterior of the helix by an electrostatic interaction with the phosphate groups. This interaction involves both dye monomers and higher aggregates depending upon the tendency of the aminoacridine to self-associate in solution[44].

Aminoacridines also interact with RNA although to a lesser extent than with DNA[27,53]. Whether the nature of the binding to both nucleic acids is identical has not yet been resolved, although evidence suggests that interaction occurs between the bases of RNA and the bound dye[53].

The aim of this study is to determine the influence of nucleic acid structure on the binding of 9-aminoacridine. Initially the study was concerned with an examination of the binding at temperatures at which

dissociation of the DNA helix occurred. The results indicated a difference in the binding to native and denatured DNA at elevated temperatures. In order to examine this observation further, the binding of 9-aminoacridine to both forms of DNA at lower temperatures was examined, together with the temperature dependence of the binding to denatured DNA. The interaction of 9-aminoacridine with several synthetic polynucleotides was also investigated with the view that these less-complex systems might yield further information on the interaction of acridine dyes with DNA.

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CHAPTER II

THE INTERACTION OF AMINOACRIDINES WITH DNA

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References.

1. Introduction

The interaction of aminoacridines with DNA has been described in terms of two distinct binding processes[1]. In the primary stage, at a low aminoacridine/DNA ratio, there is a strong binding of dye cations for which the binding energy is of the order of 6-10 K cals/mole of aminoacridine bound. In the secondary stage, at a high aminoacridine/DNA ratio, a much weaker binding predominates. The current models assign the primary binding to an interaction between dye cations and the bases of the DNA via an intercalation mechanism. The weaker process is attributed to an interaction between dye cations and the negatively charged phosphate groups on the exterior of the helix[2].

In this chapter the experimental evidence which led to these models is discussed, together with a description of the current intercalation models. Because of the vast quantity of literature published in the general area of dye-DNA interactions, for the purpose of this thesis, only those papers which are considered to have made significant contributions to the field will be discussed in this chapter.

2. Binding Curves

Determination of the number of binding sites and the strength of interaction is fundamental to any study of the interaction of small molecules with macromolecules. The interaction of small ions or molecules with a macromolecule

containing n binding sites, which are equivalent and independent, may be described by the following expression[3]

$$r = \frac{n\ k\ c}{1 + kc} \qquad \text{(II.1)}$$

where r is the number of ligands bound per molecule of macromolecule, c is the free ligand concentration, k is the intrinsic association constant.

$$k = \frac{[\text{occupied sites}]}{[\text{unoccupied sites}]\ [\text{free ligand}]}$$

If the macromolecules possesses p different types of binding sites, then

$$r = \sum_{j=i}^{j=p} \frac{n_j k_j c}{1+k_j c} \qquad \text{(II.2)}$$

where n_j is the number of binding sites of type j per macromolecule and k_j is the corresponding association constant.

For a single type of binding site, equation II.1 may be simplified to

$$r/c = kn - kr \qquad \text{(II.3)}$$

Thus a plot of r/c against r will be linear with an intercept at r = n on the r axis, and a slope of -k (Fig.II-1a). Such a plot is referred to as a Scatchard plot. Curvature of the plot can result from a variation in one or both of two factors[1]

1. Electrostatic field effects due to the variation with r of the electrostatic potential at any binding site. This effect is suppressed at high ionic

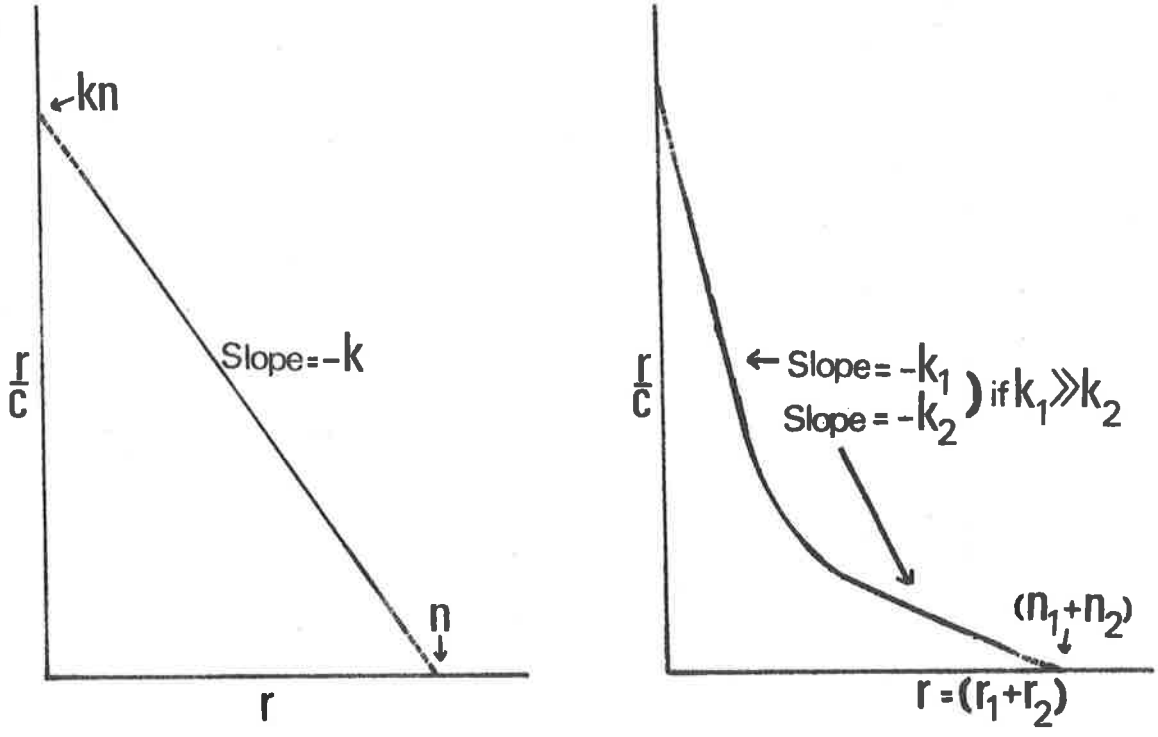

FIG. II.1 : Shape of the $^{r}/_{c}$ against r plot for:

(a) one type of binding site

(b) two types of binding sites.

strength.

2. The overlapping of the binding to more than one type of binding site.

Even when more than one type of site is present, it is sometimes possible to distinguish distinct linear portions in the Scatchard plots enabling individual n_j and k_j values to be determined. Fig. II-1b illustrates the case if there are two types of binding sites, for which equation II.2 becomes

$$r = \frac{n_1 k_1 c}{1 + k_1 c} + \frac{n_2 k_2 c}{1 + k_2 c}$$

The use of binding curves and the analysis of Scatchard plots have been extensively used to provide information on the number and nature of the aminoacridine binding sites on DNA[1,4,5].

While equations II.1 and II.2 have been derived assuming no cooperativity in binding, i.e. based on a Langmuir type binding, the possibility exists whereby bound aminoacridines facilitate the binding of further molecules. The existence of this phenomenon may be detected since the cooperative binding curves, (r versus c), are convex to the c axis, while non-cooperative curves are concave to the c axis[2].

Recently, detailed statistical mechanical treatments have been used to rigorously analyse binding curves[6-8], and this approach could add much needed sophistication to the analysis of complex binding curves.

3. The Effect of Binding on the DNA

a. Structural changes of the DNA

The binding of acridine dyes significantly increases the intrinsic viscosity of DNA[9-11]. The degree of enhancement increases with the extent of binding to a limit corresponding to the end of the primary binding process. This observation indicates that only those dye cations which are intercalated contribute to the increase in viscosity. This assertion has recently been confirmed by the detailed viscosity studies of Armstrong et al[11].

Drummond et al[10], using the worm-like chain hydrodynamic model of DNA, concluded that the increase in the viscosity, due to the binding of aminoacridines, results from an increase in the contour length of the DNA. This extension is such that the distance between an intercalated dye cation and its neighbouring bases is of the same order of magnitude as existing between the base pairs in uncomplexed DNA. According to this hypothesis, the contour length of the DNA would increase by a factor of $(1+2r_1)$, where r_1 is the number of intercalated dye molecules per nucleotide. Armstrong et al[11] have shown that the viscosity enhancement due to the binding of proflavine and acridine orange, is in fact consistent with the increase expected if the chain length was extended by this factor by the addition of extra base pairs. A similar result has also been reported for sonicated DNA-proflavine

complexes[12]. The viscosity results of Armstrong et al[11] also indicate an increase in the flexibility of the DNA molecule when intercalation of dye cations occurs. This result is in accord with a previous observation by Lloyd et al[13] using a combination of viscosity and sedimentation data.

Drummond et al[10] observed that the viscosity of denatured DNA did not increase when proflavine or 9-aminoacridine were bound, and in fact, decreased at low ionic strengths. This observation suggests that the increase in contour length of the DNA upon intercalation is a specific property of the double-helical structure of native DNA, since interaction of aminoacridines with the nucleotide bases also occurs in denatured DNA[2]. Thus, while an increase in intrinsic viscosity may be taken as evidence of an intercalation mechanism into double helical structures, it is possible that base-dye interactions may occur with other polynucleotide structures without the necessity of an increase in contour length.

The interaction of aminoacridines with native DNA also results in a decrease in the sedimentation coefficient of DNA, indicative of a decrease in the mass per unit length of the DNA strands[5,9,12,13]. This observation is compatable with the increase in contour length suggested by viscosity data. Because the insertion of a dye cation into the extended helix would contribute a mass increment less than that

of a base pair, a reduction in the mass per unit length is to be anticipated.

Further evidence of the extension of the DNA molecule when aminoacridine cations are bound has been afforded by light scattering[14], X-Ray diffraction[9,15] and autoradiography[16] studies.

b. Thermal stability of the DNA

The binding of aminoacridines to DNA stabilizes the double helical structure against thermal denaturation. This property is indicated by an increase in the temperature required to cause strand separation, i.e. an increase in the melting temperature, T_m[17-19]. At a constant ionic strength, the magnitude of the increase in T_m is a function of the extent of binding (r), and reaches a maximum at a value of r corresponding to the completion of the primary binding process[17,20]. This suggests that the enhanced stability is a result of the interaction of dye cations with the bases of the DNA. Further evidence is gained by the observation that the dye bound to the exterior of the helix is dissociated at temperatures lower than the melting temperature[17,19].

The increase in T_m indicates that a large decrease has occurred in the free energy of the native DNA-dye system over that of native DNA. This decrease must be greater than the difference in free energy between denatured DNA-dye systems and denatured DNA. The free energy calculations of Gersch and

Jordan[17] support this assumption.

Chambron et al[18] explained the increased stability as a result of an electrostatic effect due to the reduced repulsion between phosphate groups when dye cations are intercalated. However, as shown by Kleinwachter et al[19], the change in free energy of interaction results not only from electrostatic interactions, but also from the specific interactions between the dye cations and the purine and pyrimidine bases of the DNA.

4. The Effect of Binding on the Aminoacridine

a. Electronic Absorbance Spectra

The displacement of the electronic absorption spectrum of an aminoacridine when bound to DNA is the most characteristic feature of the interaction. As a rule, when the dye is bound as a monomer, a shift of the absorption maximum of the aminoacridine to longer wavelengths is observed together with a decrease in the extinction coefficient[1,4,21]. When the dye is bound as dimers or higher aggregates the spectrum is shifted to shorter wavelengths. For example, when acridine orange is bound to DNA under conditions where there is a large excess of binding sites, the wavelength of maximum absorption is displaced from 492nm to 505nm. However, as the concentration ratio of acridine orange to DNA increases, the spectrum exhibits a maximum at 465nm. The blue shift

observed is identical to that obtained with concentrated solutions of acridine orange and is indicative of the formation of dye aggregates. The concentration ratio at which this reversion in spectral shifts is observed depends upon the tendency of the various aminoacridines to self-associate. Thus acridine orange, which has a strong tendency to aggregate in solution, will exhibit this phenomenon at much lower concentrations than will proflavine which has a lower tendency to self-associate[22].

This displacement of the visible spectrum to longer wavelengths has been attributed to the interaction between dye cations and the planar bases of the DNA[4,23,24]. The interaction of proflavine with polyphosphate does not cause any such displacement[24]. Associated with the displacement of the spectrum of the dye in the visible region to longer wavelengths, is a decrease in the absorbance in the ultra-violet region. This decrease has been attributed to π-orbital interactions between the dye and the purine and pyrimidine bases, and not to interactions between adjacent dye cations[24].

When the spectrum of the aminoacridine is displaced to that of the bound monomer spectrum, it is possible to calculate the relative amounts of free and bound dye, thereby enabling the determination of a binding curve[1,2]. (see Chapter III).

Mixtures of proflavine and acid or heat denatured

DNA show spectral changes similar to those observed for native DNA[23], and may also be attributed to the coupling of the heterocyclic rings of the aminoacridines with the bases of the DNA.

Thus the changes observed in the electronic absorbance spectra of aminoacridines complexed with DNA, under conditions where self-association is minimal, indicates an interaction between the dye and the bases of the DNA. However, these observations do not exclude the possibility of binding to other sites on the DNA molecule.

b. Optical Rotatory Dispersion and Circular Dichroism Spectra

A beam of plane polarized light may be regarded as being composed of right and left circularly polarized waves. No optical rotation is observed if these components are transmitted with equal velocity through the medium. However, if the two components are transmitted with different velocities, then the plane of their resultant will be rotated. A medium is optically active if it has different refractive indices for the two components of the polarized light. Because the magnitude of the refractive indices varies with wavelength, the extent of the rotation is also wavelength dependent and thus an O.R.D. spectrum can be obtained.

If the incident right and left circularly polarized

components are absorbed unequally by the medium, the phenomenon is known as circular dichroism. Because the extinction coefficient for both components varies with wavelength, the relative amounts of each component absorbed in passage through the medium will also be wavelength dependent, enabling a C.D. spectrum to be obtained.

The optical rotation within an electronic absorption band has an interesting characteristic. It increases to a maximum near the absorption band, then decreases to zero within the band and finally increases in the other sense to a second maximum. This phenomenon was observed by Cotton in 1896 and is referred to as the Cotton Effect. However, it should be noted that not all absorption bands in a molecule are optically active.

The use of O.R.D. and C.D. in the study of amino-acridine-DNA interactions is potentially far more useful than is absorption spectroscopy, because of the greater sensitivity of O.R.D. and C.D. to environmental and structural factors.

In 1961, Neville and Bradley[25] observed an extrinsic Cotton Effect in the absorption wavelength region of acridine orange when bound to DNA. The observed rotation must have been due to an asymmetric perturbation of the dye chromophore by the DNA because by itself acridine orange is optically inactive. Induced Cotton Effects have since been observed for other aminoacridines when bound to DNA[26-29]. The induction

of optical activity in an optically inactive dye implies that a definite spatial relationship exists between the dye and its binding site. The dependence of the induced rotation on temperature, pH, ionic strength and relative concentrations of aminoacridine and DNA has thus been studied to obtain information of the structure of the dye-DNA complex[26-28].

A number of explanations have been postulated for the occurence of the optical anisotropy.

1. The ligands are orientated in a helical array. Such an arrangement produces the required asymmetry in the electronic transitions of the bound molecule for optical activity to occur. This arrangement may result from external binding or by intercalation[30]. However, denaturation of DNA does not destroy the optical activity of bound proflavine or acridine orange indicating that long segments of double helix are not necessary for optical activity to occur[27].
2. An asymmetry is induced in the bound dye by the influence of the environment[31].
3. The optical activity arises from the binding of aggregates of the ligand, which themselves have a screw orientation[32]. Because induced optical activity is observed with proflavine at concentrations where aggregation is negligible[27], this explanation can be valid only when the concentration of ligand

is large enough for self-association to occur.

The observation of Blake and Peacocke[27] that the magnitude of the induced Cotton Effect was dependent upon the DNA-proflavine ratio suggested that the optical activity was the result of nearest-neighbour interactions between bound proflavine molecules. The number of adjacent molecules needed to induce the optical activity was suggested to be approximately four with native DNA and two with denatured DNA. However, a more recent publication[33] has shown that the acquired optical activity at any wavelength for 1, 2 and 9-aminoacridines does not depend upon the degree of binding. This observation implies that the induced optical activity of bound 1, 2 and 9-aminoacridines is independent of the number of adjacent dye molecules and that these molecules bound singly are optically active. Li and Crothers[29] suggested that the weak circular dichroism induced at very low degrees of binding to proflavine arose from the environmental asymmetry of the binding site and not from dye-dye interactions. As the degree of binding increased, stronger C.D. effects were induced which were due to dye-dye interactions.

At the present time no theory exists which relates circular dichroism spectra of aminoacridine-DNA complexes to the structure of these complexes, and thus any inference based on the observed phenomena is indirect. It is not yet possible to determine conclusively the mechanism by which the inter-

action between intercalated dyes induces circular dichroism. It may be the result of an electronic interaction, as postulated by Tinoco[34], or of the intercalation of a dye molecule altering the environment of dye molecules already bound.

Thus, although circular dichroism and optical rotatory dispersion techniques are potentially extremely useful in the examination of dye-DNA interactions, the complexity of the system restricts the amount of information which can be deduced from the data. However, future developments in the understanding of the mechanism of induced optical activity can be expected to yield valuable information on the interaction of aminoacridines with DNA.

c. The Fluorescence Spectra of Bound Aminoacridines

When acridine dyes are bound to DNA a change occurs in the fluorescence spectra, intensity and polarization, with respect to the free dye. In many instances the changes in the fluorescence and absorption spectra are complementary[35]. For example, a slight red shift in the absorption spectrum is observed when acridine monomers are bound to DNA, while a corresponding blue shift occurs in the fluorescence spectrum[24]. The majority of acridine dyes studied show a decrease in the intensity of fluorescence following binding[36-38]. However, acridine orange and its derivatives exhibit an enhancement of fluorescence when bound[36,38].

The quenching of fluorescence has been used to determine the fraction of dye bound to DNA, thus enabling the determination of binding curves. The advantage of this method over spectrophotometric and equilibrium dialysis techniques is that extremely low dye concentrations can be used. The study of the dye-DNA interaction at very small extents of binding (e.g. $r = 0.02$) can thus be examined. The expressions commonly used for the determination of binding curves are

$$C = C_T \left({}^{F}/_{F_o} - p\right)/(1-p)$$

and

$$r = \frac{C_B}{P} = (C_T - C)/P$$

where F and F_o are the observed fluorescence intensities of the aminoacridine in the presence and absence of DNA, and p is the ratio of the observed quantum yield of fluorescence of the bound dye to that observed for free dye. C_T, C_B and C refer to total, bound and free dye concentrations respectively, and P is the DNA concentration.

The mechanism of fluorescence quenching is still not satisfactorily understood. A number of workers[39-41] have suggested the existence of quenching and non-quenching sites on the DNA. Non-quenching sites are assigned to AT-rich regions of the DNA into which intercalation is favoured, while for quenching to occur the presence of a G-C site adjacent to the dye is required. This suggestion is compatable with the

results of Tubbs et al[40], who observed an increase in quenching efficiency with increasing G-C content of the DNA. These workers attributed the quenching of fluorescence to a charge transfer process, with the guanine residue acting as an electron donor and the aminoacridine as the receptor. The quenching of fluorescence increases as the amount of bound dye increases, and this has been explained by a change in the relative populations of quenching and non-quenching sites[39]. Opposing the view of site specificity, Ellerton and Isenberg[42] have suggested that random dye binding, with nearest-neighbour interactions leading to quenching, is the mechanism for the decrease in emitted light with an increase in binding.

The technique of fluorescence polarization[43] has yielded information on the interaction of proflavine with DNA[42]. If the exciting radiation is polarized, the probability of absorption depends upon the orientation of the absorbing sites. Further, if the direction of the induced dipole change is parallel to the direction of oscillation of the electric field vector, then the probability of absorption is a maximum. The emitted light is polarized parallel to the orientation of the dipole moment change. Thus, if dye molecules are held rigidly within the DNA helix such that their orientations do not change in the time period between absorption and emission, then the fluorescence observed will be partially depolarized. The extent of depolarization will increase if the dye cations change their orientations during

this time period, leading to a randomization of orientation of fluorescing sites. Ellerton and Isenberg[42] have shown that the degree of depolarization of proflavine bound to DNA is greater than that expected if the dye was held rigidly within the helix, and if the DNA was itself rigid. Possible mechanisms for the depolarization have been discussed[42] and include the suggestion that a bound dye cation may alter its position within the helix during the lifetime of the excited state. Another postulate suggests that the depolarization is due to some form of local flexibility of the DNA arising from rapid oscillations between adjacent base pairs[44]. While experimental evidence is lacking for either of these postulates, it does indicate the potential usefulness of fluorescence polarization measurements as a method of examining dye-DNA interactions.

An interesting application of the fluorescence spectra of acridine orange-DNA complexes has been reported by MacInnes and McClintock[45]. These workers have calculated an expression which allows the determination of DNA base composition solely from a knowledge of the wavelength of maximum fluorescence intensity.

d. Amino Group Reactivity

Lerman[46] has shown that the chemical reactivity of the amino groups of proflavine and other aminoacridines, as reflected by diazotization rates, is markedly decreased when bound to DNA. This reduction is much greater than that

observed when these compounds are complexed with synthetic polymers, such as polyphosphate and polystyrene sulphonate. Lerman concluded that this reduced reactivity resulted from the inaccessibility of the amino groups to the electrophilic reagent because of the complete enclosure of the proflavine cation within the DNA double helix.

5. The Effect of Aminoacridine Structure on Binding

The structure of aminoacridines can be expected to be important in determining both the extent and strength of the interaction with DNA. The nature and position of groups attached to the acridine nucleus will determine the electronic distribution within the molecule and thus influence the interaction between the acridines and the bases of the DNA. Steric factors can also be expected to play an important role in the location of the bound dye.

Parallel with their antibacterial activity[47], only those aminoacridines which are in their cationic form interact strongly with DNA, indicating the involvement of electrostatic forces in the interaction. However, as discussed by Löber and Achtert[48], amino-substitution in the 3 or 3 and 6 positions decreases the charge located on the C 9 and the heteroatom in favour of the amino substituents, thus allowing interaction of the amino groups, as well as the ring nitrogen, with negatively charged groups on the polynucleotide.

Substitution at the 3 or 3 and 6 positions with electron donating groups increases the basicity of the acridines, with a resulting enhancement in their ability to bind to DNA. Löber[37] has shown that the free energy of binding to DNA of a series of acridine derivatives increases linearly with increasing pKa of the dyes. Corresponding to this increase in basicity there is a decrease in the net positive charge at the ring nitrogen. Thus the enhanced binding cannot be explained by an electrostatic interaction between the ring nitrogen and the negative phosphate groups of DNA.

Ring N-alkylation of proflavine and acridine orange with methyl, ethyl, propyl, butyl and amyl groups resulted in a steep increase in the binding tendency to DNA, relative to the parent compounds[48]. However, this increase was not significantly dependent upon the length of the alkyl chain. These observations are in marked contrast to those seen for the influence of ring N-alkylation on acridine itself[48], where the equilibrium constant for the interaction with DNA decreases with alkylation to an extent dependent upon the length of the aliphatic group. This difference in the binding behaviour of acridine base and its amino-substituted derivatives when alkyl groups are attached to the ring nitrogen, suggests that this position is not very important in the interaction of aminoacridines to DNA, but is

critical for the acridine base. Further evidence for this assumption is afforded by the observation that the attachment of a benzyl group at the ring nitrogen of acridine orange has little influence on the binding[48].

The attachment of long bulky side chains to the amino group of 9-aminoacridine, such as in atebrin, does not hinder the interaction with DNA[4,49,50]. Thus the orientation of the dye in the DNA-dye complex must be such that the long chains attached to the 9-amino position does not interfere with the interaction.

The requirement of a planar ring structure for the interaction of aminoacridines with the bases of the DNA has been shown by the binding of 9-amino-1,2,3,4 Tetrahydroacridine (T.H.A.)[4]. The binding of this compound, possessing a buckled ring, is less than one-half that obtained for the planar 9-aminoacridine. This observation indicates that while a planar ring structure is favoured, the ability of THA to bind to any extent suggests a model in which the complete insertion of the dye cation within the DNA helix is unnecessary. Such a model for THA would be one in which only the two planar rings are intercalated between the bases.

Recent work by Moller and Crothers[51] has suggested that the preferred orientation for the intercalation of 3.6 diaminoacridine derivatives is one in which the long axes of the dye and the base pairs are parallel. This assertion is

based on the observation that the attachment of t-butyl groups to the 2 and 7 positions of proflavine prevents intercalation. The alternative model in which the axes are perpendicular would permit intercalation.

Evidence of the importance of the position of the amino group(s) on the acridine structure to the binding of aminoacridines to DNA is provided by experiments employing O.R.D. techniques[27,33,50]. Acridines possessing a 3-amino group require the presence of other bound dye molecules for induced optical activity. However, acridines with amino group in positions 1, 2 or 9 have no such requirement. In view of the fact that optical properties are dependent on environment[29], these observations suggest that the exact mode of binding of aminoacridines to DNA may be influenced by the position of the amino group.

The different effects on the binding obtained by amino group substitution do not form any definite pattern. This observation has led Löber and Achtert[48] to suggest that it seems likely that no single intercalation model exists for all acridine derivatives.

6. Effect of DNA Structure on Binding

a. Influence of Base Composition of the DNA

Although a number of studies have been made of the influence of DNA base composition on the binding of

aminoacridines, no firm conclusions can as yet be provided. Most of the studies have involved fluorescence methods and from the results two opposing hypotheses have been developed. Thomes et al[39] and Bidet et al[41] state that the fluorescence measurements, taken under conditions when only intercalation is occuring, indicate two types of binding sites with intercalation being favoured in AT-rich regions of the DNA. However, other workers employing the same technique and similar conditions[42,45,52,53] have stated that the binding data indicate only one type of site. They assert that the assumptions to the contrary are due to the incorrect assumption that the quantum yield of the bound dye is independent of the extent of binding.

The theoretical calculations of Gersch and Jordan[17] indicate that the order of relative affinities of aminoacridines for binding sites in DNA is AT:TA>AT:AT>TA:AT>TA:GC> AT:GC>AT:CG>GC:CG>TA:CC>GC:GC>CG:GC. The observation by Kleinwachter and Koudelka[20] that the increase in thermal stability of DNA, as a result of the binding of aminoacridines, is greater with DNA of high AT content is in agreement with these results. However, the more refined free-energy calculations of Gilbert and Glaverie[54] fail to indicate any significant preference for a given site.

Hruska and Danyluk[55] studied the interaction of acridine orange to pyrimidine and purine derivatives using a

N.M.R. technique and concluded that intercalation is favoured in purine-rich regions of polynucleotides.

In view of the conflicting reports it is clear that the question of site-specificity is still unanswered.

b. The Effect of Denaturation

The outstanding structural feature of native DNA is the organized double helix[56]. This structure is to be compared with the disorganized random coil configuration of the separated strands in denatured DNA. In view of the vast difference in the secondary structure of native and denatured DNA, an examination of the binding of aminoacridines to both forms of DNA is useful in determining if any structural features of the DNA are necessary for interaction to occur.

A number of reports, employing a variety of techniques, have indicated that at 20 to 25^{o}C the binding of amino-acridines to denatured DNA is at least as great as to native DNA[4,13,33,57]. These results were obtained at ionic strengths greater than 0.1 when the binding to the phosphate groups of the polynucleotide is virtually absent. This observation indicates that the intact double helix is not an essential structural condition for primary binding. However, the thermodynamic data obtained by Ichimura et al[58] for the interaction of acridine orange with native and denatured DNA suggests that the modes of binding to the two forms may be

different.

Chambron et al[18] and Kleinwachter et al[19] have shown that on heating proflavine-DNA complexes, the main release of dye occurs at temperatures coincident with the destruction of the double helical DNA structure. This observation was interpreted by these workers as indicating the necessity of the double helix for the binding process. However, while this assertion may be correct at elevated temperatures, the generalised conclusion is clearly incorrect. Their results only indicate that proflavine does not bind to denatured DNA at elevated temperatures and gives no indication of the binding at lower temperatures, around 20°C. (The effect of temperature on the binding of 9-aminoacridine to native and denatured DNA is discussed in detail in Chapter IV.)

Thus while current evidence suggests that interaction of aminoacridines with the purine and pyrimidine bases of denatured DNA is possible without the requirement of the double helix, no definite evidence is as yet available to determine if the mode of binding is identical to both forms of DNA.

7. Influence of Ionic Strength

Evidence for the involvement of electrostatic forces in the interaction of aminoacridines with DNA is given by the reduction in the extent of binding with increasing ionic

strength[1,4,42,57]. Both the primary and secondary binding processes are dependent upon ionic strength, with the latter being more markedly affected. Since the primary process is believed to involve an intercalation mechanism while the secondary binding on attachment of dye to the exterior of the helix, this observation emphasizes the important role of electrostatic interactions in the intercalation process. This is apart from the specific interactions between the heterocyclic rings of the acridine cations and the DNA bases.

8. Orientation of the Bound Aminoacridine with respect to the Helix Axis and to the Nucleotide Bases

Several studies[30,59,61] using flow dichroism, flow polarized fluorescence and flow birefringence techniques have been conducted in an attempt to determine the relation of the plane of the intercalated aminoacridines to the helix axis of the DNA. All these studies indicate that the plane of the bound dye is within 30^{o} of being perpendicular to the helix axis. The flow circular dichroism results of Gardner and Mason[30], suggest that while bound acridine orange is more perpendicular than parallel to the helix axis, the deviation is sufficient to require some tilting of the bases to allow the insertion of the dye.

9. The Structure of the Complex

a. Primary Binding

The experimental observations when aminoacridines

are bound to DNA under conditions in which primary binding predominates may be summarized as follows:

1. Interaction with DNA bases is involved.
2. The contour length of native DNA is increased, while that of denatured DNA is not increased.
3. The mass per unit length of native DNA is decreased.
4. The reactivity of the amino groups of bound aminoacridines is reduced.
5. The bound dyes are orientated parallel to the bases and perpendicular to the helix axis.
6. The thermal stability of native DNA is increased.
7. Denaturation of the DNA does not cause any reduction in primary binding at 20-25^{o}C.
8. Only dye cations are bound.
9. Increasing ionic strength causes a reduction in degree of binding.
10. The attachment of long side chains to the C9 position of 9-aminoacridine does not reduce the extent of the binding.
11. The interaction of flat planar ring structures is favoured.

On the basis of observations 1-5, Lerman[9,59,62] suggested that the DNA molecule untwisted and extended such that the base pair separation increased from 3.36 to 6.72 A^{o}, thereby allowing the insertion of a dye cation between adjacent

base pairs. Lerman postulated an uncoiling of 45^{o}, resulting in the original right-handed helix becoming left-handed with an angle between neighbouring base pairs of 9^{o}. However, a more detailed investigation by Fuller and Waring[53] suggests that an uncoiling of only 12^{o} is necessary, which results in the maintenance of the right-handed configuration with an angle between neighbouring base pairs of 24^{o}. In the Lerman model, the dye is centrally located within the helix so that the positive ring nitrogen is near the central axis of the DNA, and there is an obvious structural requirement for the double helix (see Fig. II.2).

Although the Lerman model satisfactorily explains many of the observations, it cannot account either for the binding of aminoacridines to denatured DNA, or the binding of acridines possessing bulky substitutes attached to the 9-amino position. In order to account for these observations, Pritchard et al[64] proposed a modified intercalation model in which aminoacridine cations intercalate between successive bases on the same polynucleotide chain. They further suggested that the negatively charged oxygen atom of the phosphate group between the bases can swing in and take up a position close to the positive ring nitrogen of the amino-acridine cation (see Fig. II.3). This model assumes that the double helical structure is not essential for primary binding and thus allows for the binding of aminoacridines to

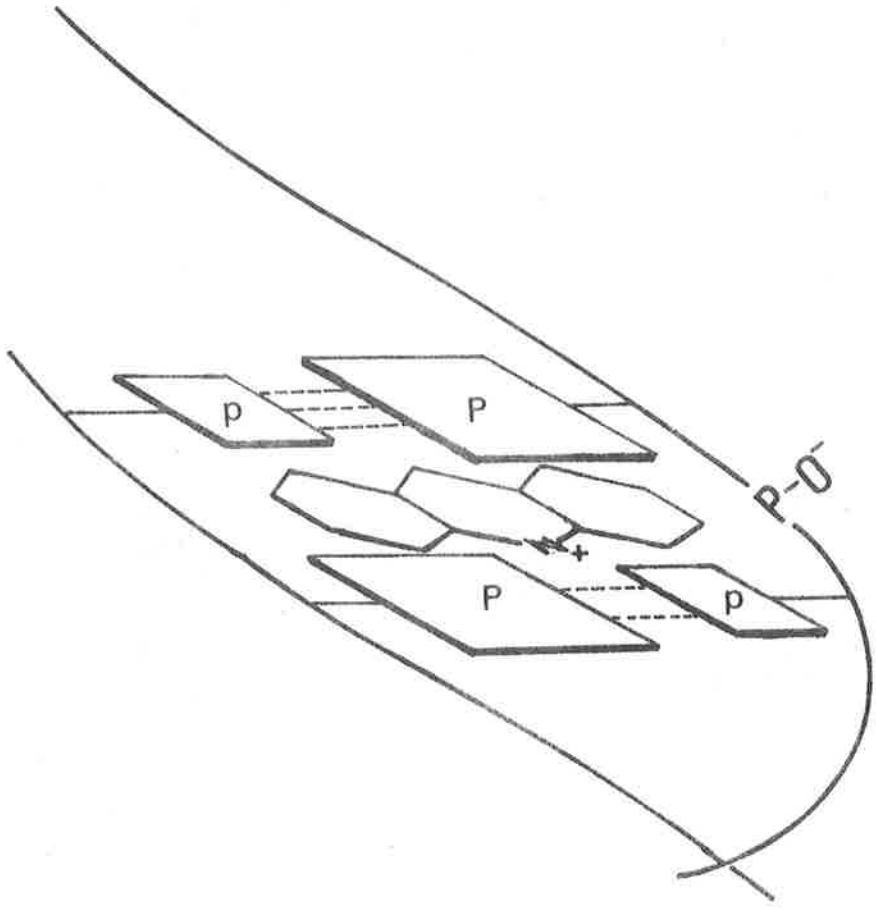

FIG. II.2 : Schematic representation of the Lerman model for the intercalation of amino-acridine cations into native DNA.

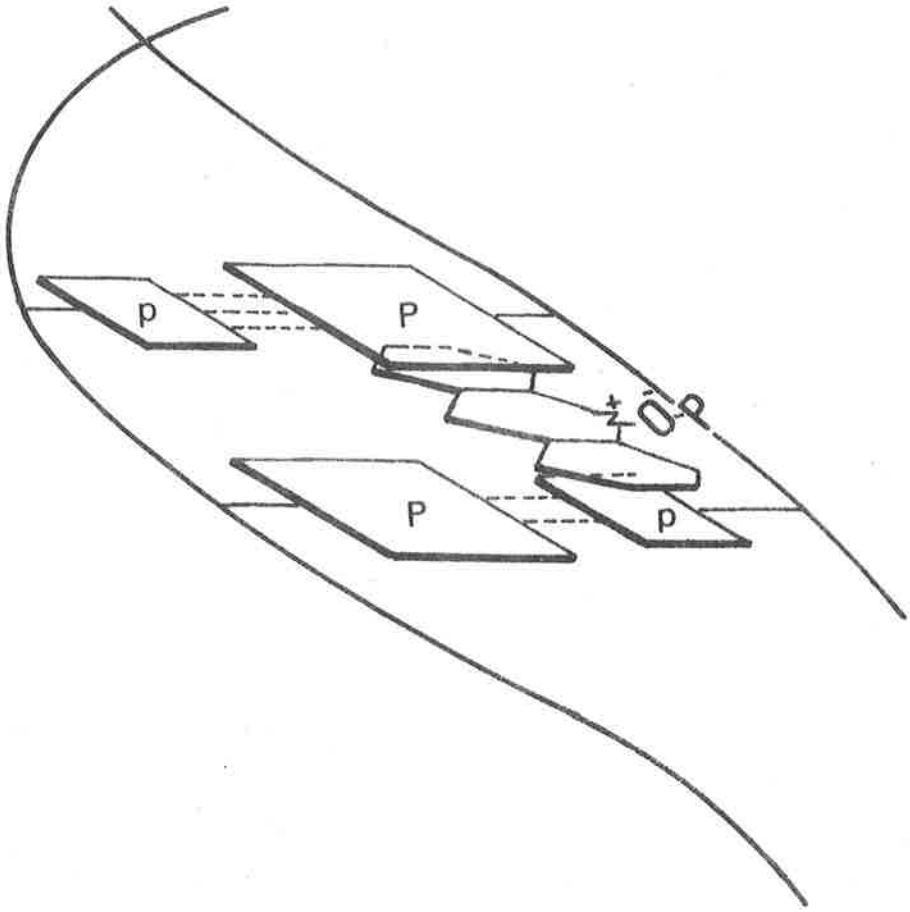

FIG. II.3 : Schematic representation of the intercalation model of Pritchard et al for the interaction of aminoacridines with DNA.

native and denatured DNA. Further, the location of the dye is such that the long chains attached to the 9-amino position do not interfere with the structure. The binding of Tetra-hydro-acridine can also be described by this model since complete insertion seems unlikely in view of the bulked nature of one ring. The binding of aminoacridine to native DNA by this modified model would still require an extension of the double helix of the same order as that postulated for the Lerman model. However, in view of the less rigid configuration of denatured DNA, insertion of dye cations may be possible without the need for further modification of the contour length, which is in accord with experimental observation.

Although these two models have in the past been regarded as alternatives, recent observations on the influence of dye structure on the binding[48,50] suggests they form the two extremes of a large number of positions. The precise position of an intercalated molecule can be regarded as that which gives the highest binding energy. This binding energy will be determined by both steric factors and the nature and strength of the intermolecular forces involved, which may vary for each aminoacridine studied.

A recent postulate by Armstrong et al[11] suggests that acridines are strongly bound in two distinct modes. Assuming the modified intercalation model of Pritchard et al[64]

they proposed that the two modes correspond to an intercalation between base pairs and to the binding of a non-intercalated acridine on to one already intercalated. Whether this model is applicable to all acridines or only to those which self-associate readily cannot be determined at this stage and must await further investigation.

b. Secondary Binding

Binding of aminoacridines to DNA by the secondary binding process has the following experimental characteristics:

1. The binding energy is very low.
2. It is electrostatic in nature and is decreased more than the primary binding process by an increase in ionic strength.
3. It can involve the interaction between bound dye molecules.
4. Since it occurs after intercalation it is probably an external binding process.

The most probable model for the aminoacridines bound by this process is one in which the dye cations are bound externally to the double helix of DNA with the positive ring nitrogen close to the negative phosphate groups. Because the helix is extended by intercalation prior to the binding by this weak process, no effect on viscosity would be anticipated. For aminoacridines which readily self-associate, the possibility exists for the external attachment of dye aggregates.

Thus the evidence currently available suggests two distinct sites for the interaction of aminoacridines with DNA, namely the purine and pyrimidine bases (constituting the primary binding site) and the external phosphate groups (constituting the site of weaker interaction). However, while the insertion of an aminoacridine dye cation between the bases is certainly the mode of strong binding, the exact location for any particular aminoacridine remains unresolved.

10. Kinetics and Mechanisms of Complex Formation

The various states of binding of aminoacridines to DNA cannot always be easily distinguished using optical absorption spectroscopy. However, an examination of the dynamics of the reaction can be useful if the reaction rates for the different states differ. Because the rates involved are very rapid, the use of modern perturbation and flow techniques is necessary[65]. The kinetic data obtained using such techniques are, in general, ambiguous since they must often be assigned to one of several possible reaction mechanisms. Thus critical attention must be paid to the mechanisms chosen. The successful elucidation of the reaction mechanism requires the application of both kinetic and static techniques.

Li and Crothers[66] have examined the interaction of proflavine with DNA using a temperature-jump relaxation kinetic technique. Most of their kinetic experiments were made at very low r values (0.04 - 0.05), when only inter-

calation occurs. The results showed two relaxation times, one of a few milliseconds, the other faster than several tenths of a millisecond. This observation was taken to indicate the presence of two forms of the complex at equilibrium. These workers suggest that the rapid process is the binding of dye monomers to the outside of the helix, while the slower reaction is intercalation. Two possible mechanisms are postulated to account for the observed data:

1. A series reaction in which intercalation occurs in two steps

$$P + DNA \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (P)_{Out} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} (P)_{In}$$

2. A parallel reaction in which interaction can occur directly between free dye and the DNA

$$P + DNA \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (P)_{Out} \qquad P + DNA \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} (P)_{In}$$

where P is proflavine, and $(P)_{Out}$ and $(P)_{In}$ are externally bound, and intercalated dye respectively. On the basis of their kinetic data, Li and Crothers[66] contend that the series reaction is the more probable mechanism. Firstly the dye is bound to the exterior of the helix by a rapid bimolecular

process ($k_{12} \simeq 10^7\ M^{-1}\ Sec^{-1}$) and then intercalated by a subsequent first-order reaction ($k_{23} \simeq 10^3 - 10^4\ Sec^{-1}$).

Whether the intercalation mechanism requires the opening of a base pair to allow the dye to enter is not clear. However, the results of Li and Crothers[66] favour a mechanism in which the dye can be inserted between base pairs without the necessity of base pair separation. This could be achieved by a longitudinal flexing of the DNA molecule occuring simultaneously with the insertion of the dye.

More recent studies on the interaction of acridine orange with DNA using a stopped-flow technique[67,68] support the mechanism in which intercalation proceeds via an outside-bound state.

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CHAPTER III

THE INTERACTION OF PROFLAVINE AND 9-AMINOACRIDINE WITH DNA AT TEMPERATURES BELOW AND ABOVE THE MELTING TEMPERATURE

1. Introduction

Investigation of the effect of temperature on the interaction of heterocyclic dye molecules with DNA can be expected to yield information on the thermodynamic properties of the binding process, and also on the problem of whether the intact double helical structure of DNA is a prerequisite for intercalation.

The increase in the thermal stability of DNA when complexed with acridine dyes at moderate ionic strengths, (as indicated by an increase of the melting temperature, T_m), is now well established[1,2,3]. This stabilization of the secondary structure has been attributed to the interaction of the dye cations with the DNA bases[4,5]. The extent to which the melting temperature is raised depends upon the degree of binding, with no further increase occurring after all available intercalation sites are occupied[4]. It has been postulated that this increase in thermal stability may be due to the decrease in the charge density of the DNA helix resulting from the increase in contour length caused by intercalation[2]. It should be noted that the same effect could also be achieved by neutralization of the negatively charged phosphate groups on the exterior of the DNA helix by the aminoacridine cations. However, because stabilization occurs at very low degrees of binding, where intercalation predominates, it seems reasonable

to assume that the enhanced stability is a result of dye-base interactions.

The release of dye from the DNA-dye complexes with increasing temperature has been studied by such techniques as equilibrium dialysis[7,8], uv-visible spectroscopy[1-4] and nuclear magnetic resonance spectroscopy[9]. The resulting decrease in the extent of binding has been partly explained by a reduction in the number of binding sites at elevated temperatures[10]. However, the enthalpy change of binding (ΔH_B) decreases with temperature[7], and thus any explanation must take into account the variation of the binding coefficients with temperature.

A marked decrease in the interaction between dye cations and DNA occurs over the relatively narrow temperature range corresponding to the dissociation of the DNA helix[1,2]. At such elevated temperatures it would appear that there is a significant difference in the primary binding to single and double-stranded DNA, the latter being much the stronger. The double helical structure would thus appear to be essential for primary binding, at least at elevated temperatures. However, this cannot be taken as evidence that the helical secondary structure of DNA is necessary for primary binding at lower temperatures.

In this chapter the influence of temperature on the

binding of 9-aminoacridine and proflavine to DNA is studied by use of techniques allowing the examination of the binding of dye cations to single and double-stranded DNA molecules under identical conditions. Use is made of the observation that the stability of the double stranded DNA is increased on interaction with aminoacridine cations[14]. 9-Aminoacridine and proflavine were both studied since their cationic forms bind differently to DNA, the former having much stronger primary binding than the latter[11].

2. Results and Discussion

Binding curves were dermined from spectral data using the following equation[12],

$$r = \frac{D_1 - D}{D_1 - D_2} \frac{T_L}{T_A} \qquad \text{(III.1)}$$

where r is the number of moles of aminoacridine bound per mole of DNA phosphorus (i.e. per nucleotide residue); D_1 is the absorbance at λ_{max} of the aminoacridine in the absence of DNA; D_2 is the absorbance at λ_{max} of the aminoacridine of a solution of the aminoacridine containing an excess of DNA; D is the absorbance at λ_{max} of the aminoacridine for the DNA-aminoacridine solution; T_L is the total concentration of the aminoacridine, and T_A is the total concentration of the DNA. For the determination of r at a given temperature all spectral measurements were carried out at that temperature. This

equation is valid only when certain conditions are fulfilled[10]. Experimental evidence suggests that these conditions are satisfied if there is an isosbestic point through which all the spectra of mixtures of varying T_L/T_A ratios pass.

Two different methods were used to study the binding of 9-aminoacridine and proflavine to DNA at various temperatures -

1. Aminoacridine and DNA solutions were mixed in the required proportions at 20°C and then placed in the variable temperature compartment of the spectrophotometer and raised to the desired temperature. Absorbance measurements were then made at the appropriate wavelength (viz. 400 nm for 9-aminoacridine and 444nm for proflavine).
2. The DNA solution was heated to the temperature at which measurements were to be taken and the amino-acridine solution was then added.

By use of these methods, comparison of dye binding to native and denatured DNA can be made at the same temperature because in method (1) the presence of bound dye stabilizes the double helix at temperatures above the normal melting temperature. All solutions were prepared in 10^{-3}M NaCl because at this neutral salt concentration a wide variation in the melting temperature for varying T_L/T_A ratios can be obtained[1].

The spectra of solutions of varying ratios of T_L/T_A

for 9-aminoacridine prepared by method (1) at temperatures of 25, 73 and 83°C are shown in Figs. 1-3 respectively. At the three temperatures studied the spectra were characterized by a displacement to longer wavelengths (red shift), relative to the spectrum of 9-aminoacridine, and the appearance of an isosbestic point at 427 nm. These spectra may be regarded as the spectra of monomeric 9-aminoacridine cations because the concentration of dye used in this study is well below that at which the formation of aggregates occurs[4]. The observed red shifts have been attributed to the interaction of the amino-acridine cations with the bases of the DNA, rather than to the binding of dye to the charged phosphate groups on the exterior of the helix[2,13]. It can thus be assumed that the changes in the spectra as shown in Figs. III.1-3 result from the inter-action of monomers of 9-aminoacridine cation with the purine and pyrimidine bases of the DNA.

The spectra at 83°C for varying T_L/T_A ratios for solutions prepared by method (2) are shown in Fig. III.4. These spectra differ from thoseobtained at 83°C for solutions of identical T_L/T_A ratios prepared by method (1). (Refer Fig. III.3) No clear isosbestic point is observed which may mean that equation III.1 cannot be applied under these conditions. The observed decrease in the extent of hypochromicity and spectral shift as compared to those in Fig. III.3, is also indicative of a reduced interaction between dye cations and the

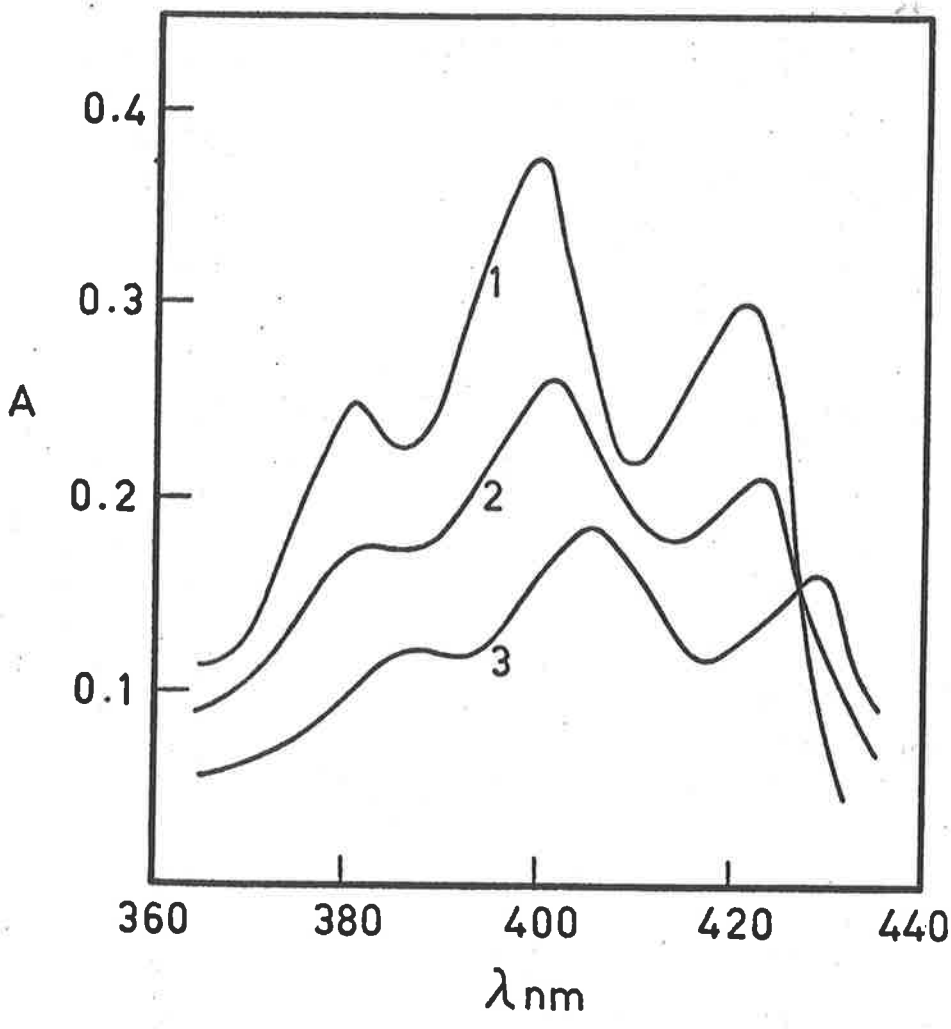

FIG. III.1 : Spectra at 25°C in 10^{-3}M NaCl of 9-amino-acridine (curve 1) with DNA T_L/T_A = 0.683 (curve 2); T_L/T_A = 0.228 (curve 3). Solutions prepared by method (1) (see text).

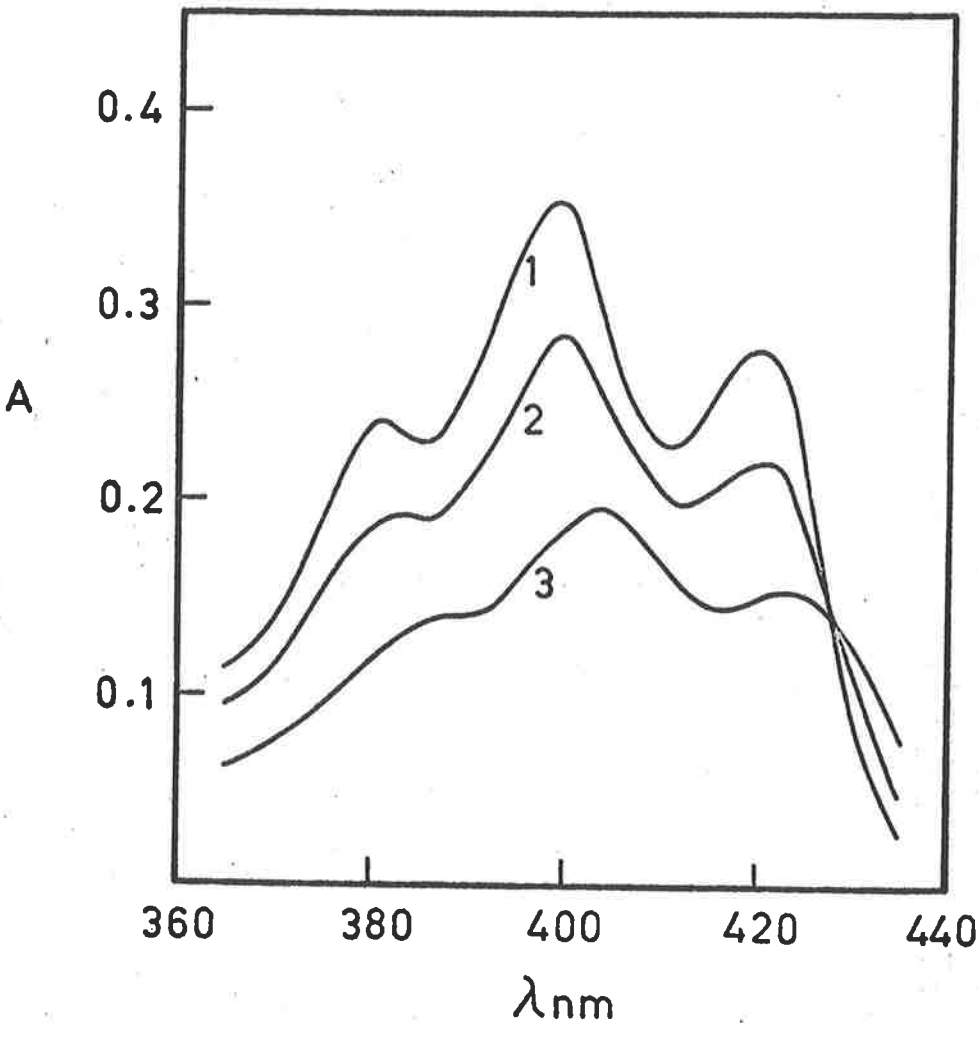

FIG. III.2 : Spectra at 73°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with DNA T_L/T_A = 0.683 (curve 2); T_L/T_A = 0.228 (curve 3).
Solutions prepared by method (1) (see text).

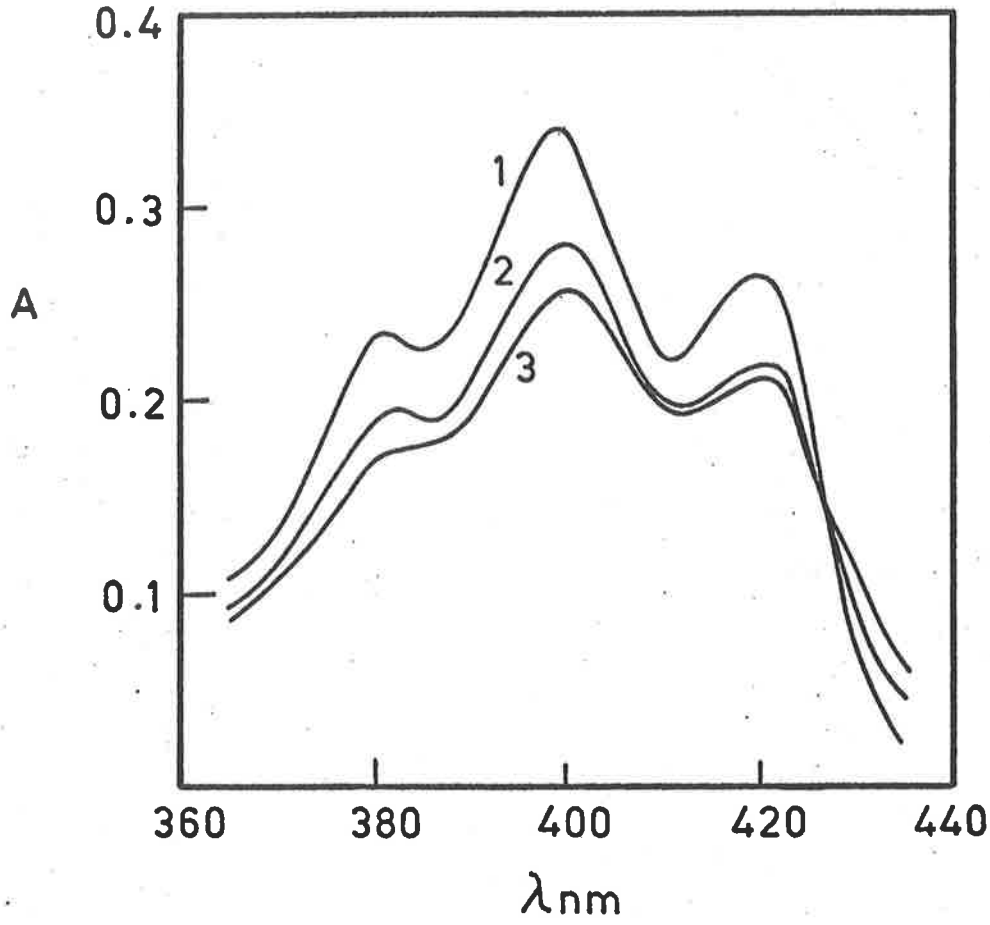

FIG. III.3 : Spectra at 83°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with DNA T_L/T_A = 0.683 (curve 2); T_L/T_A = 0.228 (curve 3).
Solutions prepared by method (1) (see text).

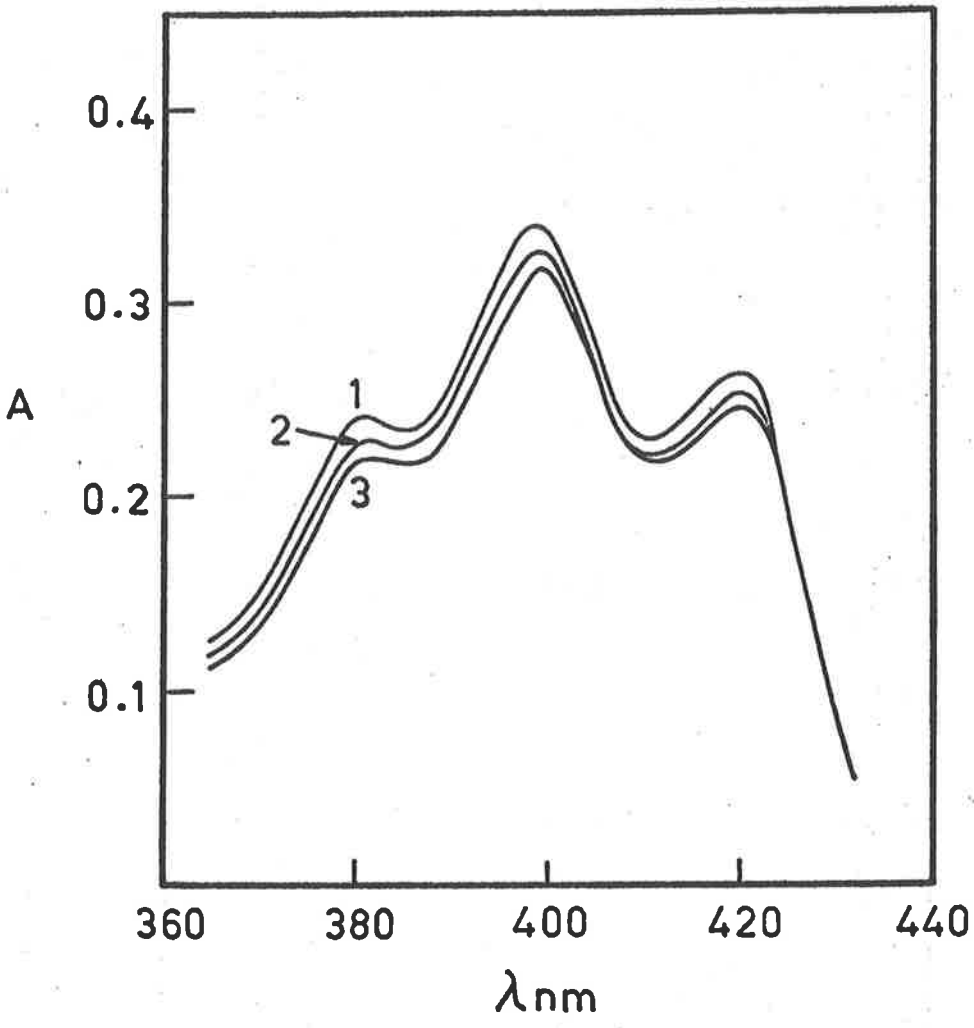

FIG. III.4 : Spectra at 83°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with DNA T_L/T_A = 0.683 (curve 2); T_L/T_A = 0.228 (curve 3).
Solutions prepared by method (2) (see text).

DNA bases at this elevated temperature when complexes are formed by method (2).

The variation of the function $(D_1 - D_2)$ with temperature is shown in Fig. III.5, curve 2. This function measures the difference in absorption at 400 nm of 9-amino-acridine cations in the absence of DNA and in the presence of an excess of DNA when no further change in absorbance is observed, the latter situation representing a limiting dye to DNA concentration ratio. Thus curve 2, Fig. III.5 shows the influence of temperatures on the primary binding of 9-aminoacridine cations to DNA. The rapid decrease in $(D_1 - D_2)$ occurs over the same temperature range as the dissociation of the DNA-aminoacridine complex. (Fig. III.5, curve 3).

The binding curves determined using equation III.1 for 9-aminoacridine in 10^{-3}M NaCl at various temperatures are shown in Fig. III.6. For these measurements the solutions were prepared using method (1), all absorbance measurements being made at the required temperature. Up to about 75^{o}C, the value of r for a given concentration of unbound 9-aminoacridine decreases with increasing temperature, but above this temperature the value of r increases as the temperature increases. This temperature region corresponds to that range over which the function $(D_1 - D_2)$ rapidly decreases (Fig. III.5, curve 2). The observation that the

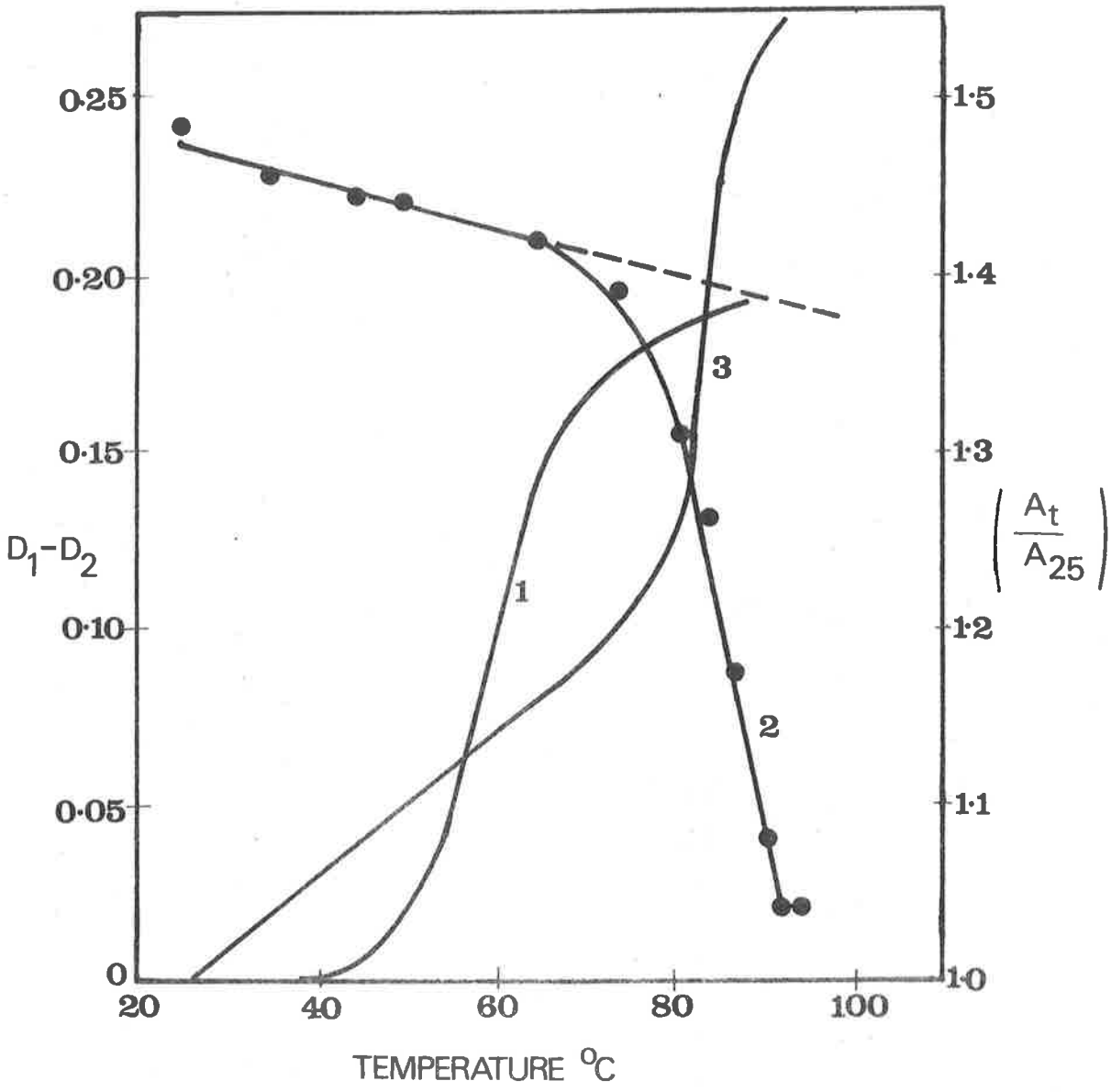

FIG. III.5 : Curve 1. Melting profile of E.coli DNA in 10^{-3}M NaCl.
Curve 2. Variation of $(D_1 - D_2)$ with temperature for E. coli DNA (initial concentration 1.0 x 10^{-3}M) with 9-aminoacridine (initial concentration 4.403 x 10^{-5}M) in 10^{-3}M NaCl.
Curve 3. Melting profile of the DNA - 9-aminoacridine complex in 10^{-3}M NaCl; $r = 0.204$, $T_L = 2.0 \times 10^{-5}$M, $T_A = 8.4 \times 10^{-5}$M. Curves 1 and 3 right hand scale, curve 2 left hand scale.

FIG. III.6 : Variation of r with concentration of unbound 9-aminoacridine (c) at various temperatures, D_1 and D_2 being measured at each temperature.
X, 25°; ●, 35°; ○, 65°; ▲, 74°; □, 81°; ■, 84°C.

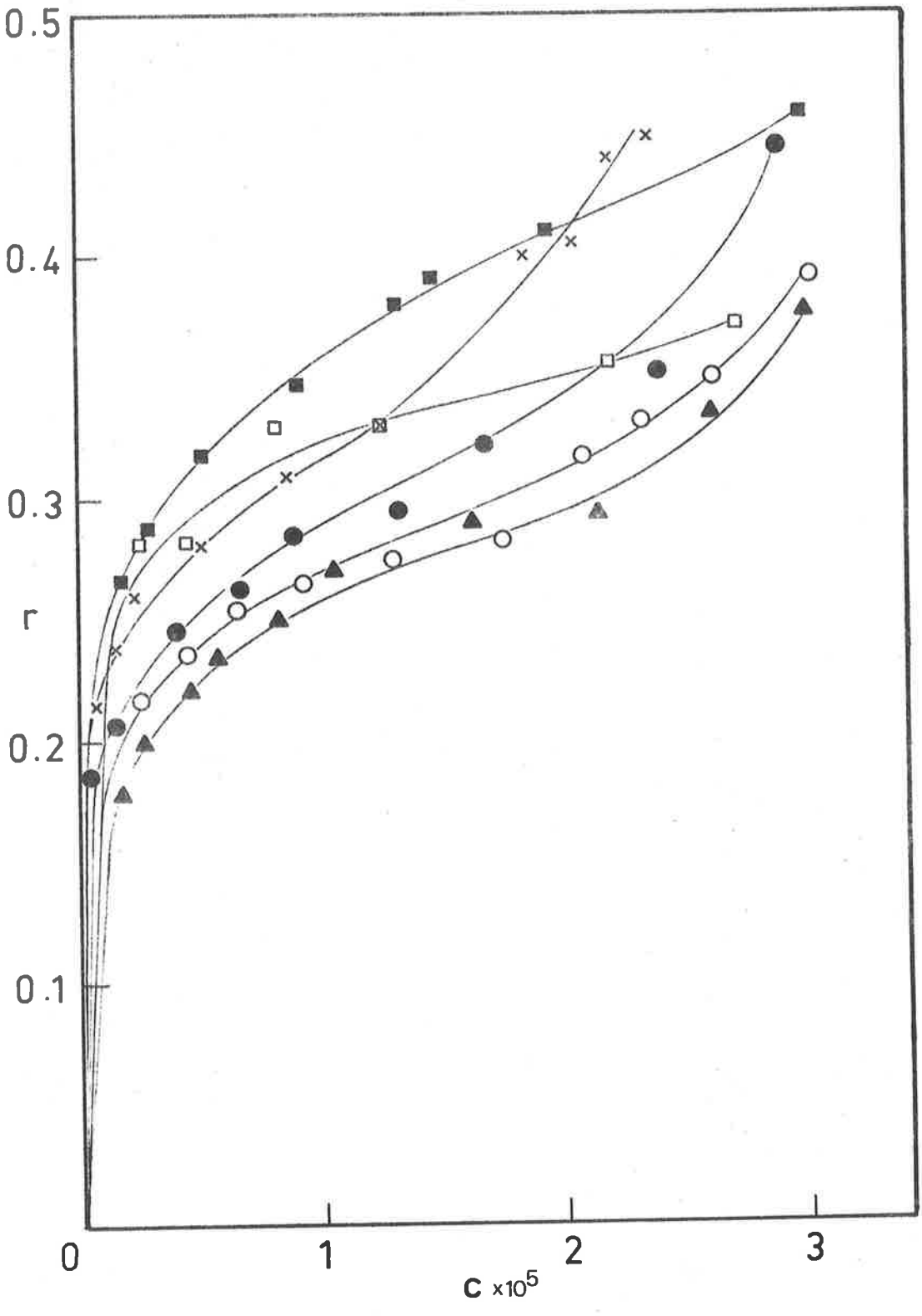

FIG. III.6

value of r increases with increase in temperature above a critical temperature, which for the case considered is about 75°C, may be explained in terms of the inadequacy of equation III.1. The increase in r, as calculated by this equation, must be due to a greater increase in D_2 than in D as the temperature is raised. Thus at temperatures above 75°C, the function $(D_1 - D_2)$ decreases to a greater extent than does the function $(D_1 - D)$, with a resultant increase in the magnitude of r as calculated using equation III.1.

For use in equation III.1 the value of $(D_1 - D_2)$ may be calculated in three ways -

1. The absorbance values may be measured at 25°C, $(D_1 - D_2)_{25}$, and may be assumed invariant with temperature, and the variation of r is dependent only on the variation of $(D_1 - D)$ with temperature.
2. The absorbance values may be measured at the temperature of the experiment, $(D_1 - D_2)_t$.
3. $(D_1 - D_2)_{ext}$ may be calculated from the extrapolation of the variation of $(D_1 - D_2)$ below 70°C (see broken line in Fig. III.5).

The variation of r with temperature for 9-aminoacridine using the values of $(D_1 - D_2)$ calculated by the three different methods is shown in Fig. III.7. The use of $(D_1 - D_2)_{25}$, curve 3, Fig. III.7, in view of the variation of $(D_1 - D_2)$ with temperature, is clearly incorrect, although both

FIG. III.7 : Variation of r with temperature using the function $(D_1 - D_2)$ measured in different ways.

Curve 1, ○ , $(D_1 - D_2)_t$

Curve 2, □ , $(D_1 - D_2)_{ext}$

Curve 3, △, $(D_1 - D_2)_{25}$

For significance of functions see text.

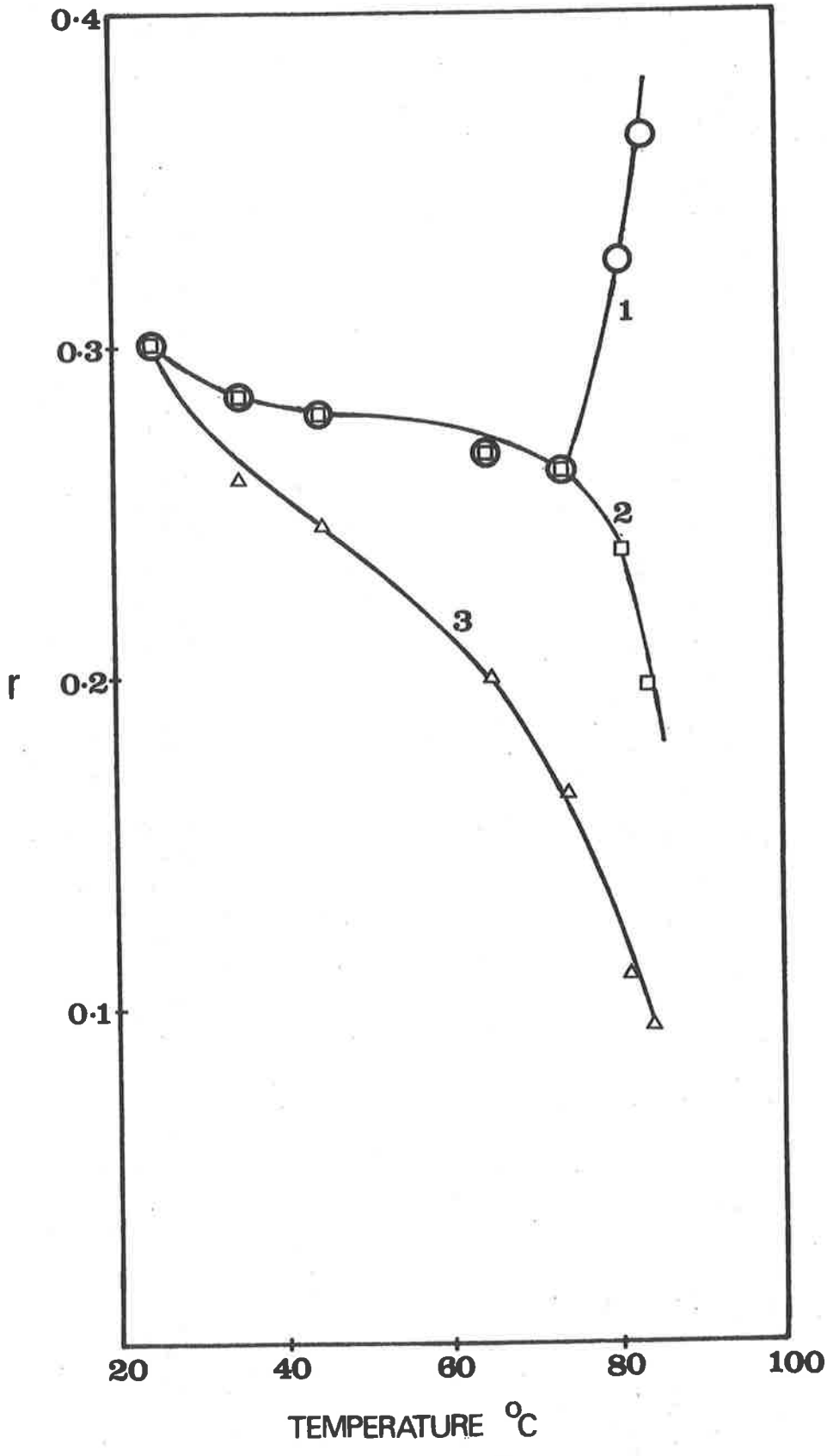

FIG. III.7

Kleinwachter et al[3] and Chambron et al[1] appear to have used this value in their calculations of r at various temperatures. Thus the data presented by these two groups of workers do not give a true indication of the temperature dependence of r.

The correct value of $(D_1 - D_2)$ to use might appear to be the value measured at the appropriate temperature, viz. $(D_1 - D_2)_t$. However, this function leads to a rapid increase in r as shown in Fig. III.6 and more clearly in Fig. III.7, curve 1. This increase was unexpected in view of the liberation of dye from the complex but may be explained by the fact that the extent of stabilization of the double helix is dependent upon the degree of binding. If Tm(D) is used to designate the melting temperature of the complex for a finite T_L/T_A ratio and $Tm(D_2)$ to designate the melting temperature for very low T_L/T_A ratios, then as has been shown, $Tm(D) > Tm(D_2)$[6,14]. The value of $Tm(D_2)$ approaches the Tm of the native DNA as the T_L/T_A ratio approaches zero. Therefore, at a given temperature between $Tm(D_2)$ and Tm(D), the extent of denaturation will be different when measuring D and D_2, with more denatured DNA being present in the case of very low ratios, i.e., equivalent to an absorbance of D_2. In equation III.1 the ratio

$$(D_1 - D) / (D_1 - D_2) \equiv \alpha \qquad \text{(III.2)}$$

where α was defined by Peacocke and Skerrett[12] as the fraction of the total dye bound, is meaningful only if D and D_2 are measured under conditions such that in each case the DNA is

either native, denatured, or has the same proportion of native to denatured material. However, in the temperature range 65 to 90°C, over which the rapid decrease in the function $(D_1 - D_2)$ occurs, there will be a different proportion of native to denatured DNA when D_2 and D are measured at the same temperature. Therefore the ratio given in equation III.2 cannot be used for the determination of r by equation III.1 for temperatures between Tm(DNA) and Tm(D), even though an isosbestic point is shown for temperatures above $Tm(D_2)$. (Refer Fig. III.3)

The function $(D_1 - D_2)_{ext}$ gives values of $(D_1 - D_2)$ for double stranded DNA at temperatures above the melting temperature. Use of this function gives an acceptable form of the variation of r with temperature (Fig. III.7, curve 2) only because the binding of the 9-aminoacridine cation to single-stranded DNA at elevated temperatures is so low as to be almost negligible.

Because any function including D_2 will be unsatisfactory to study dye binding to DNA at temperatures greater than $Tm(D_2)$, the function D_1/D will be used to represent the relative degree of binding at elevated temperatures. The variation of D_1/D with T_L/T_A at various temperatures for 9-aminoacridine and DNA in 10^{-3}M NaCl is shown in Figs. III.8 and 9. Corresponding data for proflavine are given in Fig. III.10. The data are presented for solutions prepared by the two different methods described earlier in this chapter.

The variation of $^{D_1}/_{D}$ with $^{T_L}/_{T_A}$ for 9-aminoacridine at 25, 35 and 45°C is shown in Fig. III.8. For a given temperature, the values of D_1/D are independent of the method of preparation of the solutions. These temperatures are well below the melting temperature of DNA in 10^{-3}M NaCl (Tm = 59°C) (see Fig. III.5, curve 4) and the DNA is thus present in the double-helical form in all cases. In Fig. III.9, corresponding data are shown for the temperatures 65, 74 and 84°C, which are all above the Tm of the DNA. Therefore, solutions prepared using method (2) under these conditions involved the addition of 9-aminoacridine to solutions of DNA which is already denatured. At these temperatures the function $^{D_1}/_{D}$ is no longer independent of the method of preparation of the solutions. When the dye is added to DNA which has been heated to the temperature of measurement, the extent of binding for a given value of $^{T_L}/_{T_A}$ decreases rapidly with temperature as the degree of denaturation increases (Fig. III.9, curves 4,5,6). If the dye and DNA are mixed at 20°C and then heated to the required temperature, (i.e. method (1)), the values of $^{D_1}/_{D}$ at a particular value of $^{T_L}/_{T_A}$ show a much smaller variation with temperature (Fig. III.9, curves 1,2,3). The difference between curves 1 and 4, 2 and 5 and 3 and 6, is significant and shows that the relative binding, at these temperatures, of 9-aminoacridine to denatured DNA is markedly less than to native DNA. At the highest temperature studied, 84°C, DNA is present in the single-stranded form, so it

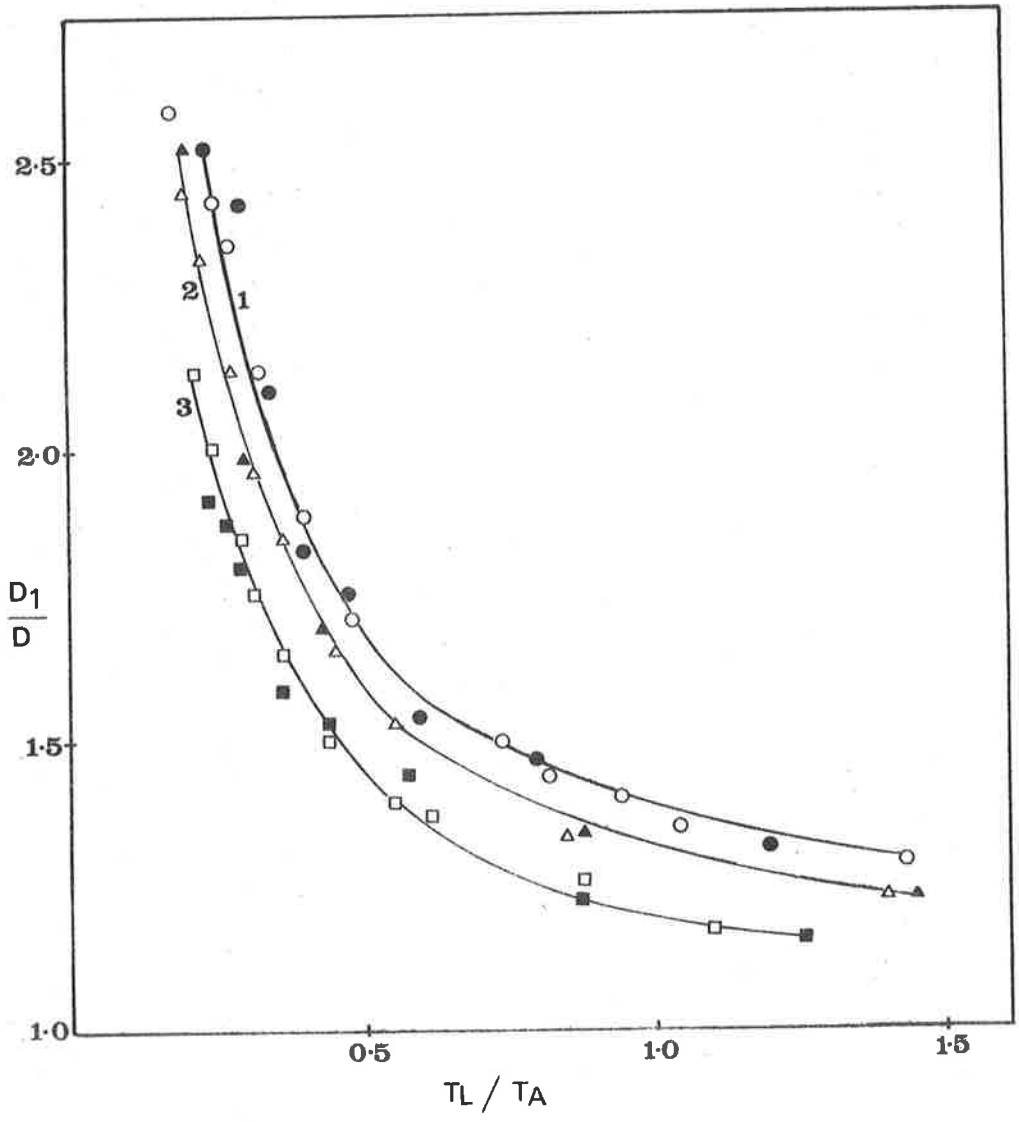

FIG. III.8 : Variation of D_1/D at 400 nm with T_L/T_A for 9-aminoacridine at temperatures below T_m of native DNA.

Curve 1, 25°; curve 2, 35°; curve 3, 45°C.

○, △, □ solutions prepared by method (1)

●, ▲, ■ solutions prepared by method (2)

(see text).

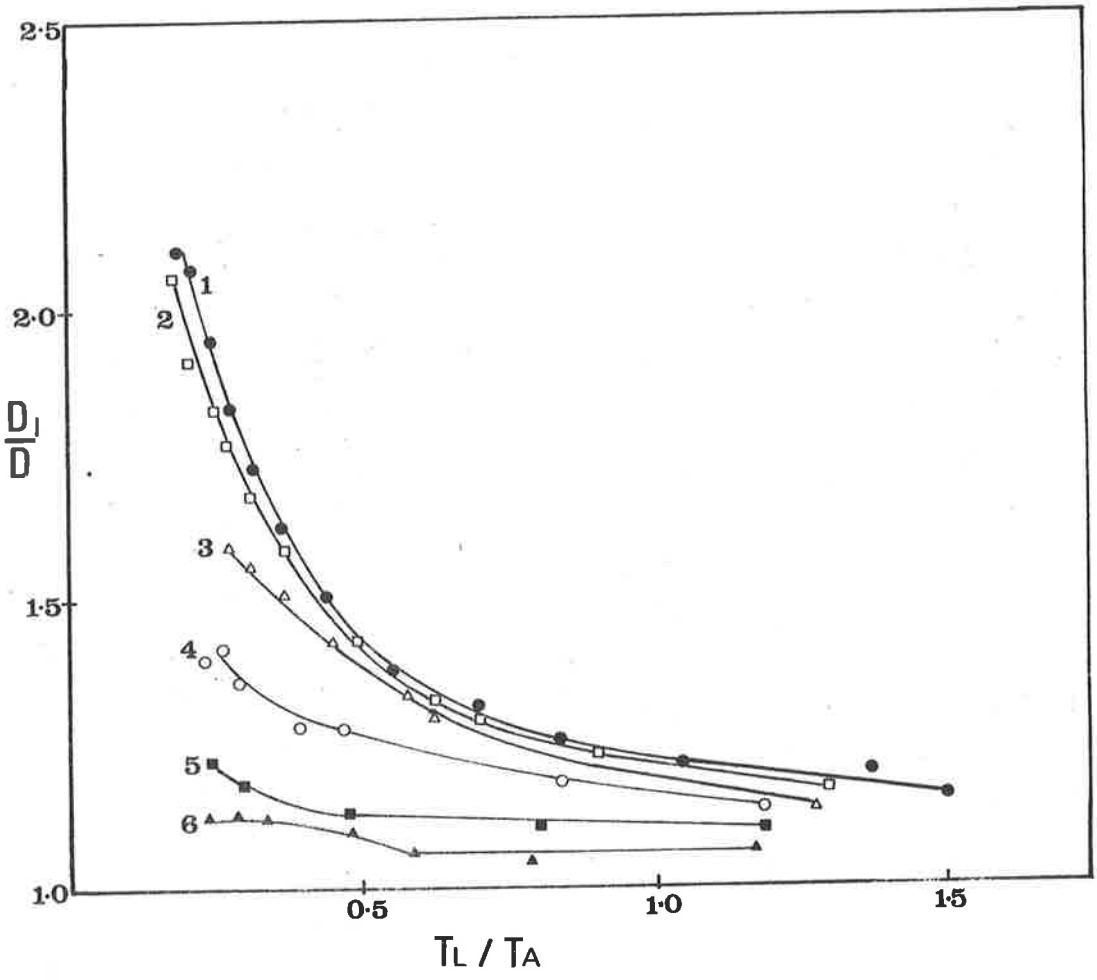

FIG. III.9 : Variation of D_1/D at 400 nm with T_L/T_A for 9-amino acridine at temperatures above T_m of native DNA.

Curves 1 and 4, 65°; curves 2 and 5, 74°; curves 3 and 6, 84°C.

Curves 1, 2 and 3 for solutions prepared by method (1). Curves 4, 5 and 6 for solutions prepared by method (2) (see text).

can be concluded that very little binding to this species occurs at this high temperature, even though the binding to native DNA may be considerable at this temperature. These results are in agreement with those obtained by Ichimura et al[8] for the binding of acridine orange to single-stranded DNA at elevated temperatures.

In Fig. III.10 similar data are presented for proflavine which is less strongly bound to DNA than 9-aminoacridine and measurements can be made at lower values of $^{T_L}/_{T_A}$. These results are similar to, and thus confirm, those obtained with 9-aminoacridine in that at temperatures below Tm of native DNA the values of $^{D_1}/_{D}$ are independent of the method used for preparing the solutions, while above Tm there is a marked difference between the two sets of values. The sharp decrease in $^{D_1}/_{D}$ in Fig. III.10, curve 4 ($85^{o}C$) at values of $^{T_L}/_{T_A}$ below 0.1, may be explained in terms of the decreasing stabilization of the helix as the $^{T_L}/_{T_A}$ ratio is decreased. The Tm for the proflavine-DNA complex at varying ratios of $^{T_L}/_{T_A}$ in 10^{-3}M NaCl decreases rapidly for ratios less than 0.2, and at a ratio of 0.1, the Tm was found to be approximately $75^{o}C$[14]. At this ratio, the Tm is now less than the temperature of measurement, viz. $85^{o}C$ and a decrease in the ratio $^{D_1}/_{D}$ is to be expected, and is compatible with the earlier conclusions on the deficiencies of equation III.1 for the measurement of the extent of binding at elevated temperatures. A corresponding

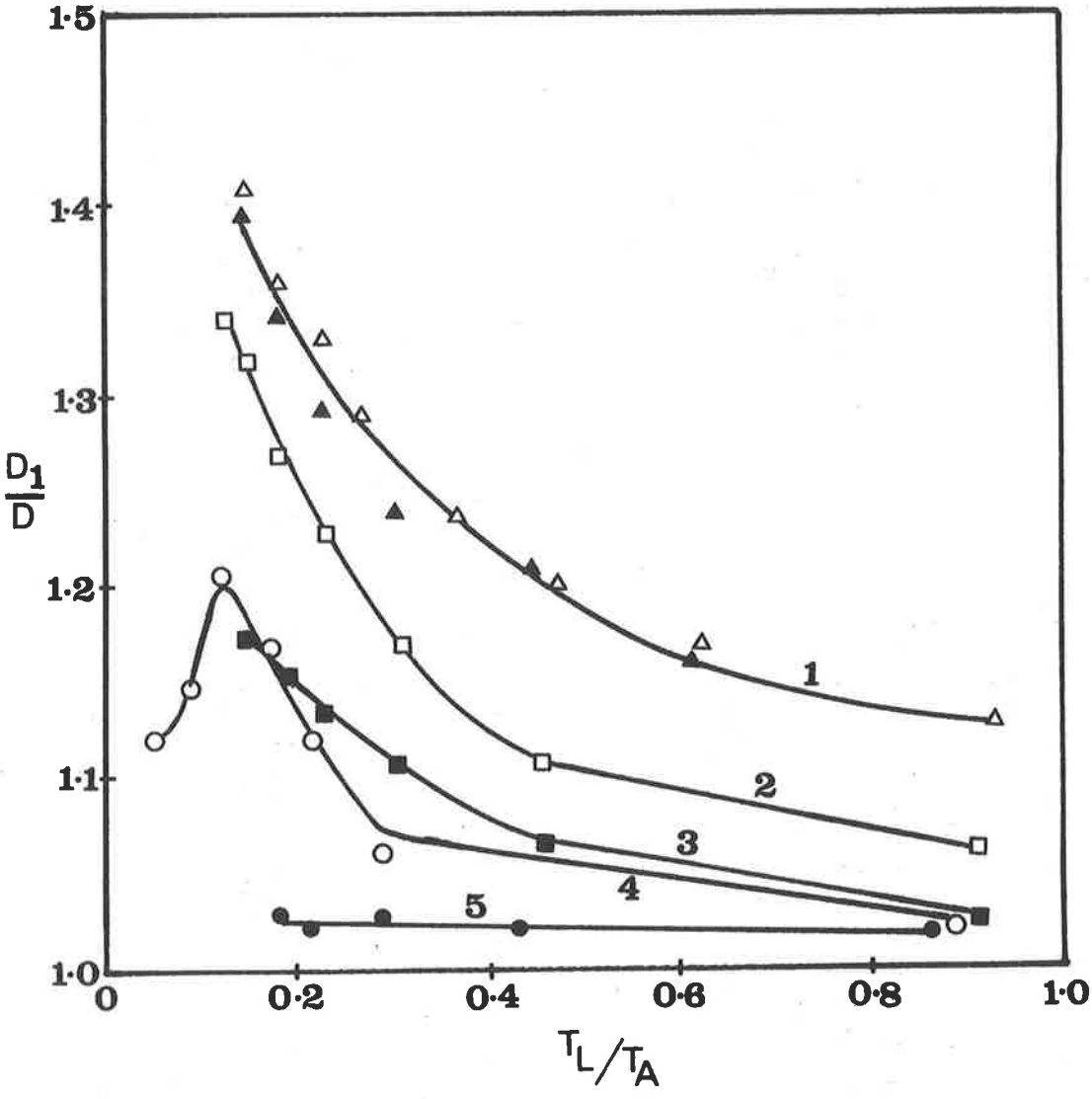

FIG. III.10 : Variation of D_1/D at 444 nm with T_L/T_A for proflavine at temperatures below and above the T_m of native DNA.

Curve 1, 25°; curves 2 and 3, 65°; curves 4 and 5, 85°C.

Curves 1Δ, 2 and 4 for solutions prepared by method (1)
Curves 1▲, 3 and 5 for solutions prepared by method (2) (see text).

decrease in $^{D1}/_{D}$ is not observed in curves 1 and 2, Fig. III.10. Curve 1 is obtained at 25°C, i.e. below the Tm for native DNA, and curve 2 is obtained at 65°C, a temperature just above the Tm of native DNA in 10^{-3}M NaCl, and in order to obtain a decrease in $^{D1}/_{D}$ at 65°C, extremely low ratios of $^{T_L}/_{T_A}$ would need to be examined.

The stabilization of the double-helical DNA is achieved at much lower $^{T_L}/_{T_A}$ values with 9-aminoacridine, and thus Fig. III.9, curves 1, 2 and 3, do not show any decrease in $^{D1}/_{D}$ with decreasing $^{T_L}/_{T_A}$, down to the values studied.

3. Summary

From these results it can be concluded that for both the aminoacridines studied there is a marked difference in the binding to double and single-stranded DNA at elevated temperatures and that at the melting temperature of the DNA-dye complex there is a considerable release of dye as concluded by Chambron et al[1] and Kleinwachter et al[3].

Further, the use of the equation III.1 to calculate the degree of binding from spectrophotometric data[12], is inappropriate under those conditions where varying amounts of a given species of DNA, i.e. native or denatured, are present.

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CHAPTER IV

THE INTERACTION OF 9-AMINOACRIDINE WITH NATIVE AND DENATURED DNA

1. Introduction.
2. Characterization of Denatured DNA.
3. Spectra of 9-aminoacridine-denatured DNA complexes.
4. Binding Curves for the interaction of 9-aminoacridine with native and denatured DNA.
   a. Results.
   b. Discussion.
5. Influence of Temperature on the binding of 9-aminoacridine to denatured DNA.
   a. Results.
   b. Discussion.
6. Sedimentation of DNA - 9-aminoacridine complexes.
   a. Introduction.
   b. Results and Discussion -
      (i) Native DNA.
      (ii) Denatured DNA.

References

1. Introduction

Knowledge of any influence which denaturation of DNA may have on the binding of aminoacridines is important in elucidating whether or not the intact double helix is an essential structural requirement for intercalation.

The intercalation model proposed by Lerman[1,2], in which the aminoacridine cations are inserted between adjacent base pairs of the DNA, requires the existence of a double helical structure. However, more recently there have been a number of reports[3-6] using a variety of experimental techniques in which the binding of proflavine to denatured DNA at 20-25^{o}C is at least as great as to native DNA. Drummond et al[7], using a spectrophotometric technique, found that the extent of binding of proflavine and 9-aminoacridine to denatured DNA was increased relative to the binding to native DNA. They postulated that this enhancement was due to an increase in the number of sites available for intercalation. However, a subsequent publication[8] has since reported that a systematic error was involved in the calculation of the binding curves, with the result that the difference in the binding of proflavine to native and denatured DNA is no longer significant, although denaturation still appears to cause some increase in primary binding for 9-aminoacridine.

The observation that the double helical structure

was not essential for the binding of proflavine or 9-aminoacridine led to the modified intercalation model proposed by Pritchard et al[9], in which the dye cations are intercalated between bases on the same polynucleotide chain, thus negating the requirement of the double helix for intercalation.

Chambron et al[10], on the basis of elevated temperature studies on the binding of proflavine to DNA, concluded that because binding decreased markedly over the temperature range coincident with the destruction of the double helix, the helical structure of DNA was a necessary condition for intercalation. However, as shown in Chapter III, there is a marked difference in the binding to native and denatured DNA at elevated temperatures for both proflavine and 9-amino-acridine. These observations at elevated temperatures cannot therefore be used to predict the binding behaviour of denatured DNA at lower temperatures. This difference in the binding of dye to the two states of DNA at high compared to low temperatures could be due either to a difference in the mode of binding at the two temperatures or to a marked difference in the variation of the binding coefficients with temperature for the two different DNA structures.

Ichimura et al[11] have shown that while the changes in free energy for the binding of acridine orange to native and denatured DNA are essentially the same, the changes

in enthalpy and entropy are quite different. Larger values of $-\Delta H^{o}$ and $-\Delta S^{o}$ were obtained for the interaction with denatured DNA. This observation suggests that the mode of binding of acridine orange to native and denatured DNA may be different.

In this chapter the interaction of 9-aminoacridine with native and denatured DNA is studied at $25^{o}C$ and at varying ionic strengths to determine the influence of the secondary structure on the extent of binding. The temperature dependence of the binding to denatured DNA is also examined in an attempt to investigate the observation that at elevated temperatures the binding of 9-aminoacridine to denatured DNA is much less than to native DNA.

2. Characterization of Denatured DNA.

Denatured DNA was obtained by using a combination of dilution and heat denaturation techniques[12,13]. Dilute solutions of DNA (0.005%) were prepared in double-distilled water of specific conductance less than 2×10^{-6} ohm^{-1} cm^{-1}. Under these conditions partial strand separation occurs[12]. Solutions were then heated at $100^{o}C$ for 10 minutes and shock-cooled at $0^{o}C$. Concentrated NaCl solution was then added to give the required concentration of the neutral salt.

In Fig. IV.1, curves 1-3 is shown the relative absorbance change with temperature at 260 nm for denatured

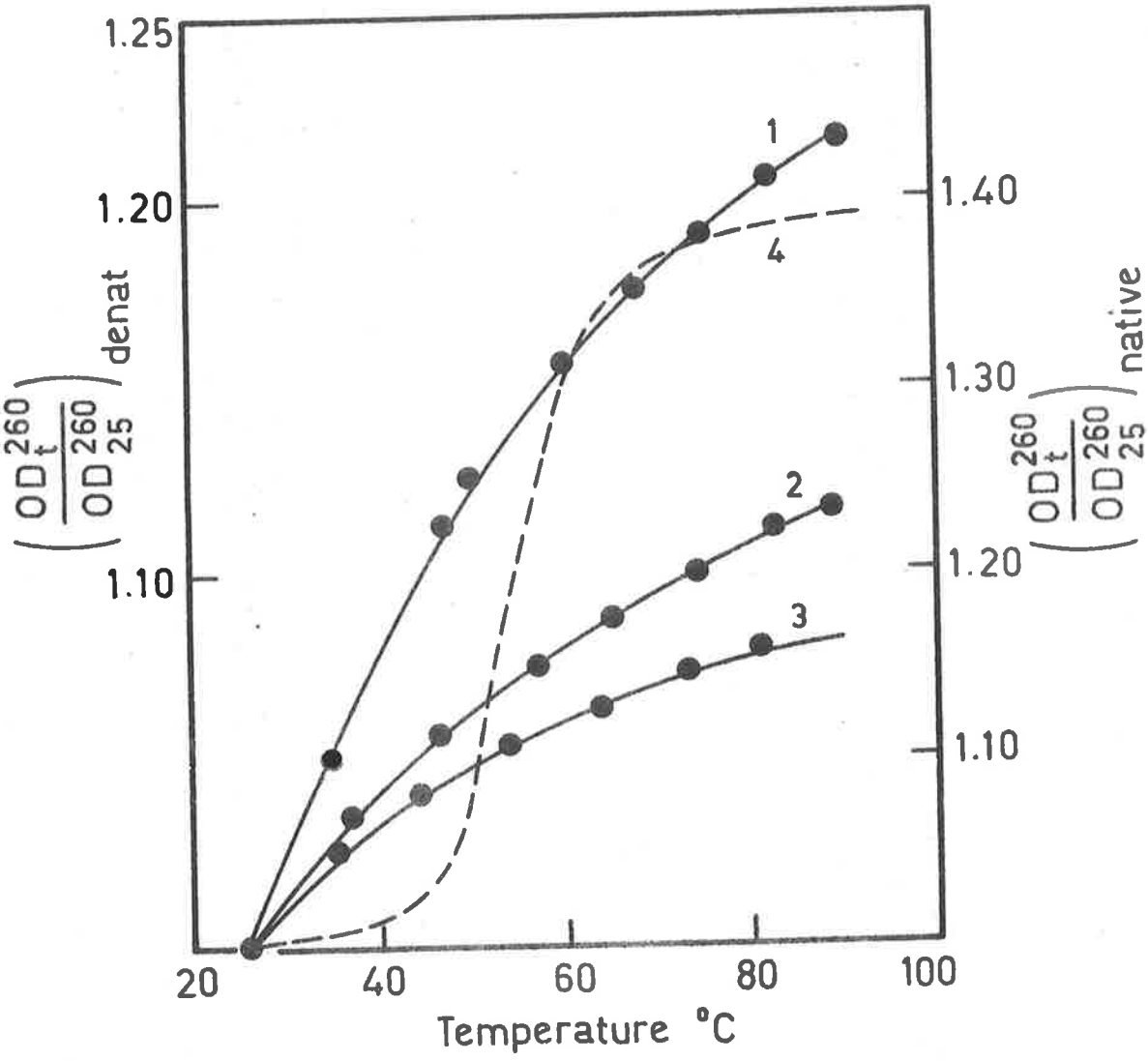

FIG. IV.1 : Relative absorbance change at 260 nm with temperature for denatured DNA (curves 1-3) and native DNA (curve 4).

Curve 1. 10^{-1}M NaCl.
Curve 2. 10^{-2}M NaCl.
Curves 3 and 4. 10^{-3}M NaCl.

Curves 1-3 left hand scale, curve 4 right hand scale.

DNA in the NaCl concentrations 10^{-1}M, 10^{-2}M and 10^{-3}M respectively. A steady increase in the absorbance is observed over the temperature range of 25^{o} - 90^{o}C. Fig. IV.1, curve 4, shows the relative absorbance change with temperature for native DNA in 10^{-3}M NaCl, and the sudden change in absorbance observed is typical of the melting profile for double helical DNA[14]. The lack of any such profile for the solutions of denatured DNA is taken to indicate the absence of any substantial renaturation and double-helix formation. The steady increase in relative absorbance with temperature has been attributed to a decrease in partial stacking of the purine and pyrimidine bases in single-stranded DNA with increasing temperature[15]. The magnitude of the relative absorbance change observed up to 90^{o}C, increases with increasing ionic strength, being 8% for solutions in 10^{-3}M NaCl and 22% for solutions in 10^{-1}M NaCl. This increase in relative absorbance is due to a greater degree of base-base interaction at lower temperatures in higher ionic strengths as a result of a reduction in the charge repulsion between neighbouring phosphate groups[16].

In view of the difference observed in the absorbance versus temperature graphs for native and denatured DNA, it has been assumed that the DNA obtained after dilution denaturation and heat treatment is essentially single-stranded with little, if any, double-helical structure.

3. Spectra of 9-aminoacridine-denatured DNA complexes.

In Figs. IV, 2-4 are shown the spectra of solutions of varying T_L/T_A ratios for 9-aminoacridine and denatured DNA at the temperatures 25°, 60° and 74°C respectively in 10^{-3}M NaCl. At 25° and 60°C small red shifts in the spectra are observed together with clear isosbestic points at 427 nm. These spectra are similar to those obtained for complexes of 9-aminoacridine and native DNA at temperatures below 84°C. (Refer Figs. III, 1-3.) The spectra observed at 74°C show no clear isosbestic point. This observation is in accord with that obtained when native DNA in 10^{-3}M NaCl is heated above the melting temperature before the addition of dye (refer Fig. III.4). Fig. IV.5 shows the spectra obtained for 9-aminoacridine-denatured DNA complexes at 25°C in 2×10^{-2}M NaCl. A distinct isosbestic point is again observed at 427 nm. The appearance of isosbestic points at low ionic strengths at 25°C for 9-aminoacridine is unlike the observation reported for the spectra of proflavine-denatured DNA complexes, in which clear isosbestic points are only obtained at relatively high ionic strengths[8] (> 0.1). This difference may be the result of the greater degree of primary binding observed for 9-aminoacridine[7].

The occurrence of a spectral shift and clear isosbestic points at 25°C for complexes of 9-aminoacridine and

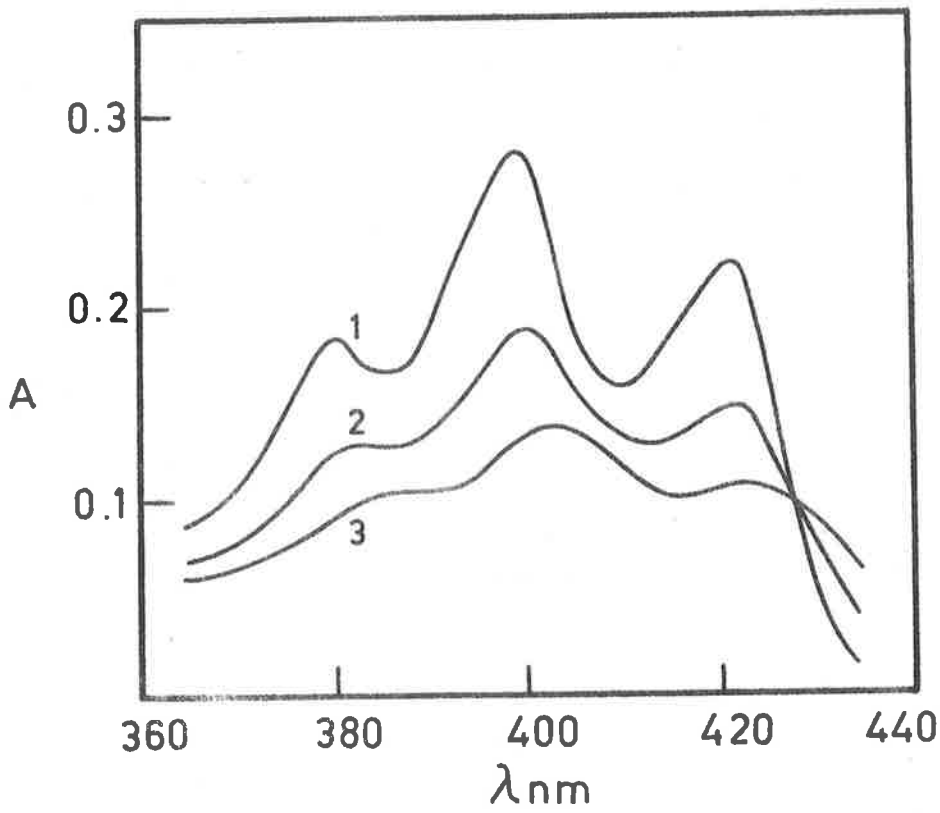

FIG. IV.2 : Spectra at 25°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with denatured DNA $^{T}L/_{T_A}$ = 0.962 (curve 2); $^{T}L/_{T_A}$ = 0.412 (curve 3). Constant dye concentration = 3.105 x 10^{-5}M.

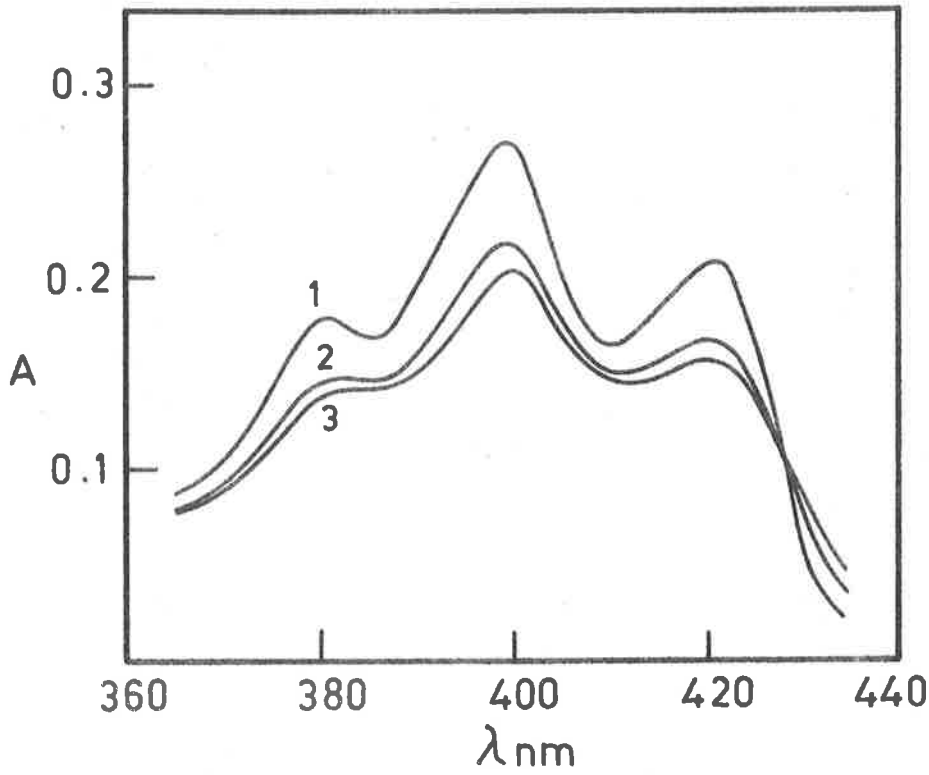

FIG. IV.3 : Spectra at 60°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with denatured DNA $^{T_L}/_{T_A}$ = 0.962 (curve 2); $^{T_L}/_{T_A}$ = 0.412 (curve 3). Constant dye concentration = 3.105 x 10^{-5}M.

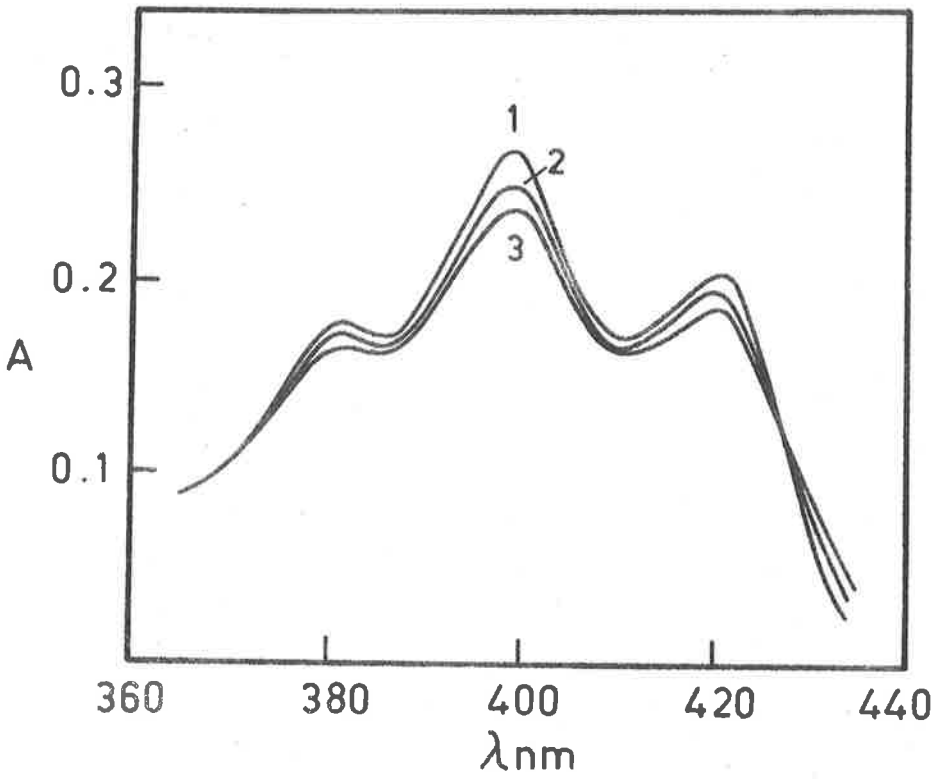

FIG. IV.4 : Spectra at 74°C in 10^{-3}M NaCl of 9-aminoacridine (curve 1) with denatured DNA $^{T_L}/_{T_A}$ = 0.962 (curve 2); $^{T_L}/_{T_A}$ = 0.412 (curve 3). Constant dye concentration = 3.105 x 10^{-5}M.

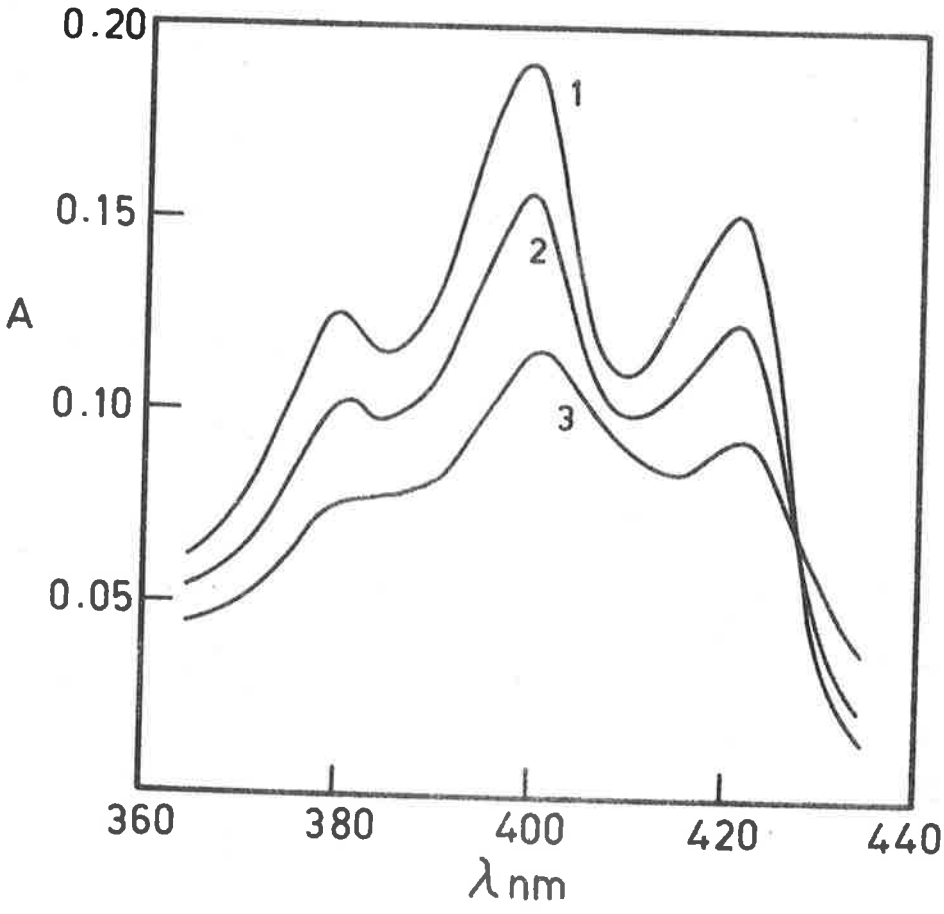

FIG. IV.5 : Spectra at 25°C in 2 x 10^{-2}M NaCl of 9-aminoacridine (curve 1) with denatured DNA T_L/T_A = 1.00 (curve 2); T_L/T_A = 0.375 (curve 3).
Constant dye concentration = 2.129 x 10^{-5}M.

denatured DNA allows the use of the Peacocke and Skerrett equation[17] for the spectroscopic determination of the extent of binding.

4. Binding Curves for the Interaction of 9-aminoacridine with Native and Denatured DNA.

a. Results

In Fig. IV.6, a-e, is shown the binding curves of the number of moles of dye bound per mole of DNA-phosphorus (r) plotted with the concentration of free or unbound dye (c), for the binding of 9-aminoacridine to native and denatured DNA in 10^{-3}, 5×10^{-3}, 10^{-2}, 2×10^{-2} and 10^{-1}M NaCl respectively. A greater extent of binding to denatured relative to native DNA is observed at all neutral salt concentrations studied. This increase is evident over the whole range of free dye concentrations studied including very low values where primary binding is expected to be dominant.

For a given salt concentration the shape of the binding curves is similar for the two DNA species. In 10^{-3}M NaCl, no plateau in the value of r is obtained for either species while in 10^{-1}M NaCl both binding curves exhibit distinct plateau regions in the variation of r with c.

A decrease in the extent of binding with increasing

FIG. IV.6 : Spectrophotometric binding curves for the interaction at 25°C of 9-aminoacridine with native DNA (□) and denatured DNA (■).

(a) 10^{-3}M NaCl, (b) 5 x 10^{-3}M NaCl, (c) 10^{-2}M NaCl, (d) 2 x 10^{-2}M NaCl, (e) 10^{-1}M NaCl.

(Average pH = 6.6)

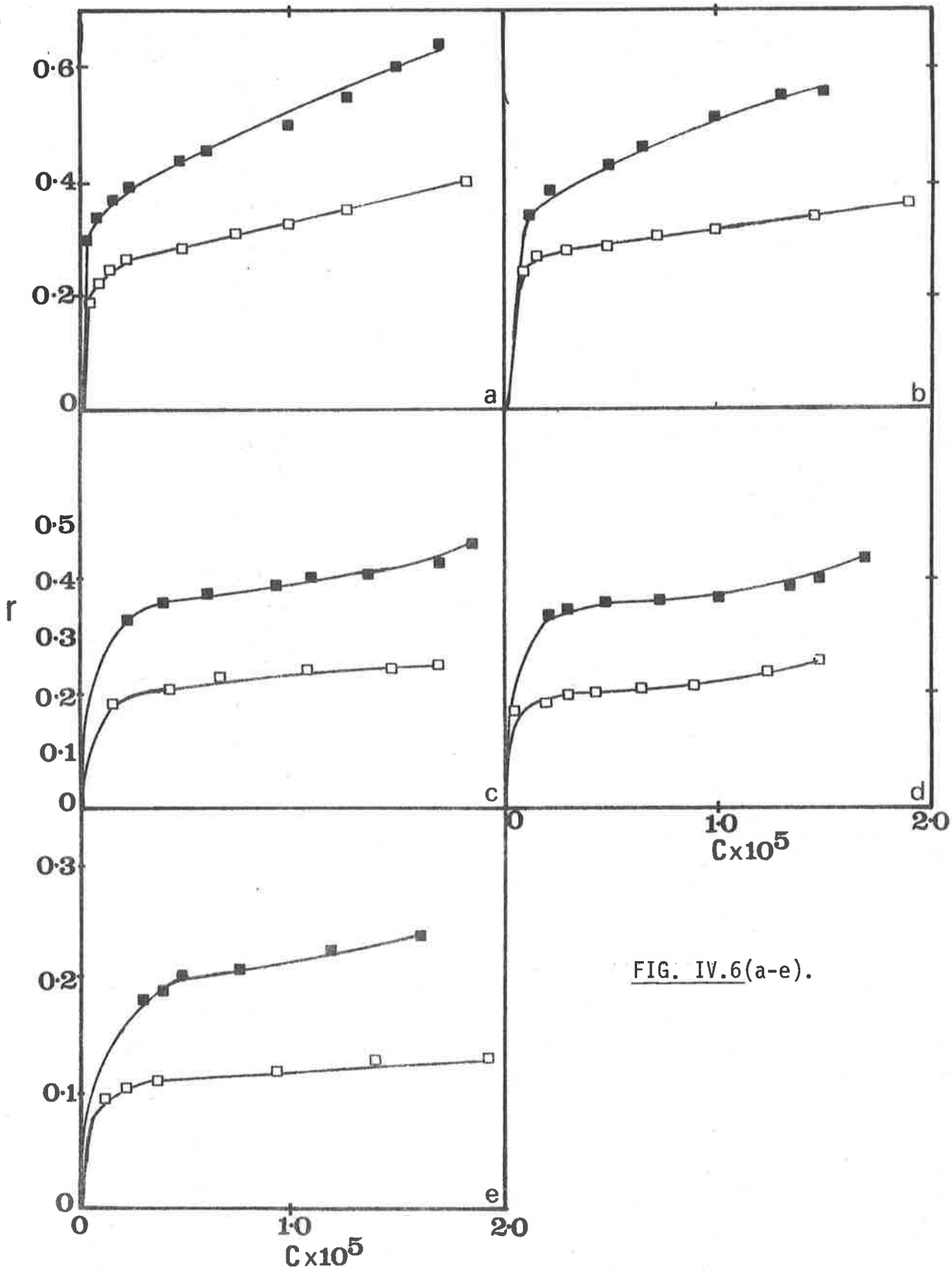
r
0·6
0·4
0·2
0
a
b
0·5
0·4
0·3
0·2
0·1
0
c
d
0
1·0
2·0
C x10^5
0·3
0·2
0·1
0
e
0
1·0
2·0
C x10^5

FIG. IV.6(a-e).

ionic strength is observed for both native and denatured DNA indicating the involvement, at least in part, of electrostatic interactions in the binding process to both species. A similar observation has been reported previously[7].

b. Discussion

The increase in the extent of binding upon denaturation can result from an increase in either one or both of the following parameters -

1. The maximum number of binding sites (n)
2. The apparent binding constant (k).

As reported by Drummond et al[7], the significance of these two parameters may be distinguished because an increase in site number would enhance the degree of binding at all free-dye concentrations, whereas this effect would not be achieved by an increase in the binding constant. As discussed in Chapter II, one method of obtaining the value of these parameters is by the use of Scatchard Plots[18], which for the systems studied are shown in Fig. IV.7, a-e. The values of n and k obtained are given in Table IV.1. The apparent binding constant (k) can be seen to be relatively insensitive to the change in DNA structure and thus cannot account for the increase in the extent of binding to denatured DNA. However, the enhanced binding is explainable in terms of the increase in site number

FIG. IV.7 : The variation in $^{r}/_{c}$ with r for 9-aminoacridine and native DNA (□) and denatured DNA (■) at 25°C.

(a) 10^{-3}M NaCl, (b) 5 x 10^{-3}M NaCl, (c) 10^{-2}M NaCl, (d) 2 x 10^{-2}M NaCl, (e) 10^{-1}M NaCl.

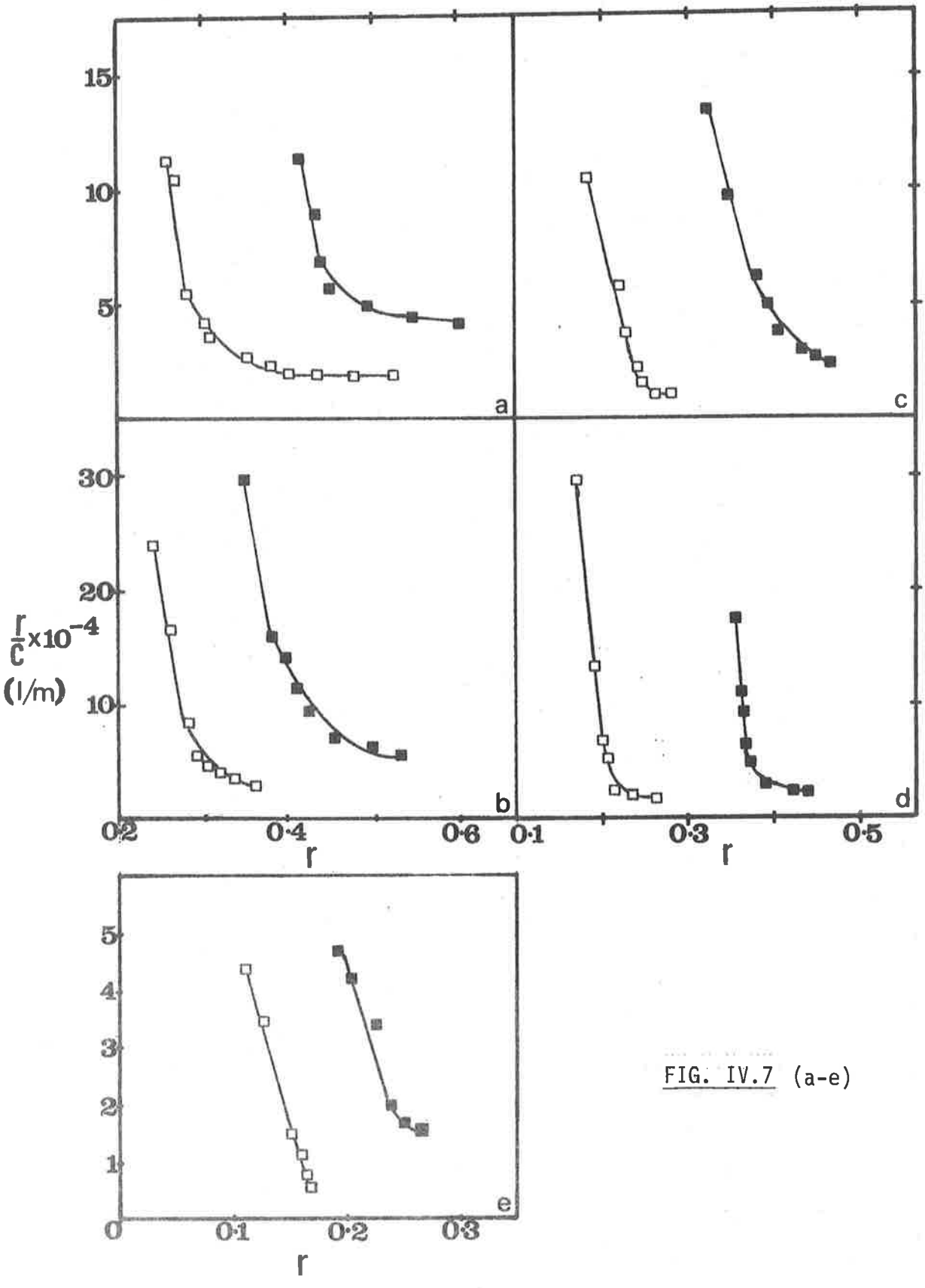
15
10
5
a
c
30
20
10
b
d
r/c x10^-4 (l/m)
0·2
0·4
0·6
r
0·1
0·3
0·5
r
5
4
3
2
1
0
e
0·1
0·2
0·3
r

FIG. IV.7 (a-e)

	NATIVE DNA		DENATURED DNA		
NaCl Concentration	n_1	k_1 (l/mole)	n_1	k_1 (l/mole)	$\frac{n(\text{denatured})}{n(\text{native})}$
10^{-3}M	0.32	2.4×10^6	0.49	1.8×10^6	1.53
5×10^{-3}M	0.29	4.0×10^6	0.45	2.8×10^6	1.55
1×10^{-3}M	0.26	1.8×10^6	0.42	1.5×10^6	1.62
2×10^{-2}M	0.22	4.0×10^6	0.37	4.6×10^6	1.65
10^{-1}M	0.17	0.75×10^6	0.27	0.70×10^6	1.59

Temperature 25°C

TABLE IV.1 : The values of n and k for the primary binding process of 9-aminoacridine to native and denatured DNA at various ionic strengths at 25°C.

observed at all neutral salt concentrations studied. If the increase in the extent of binding to denatured DNA results from a greater number of externally bound dye cations, then the ratio $n_{denatured}/n_{native}$ would be expected to decrease with increasing ionic strength, because binding to these external sites is much more sensitive to changes in ionic strength than is intercalation[7,10,17]. However, as shown in Table IV.1, the increase in site number is relatively independent of ionic strength, at least for NaCl concentrations up to 0.1M, and it may therefore be postulated that the increase in binding observed for denatured DNA is a result of a greater number of sites becoming available for the intercalation of dye cations.

Armstrong et al[19] have proposed a restriction on the primary site number in native DNA by postulating that intercalation does not occur at sites immediately adjacent to sites already occupied. This postulate assumes that the region between two successive base pairs can be occupied by only one intercalated dye cation, and thus limits intercalation to a theoretical maximum of one dye cation per four DNA phosphate groups. In the intercalation model proposed by Pritchard et al[9], the binding site is the region between two adjacent bases on the same polynucleotide chain. Thus, ignoring any steric restrictions, the region between two successive base pairs in native DNA should constitute

two intercalation sites, one on each chain of the double helix, resulting in a theoretical limit for intercalation of twice that proposed by Pritchard et al[9]. That such a limit has not been observed for native DNA, suggests that the presence of a dye cation in an intercalation site on one chain, imposes a restriction on the potential site existing between the complimentary bases in the adjacent polynucleotide chain. This particular restriction is not relevant if intercalation occurs between the base pairs with the dye centrally located within the helix, as has been proposed by Lerman[2], because in this model the dye effectively occupies both sites.

Two possibilities exist concerning the mode of binding of 9-aminoacridine to native and denatured DNA.

(a) The mode of binding is exactly the same to both states of DNA. This possibility excludes the Lerman model as a general model for intercalation because denaturation destroys the double helix which is essential in this model.

(b) The mode of binding is different for the two states; for example, Ichimura et al[11] has postulated that the binding of acridine orange to native DNA is an intercalation between adjacent base pairs, while the binding to single-stranded DNA is an intercalation between adjacent bases.

On denaturation and strand separation of native DNA, the restriction on the occupancy of the 'potential' site imposed by the dye cation bound in the opposite site would no longer exist and thus a two-fold increase in site number would be expected. (Fig. IV.8). Any strand recombination forming double helical segments due to renaturation or folding of the molecule, would effectively reduce the number of 'potential' sites being made available for intercalation, and thus the two-fold increase would be the theoretical maximum attainable.

Irrespective of whether the Lerman or the Pritchard model is chosen for the interaction of 9-aminoacridine with native DNA an increase in site number on denaturation is to be expected on the basis of the above discussion. (See Fig. IV.8). Therefore, it is not possible to determine whether the mode of binding of the dye to native and denatured DNA is different using the results presented.

5. Influence of Temperature on the binding of 9-aminoacridine to Denatured DNA.

a. Results

The temperature dependence of the binding of aminoacridines to native DNA has been extensively studied with the observation that the majority of the dye is released on the dissociation of the double helix[10,20,21].

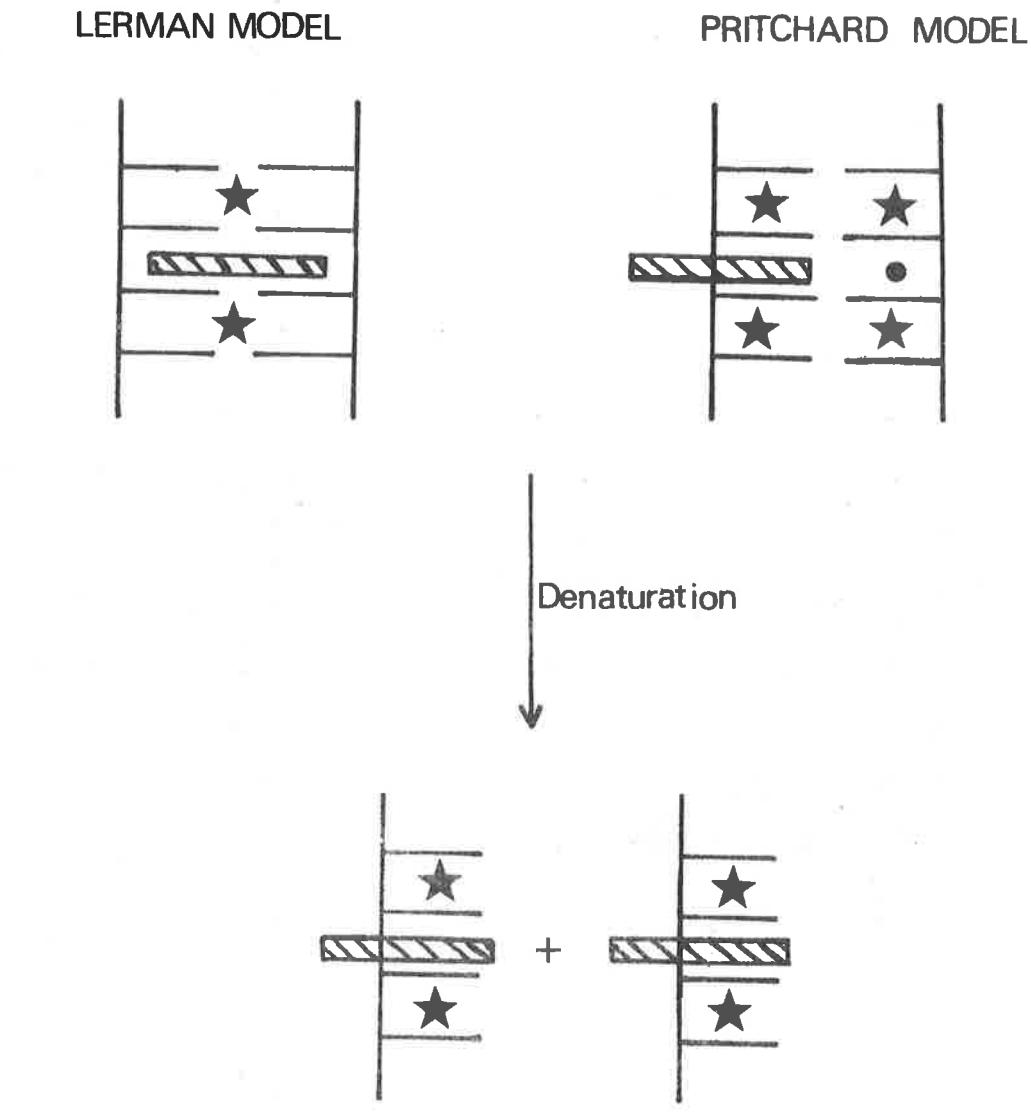

FIG. IV.8 : Schematic representation of the increase in the number of intercalation sites when DNA is denatured.

This indicates that the double helical structure stabilizes the aminoacridine-DNA complex against thermal dissociation, but cannot be taken as evidence for the structural requirement of the double helix for intercalation. Examination of the influence of temperature on the binding to denatured DNA can be expected to yield information not only on the nature of the binding to single stranded DNA but also on the thermal stability afforded by the double helical structure to aminoacridine-native DNA complexes.

Fig. IV.9, curves 1-4, shows the influence of temperature on the absorbance of mixtures of varying ratios of 9-aminoacridine and denatured DNA at 400 nm in 10^{-3}M NaCl. A large increase in absorbance is observed over the temperature range of 50^{o} - 70^{o}C, with the midpoint of the transition being independent of the T_L/T_A ratio. Fig. IV.9, curve 5, shows the absorbance at 400 nm as a function of temperature of a native DNA - 9-aminoacridine complex in 10^{-3}M NaCl. Comparison of curves 1 and 5, for which the T_L/T_A ratio is identical (0.45), illustrates the large difference in the temperature dependence of the binding of 9-aminoacridine to native and to denatured DNA. The dissociation of the native-DNA dye complex occurs at a temperature at least 20^{o}C above that observed for the complex with denatured DNA.

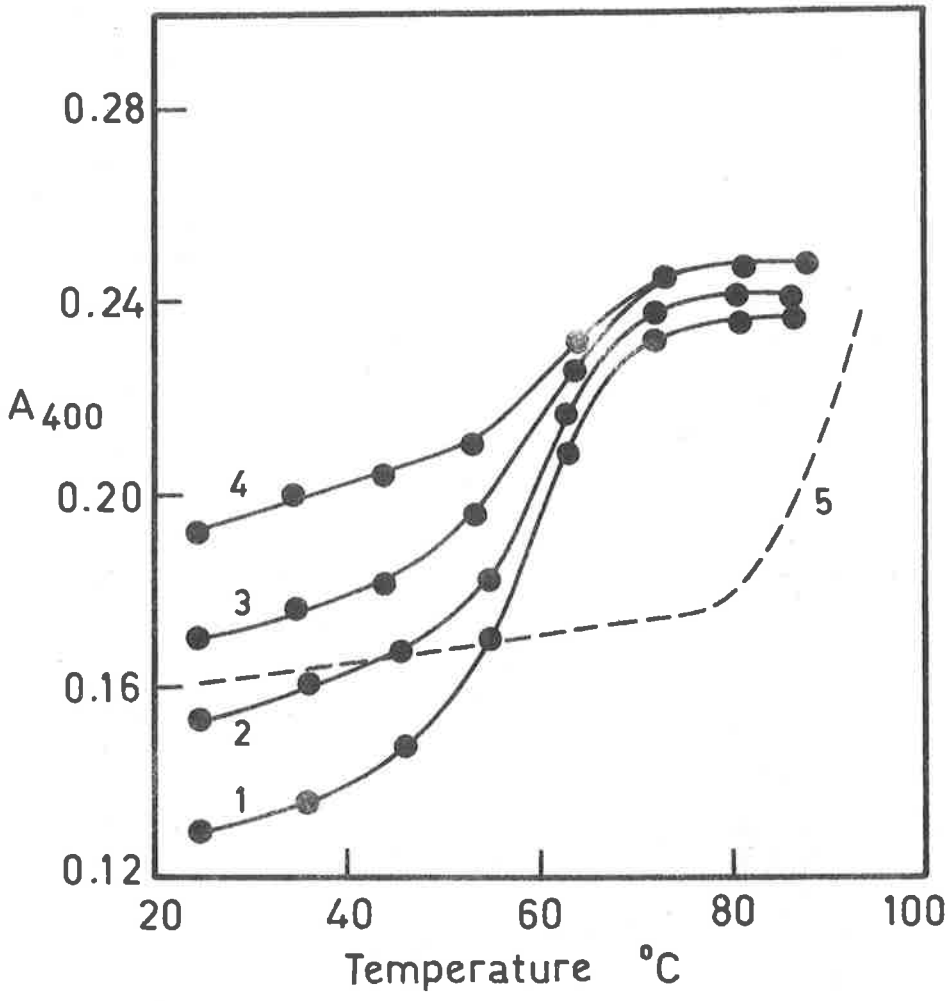

FIG. IV.9 : Absorbance at 400 nm as a function of temperature of 9-aminoacridine-denatured DNA complexes (curves 1-4) and of a 9-aminoacridine-native DNA complex (curve 5) in 10^{-3}M NaCl.
Curve 1, $^{T_L}/_{T_A}$ = 0.452; curve 2, $^{T_L}/_{T_A}$ = 0.603;
curve 3, $^{T_L}/_{T_A}$ = 0.755; curve 4, $^{T_L}/_{T_A}$ = 1.13;
curve 5, $^{T_L}/_{T_A}$ = 0.451.
Constant dye concentration = 3.05 x 10^{-5}M (curves 1-4)
Constant dye concentration = 2.79 x 10^{-5}M (curve 5)

In Figs. IV.10 and 11 are shown the temperature dependence of the absorbance at 400 nm for mixtures of varying T_L/T_A ratios of 9-aminoacridine and denatured DNA in 10^{-2} and 10^{-1}M NaCl respectively. At these salt concentrations the dissociation of the complexes also occurs at much lower temperatures than expected for complexes with native DNA, for which the dissociation temperature is at least as high as the melting temperature of the native DNA[10,22].

The shape of the absorbance versus temperature graphs alter with varying neutral salt concentration. At the lowest salt concentration studied, 10^{-3}M NaCl, the curves may be partitioned into two distinct regions with the majority of the absorbance increase, indicative of dye release, being obtained at temperatures above 50°C. However, in 10^{-1}M NaCl approximately 50% of the total absorbance change is achieved before 45°C. The shape of the curves obtained in 10^{-2}M NaCl is intermediate between those observed for 10^{-3} and 10^{-1}M NaCl. The difference observed in the shape of the graphs at varying NaCl concentrations may be the result of an interaction of 9-aminoacridine cations with the negatively charged phosphate groups on the polynucleotide chains. This interaction will be suppressed at higher salt concentrations and it may be postulated that in 10^{-3}M NaCl these externally bound dye cations prevent the release of dye from the

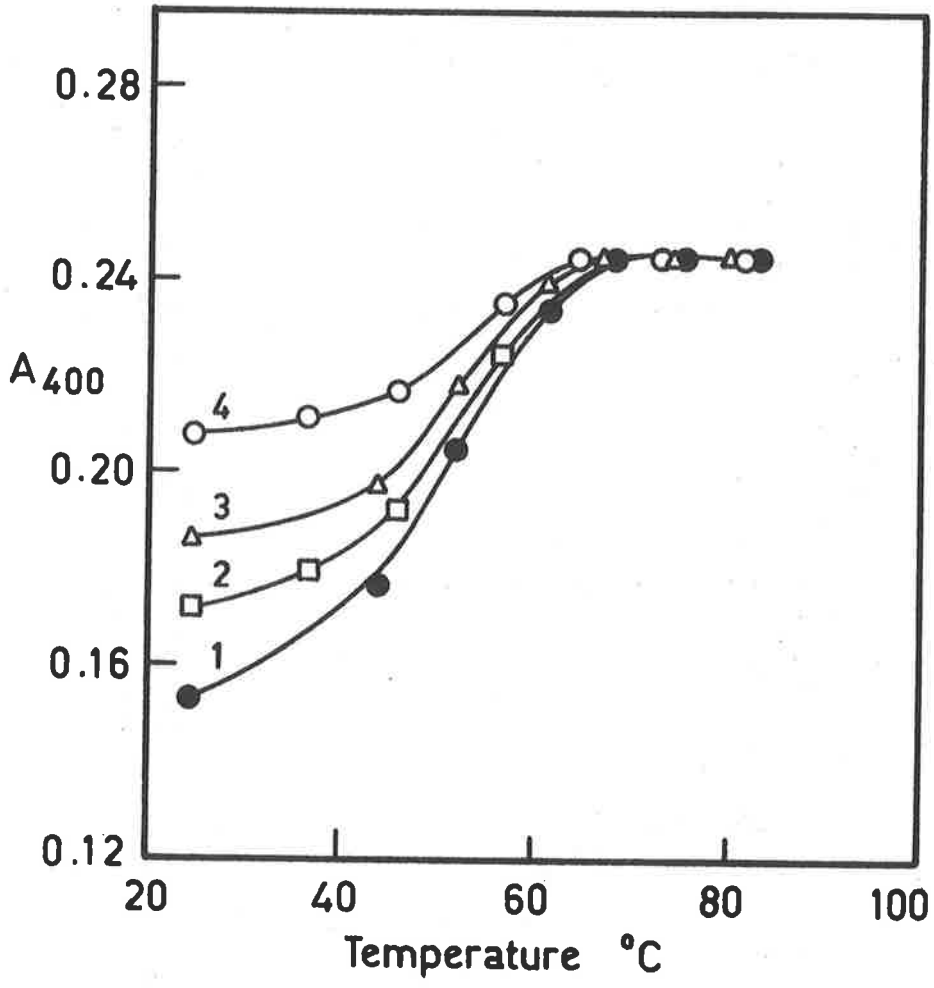

FIG. IV.10 : Absorbance at 400 nm as a function of temperature of 9-aminoacridine-denatured DNA complexes in 10^{-2}M NaCl.
Curve 1, $^{T}L/T_A$ = 0.459; curve 2, $^{T}L/T_A$ = 0.612;
curve 3, $^{T}L/T_A$ = 0.765; curve 4, $^{T}L/T_A$ = 1.147.
Constant dye concentration = 3.06 x 10^{-5}M.

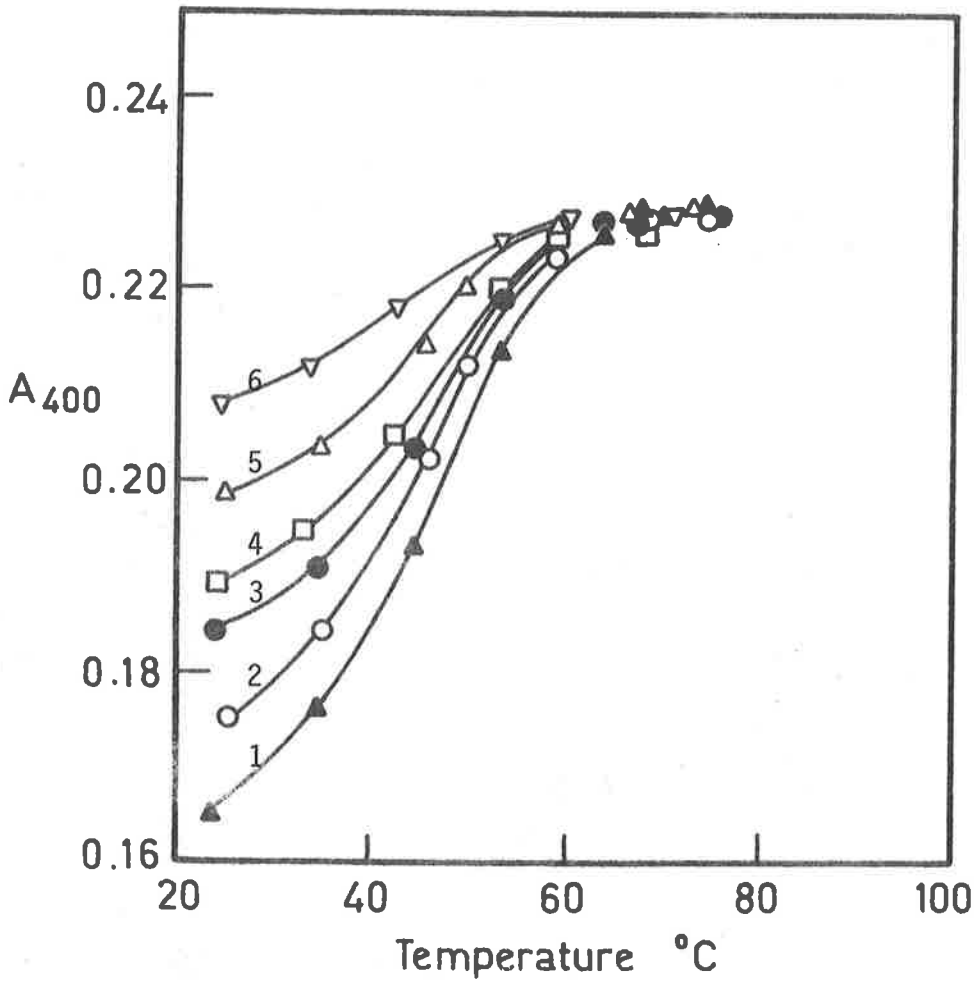

FIG. IV.11 : Absorbance at 400 nm as a function of temperature of 9-aminoacridine-denatured DNA complexes in 10^{-1}M NaCl.
Curve 1, T_L/T_A = 0.342; curve 2, T_L/T_A = 0.466;
curve 3, T_L/T_A = 0.513; curve 4, T_L/T_A = 0.617;
curve 5, T_L/T_A = 0.777; curve 6, T_L/T_A = 1.15.
Constant dye concentration = 2.70 x 10^{-5}M.

intercalation sites of denatured DNA until a temperature is reached at which they are released. This postulate will be examined in more detail in the following discussion.

(b) Discussion

The observation that 9-aminoacridine is released from denatured DNA complexes at much lower temperatures than from complexes with native DNA is explainable in terms of a model in which the purine and pyrimidine bases in the denatured DNA molecule are able to oscillate about the deoxyribose-phosphate backbone with a greater degree of freedom than they can in native, double-helical, DNA.

The interaction between neighbouring bases is important in determining the configuration of the DNA molecule. Formerly, hydrogen bonding between matched bases in the DNA double helix was considered to be the major factor contributing to the stability of the helix[23]. However, it is now generally accepted that the helix stability results from the interaction between neighbouring bases on a single strand, leading to a stacked configuration[24], whilst hydrogen bonding is responsible for base specificity. The type of intermolecular forces involved in base stacking are

(1) Electrostatic forces, which tend to stack the bases in such a manner as to give the greatest

overlap; e.g., London forces, dipole-dipole and dipole-induced dipole interactions

(2) Hydrophobic bonding which tends to minimize the contact of the bases with the solvent. The result of this bonding is identical to that achieved by London forces.

These forces, subject to structural constraints of the sugar-phosphate backbone, determine the relative position of the bases by stacking them in such a conformation so that maximum overlap is achieved. This conformation is not a static one, as it has been shown that base-pair separation and subsequent orientation of the bases away from the helix centre can occur[25,26]. However, this oscillatory freedom is very small in double helical DNA due to the presence of the hydrogen bonds between the two strands[27], with the result that stacking of the bases is not substantially influenced[28]. In single-stranded DNA such restraints are not present and there is considerable freedom for base oscillation, although at low temperatures the bases would be expected to be in a rigidly stacked configuration[29]. However, with increasing temperature as the structure becomes less rigid, the relative oscillations of the bases would increase.

A corollary can be proposed for double helical DNA at temperatures below and above the melting temperature (T_m).

The rigidity of the double helix and the presence of hydrogen bonds constrain the base oscillations at temperatures below T_m. At temperatures above T_m, rigidity is lost and the degree of base oscillation would be expected to increase dramatically, not only as a result of strand separation but also because of the high thermal energy possessed by the system at elevated temperatures. As a consequence of increasing base oscillation, the interaction between neighbouring bases decreases giving rise to the large hyperchromic effect observed at 260 nm when disruption of the helix occurs at the melting temperature[14].

The location of an intercalated dye cation in the DNA molecule will be in that position for which the highest binding energy occurs as a result of molecular interactions and steric factors[30]. The extent to which intermolecular forces, such as London forces, Dipole-Dipole and Dipole-induced Dipole interactions contribute to the binding energy, will depend upon the degree of interaction between the dye cations and their nearest neighbour purine and pyrimidine bases. The interaction will be influenced by both the extent of overlap of the dye with the bases, and the time spent by the bases in sufficient overlap position to allow significant interaction to occur.

A model may be postulated in which a sufficient lifetime of conformations, for which the overlap is suitable

for the formation of an intercalation site, is necessary in order that interaction may occur between the bases and the dye cations. If this lifetime is too short, then the base-dye interactions are insufficient to maintain the dye cation in that configuration and therefore it will be released.

The observation that the majority of the dye is released from native DNA-dye complexes at a temperature corresponding to destruction of the double helix may be explained using the above model. On strand separation, the extent and frequency of base oscillations would greatly increase, with the result that the lifetime of the suitable conformations will be too short for sufficient interaction to occur. The binding energy will be greatly reduced resulting in the release of the dye from the complex.

Because the oscillations of the bases in single-stranded DNA are not subject to the constraints imposed by the double helix, it is anticipated that the extent and frequency of the oscillations for which the lifetime of the suitable conformations becomes insufficient, will occur at lower temperatures than required to dissociate the double helix of native DNA. Thus, dye release from denatured DNA complexes takes place over a temperature range lower than the release from native DNA complexes.

The observation that the dissociation of 9-aminoacridine-denatured DNA complexes occurs at lower temperatures with increasing ionic strength cannot be satisfactorily explained at this stage. A possible explanation is that externally bound dye cations may in some manner restrict base oscillations, and because the degree of external binding increases with decreasing ionic strength, higher temperatures are required to cause dissociation of the complexes.

6. Sedimentation of DNA - 9-aminoacridine Complexes.

a. Introduction

The sedimentation coefficient has been shown to be a sensitive measure of size and shape of all forms of DNA[16,31], and therefore provides a means of detecting changes in the configuration of the macromolecule under varying conditions.

The intercalation models proposed for the interaction of aminoacridines with native DNA[2,9] involve some extension and local untwisting of the DNA double helix, to an extent dependent upon the number of dye cations intercalated. Because the increase in length due to the intercalation of a dye cation is about the same as the distance between neighbouring bases in uncomplexed native DNA[1,32,33], while the mass increment is less than one-half

of a nucleotide pair, the average mass per unit length of the DNA would decrease. Lerman therefore predicted that the sedimentation coefficient of DNA should decrease upon the intercalation of dye cations, and subsequently observed this effect with proflavine[1]. This decrease in sedimentation coefficient is consistent with an increase in contour length of the DNA double helix[1,4,34]. That such an increase does occur, has been demonstrated by Cairns[35] using an autoradiographic technique, by which he was able to demonstrate an increase in the length of DNA fibres when proflavine was bound.

Lerman[1] has suggested that the binding of dye leads to both lengthening and stiffening of the DNA structure. However, when viscosity and sedimentation coefficient data for DNA-proflavine complexes were combined, Lloyd et al[4] found that the value of the Scheraga-Mandelkern coefficient[36], β, (see Footnote 1), decreased by about 20% on the binding of proflavine. Using the worm-like model for DNA of Eizner and Ptitsyn[37], in which β is related to the persistence length of the chain and to the length of the hydrodynamically independent segments[38], they concluded that while an increase in contour length did occur, the persistence length decreased indicating an increase in the flexibility of the DNA. This conclusion has been more recently confirmed by Armstrong et al[19] on the basis of the ionic strength behaviour of the intrinsic

viscosity of proflavine-DNA complexes. The decrease in stiffness of the DNA molecule results in a steeper decrease in the sedimentation coefficient than that predicted solely on the basis of a reduction in mass per unit length. These conclusions of Lloyd et al[4] are unlike those of Cohen and Eisenberg[35] who, using sedimentation and viscosity data of sonicated-DNA-proflavine complexes, found that the parameter β was only weakly dependent on the degree of binding. The difference in the observations may result from the fact that the small fragments of DNA, obtained by sonic irradiation of native DNA, have a persistence length three or four times that of high molecule weight DNA and thus any change may not be readily detected.

Footnote 1

The equation relating to sedimentation coefficient (s) and the intrinsic viscosity $[\eta]$ is

$$\beta = N S_{20}^{0} [\eta]^{1/3} \eta_o / M^{2/3} (1 - \bar{v} \rho)$$

where N is Avagadro's number, S_{20}^{0} is the sedimentation coefficient at infinite dilution measured at $20^{o}C$, $[\eta]$ is the intrinsic viscosity at $20^{o}C$, η_o and ρ are respectively the specific viscosity and density of the solvent at $20^{o}C$, $\bar{v}$ is the partial specific volume of native DNA, and M is the molecular weight of the DNA.

In view of the binding of 9-aminoacridine to denatured DNA, discussed earlier in the chapter, it is of interest to determine whether the sedimentation coefficient of denatured DNA alters when dye is bound.

b. Results and Discussion

(i) Native DNA

In view of the large concentration dependence of the sedimentation coefficient of DNA[14], it is desirable to determine the variation with r of S^0, the sedimentation coefficient of the DNA - 9-aminoacridine complex at infinite dilution. In order to obtain values of S^0, solutions with varying DNA concentration, but with the value of r constant, were prepared by using the following relationship -

$$T_L = rT_A + c$$

where T_L is the total concentration in moles/l of 9-aminoacridine; T_A is the total concentration of DNA (moles P/l); c is the concentration in moles/l of free 9-aminoacridine; and r is the number of moles of dye bound per mole of DNA-phosphorus. For a given value of r, the concentration of free dye (c) can be determined from the binding curve of r versus c at the required ionic strength. Thus the total concentration of dye to be added can be determined once the value of T_A is fixed.

All experiments were conducted at 25°C and in the

presence of 0.1M NaCl. The binding curve for 9-aminoacridine under these conditions is shown in Fig. IV.6e, in which the maximum binding observed is r = 0.17, in agreement with that obtained by Drummond et al[7] for 9-aminoacridine and Calf Thymus DNA under identical conditions.

The sedimentation coefficients were determined for a series of solutions, prepared by reference to the binding curve, of various DNA solutions, such that the value of r remained constant within each series.

The results are expressed in terms of $^1/_S$ versus DNA concentration for a series of r values and are shown in Figs. IV.12-18. The least-squares lines were extrapolated to zero concentration to obtain the values of the sedimentation coefficients at infinite dilution for a particular extent of binding ($S^0_{25,r}$). In Fig. IV.19 is shown the variation of $S^0_{25,r}$ with r. The observed decrease in the sedimentation coefficient with increasing r is similar to that previously reported for proflavine[4]. This similarity is in agreement with Lerman's interpretation[1] of the decrease in sedimentation coefficient, because at the same value of r, the decrease in mass per unit length is expected to be very similar for both aminoacridines; for example at r = 0.1, the ratio $S^0_{25,r}/S^0_{25,r=o}$ is 0.81 for 9-aminoacridine complexes, while from the data presented for proflavine by Lloyd et al[4], the ratio is 0.80.

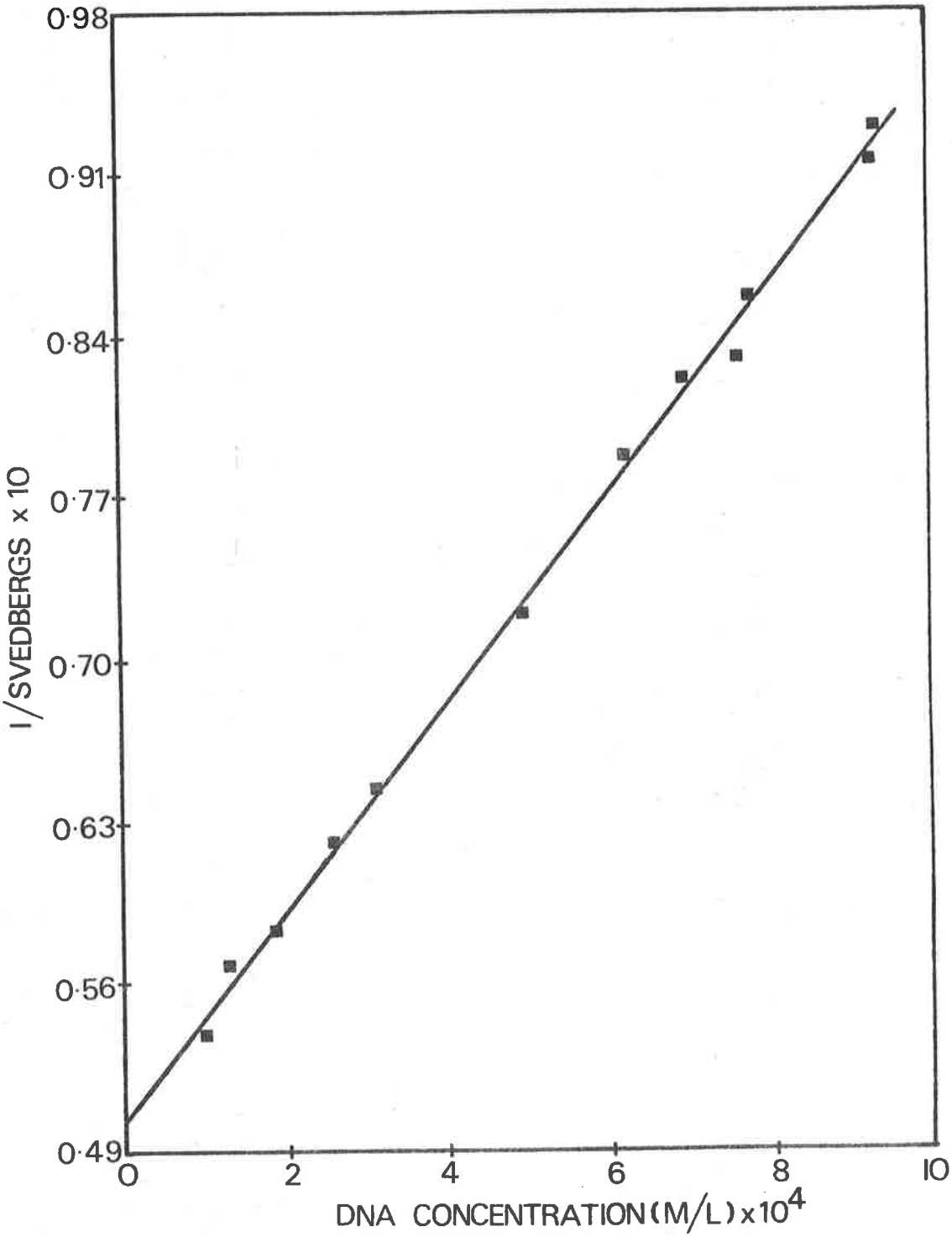

FIG. IV.12 : The concentration dependence of $^{1}/_{s}$ for DNA in 10^{-1}M NaCl at 25°C.

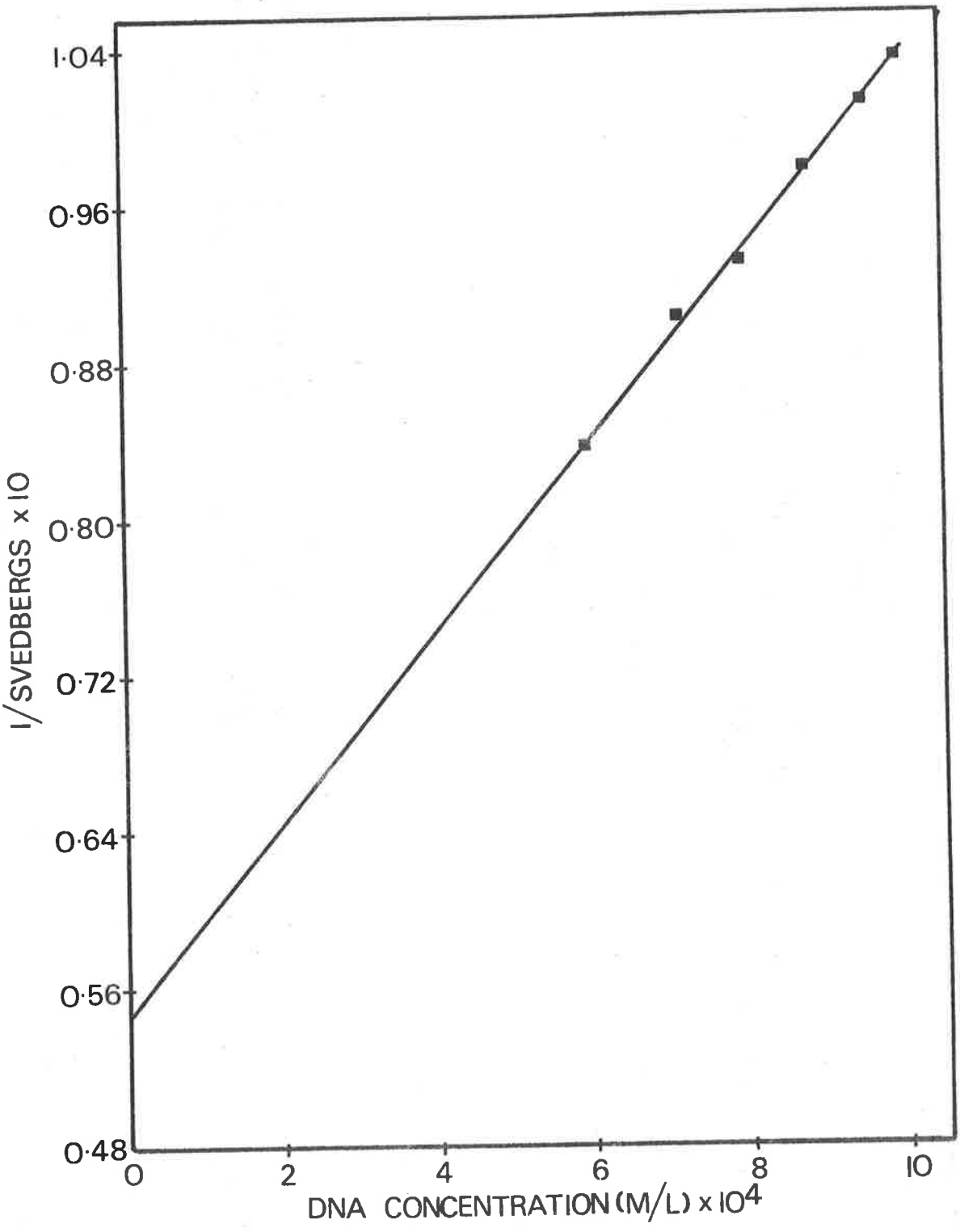

FIG. IV.13 : The concentration dependence of $^{1}/_{S}$ for the 9-aminoacridine-DNA complex at r = 0.05 in 0.1M NaCl at 25°C.

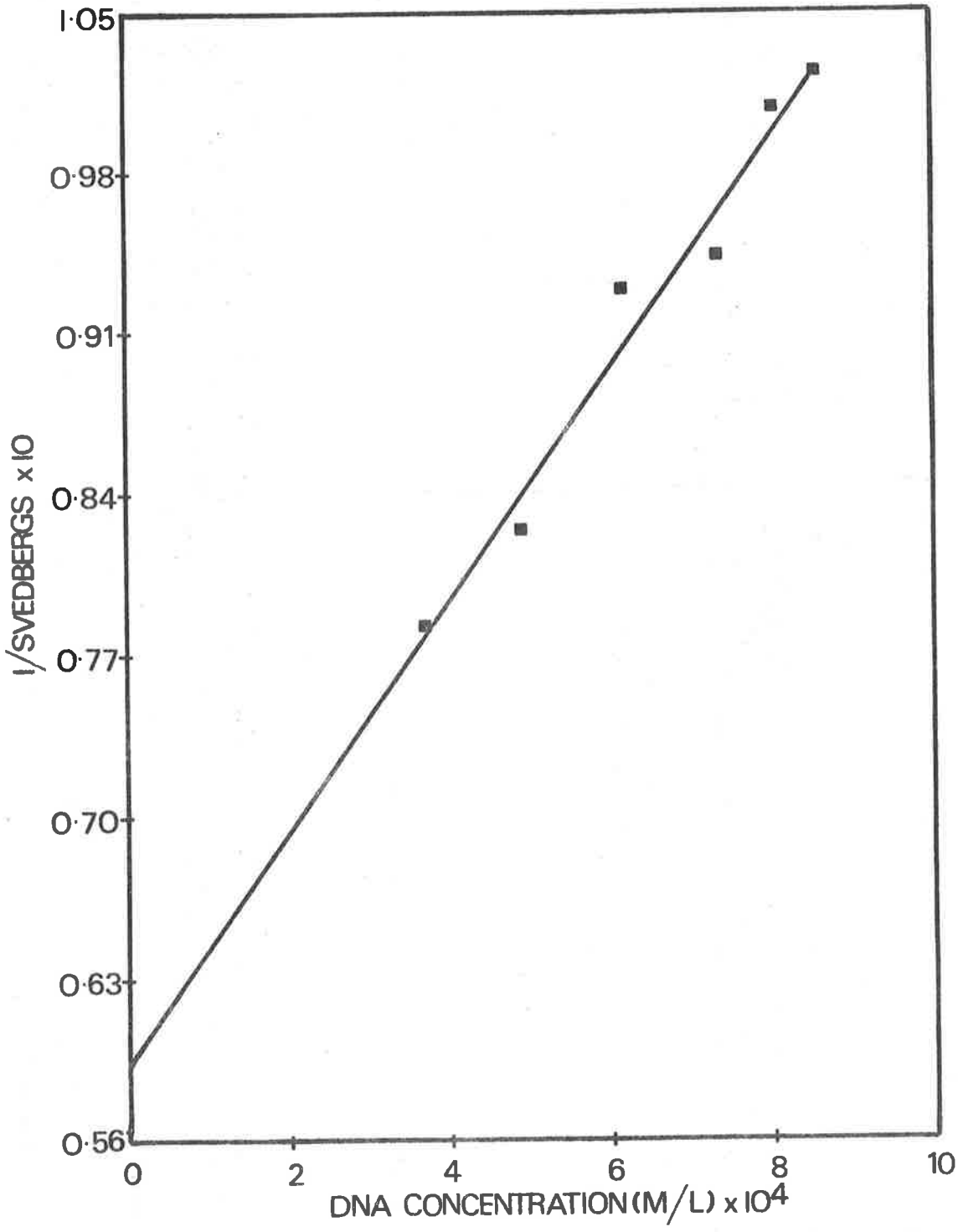

FIG. IV.14 : The concentration dependence of $^{1}/_{S}$ for the 9-aminoacridine-DNA complex at r = 0.075 in 0.1M NaCl at 25°C.

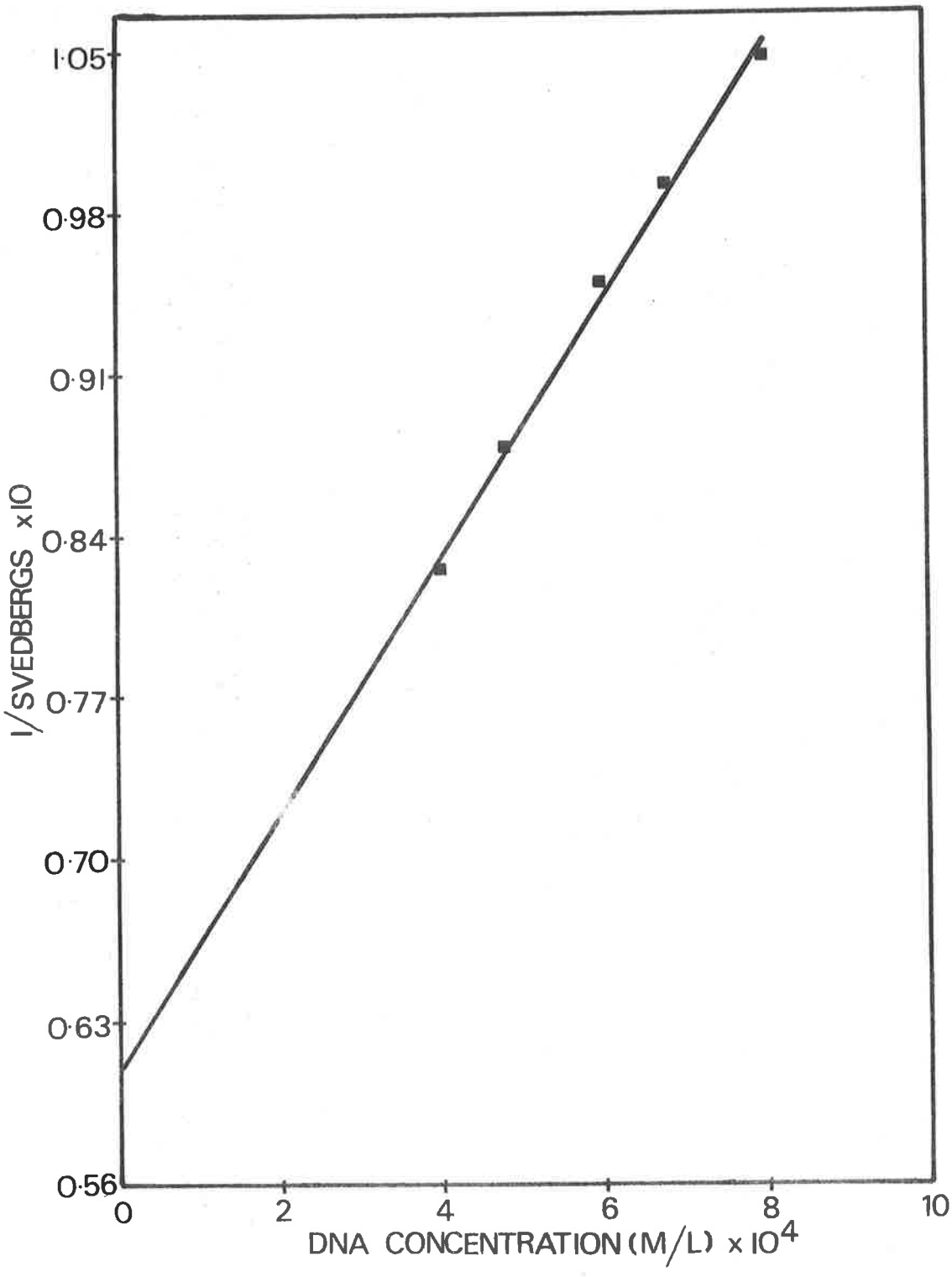

FIG. IV.15 : The concentration dependence of $^1/_S$ for the 9-aminoacridine-DNA system at r = 0.09 in 0.1M NaCl at 25°C.

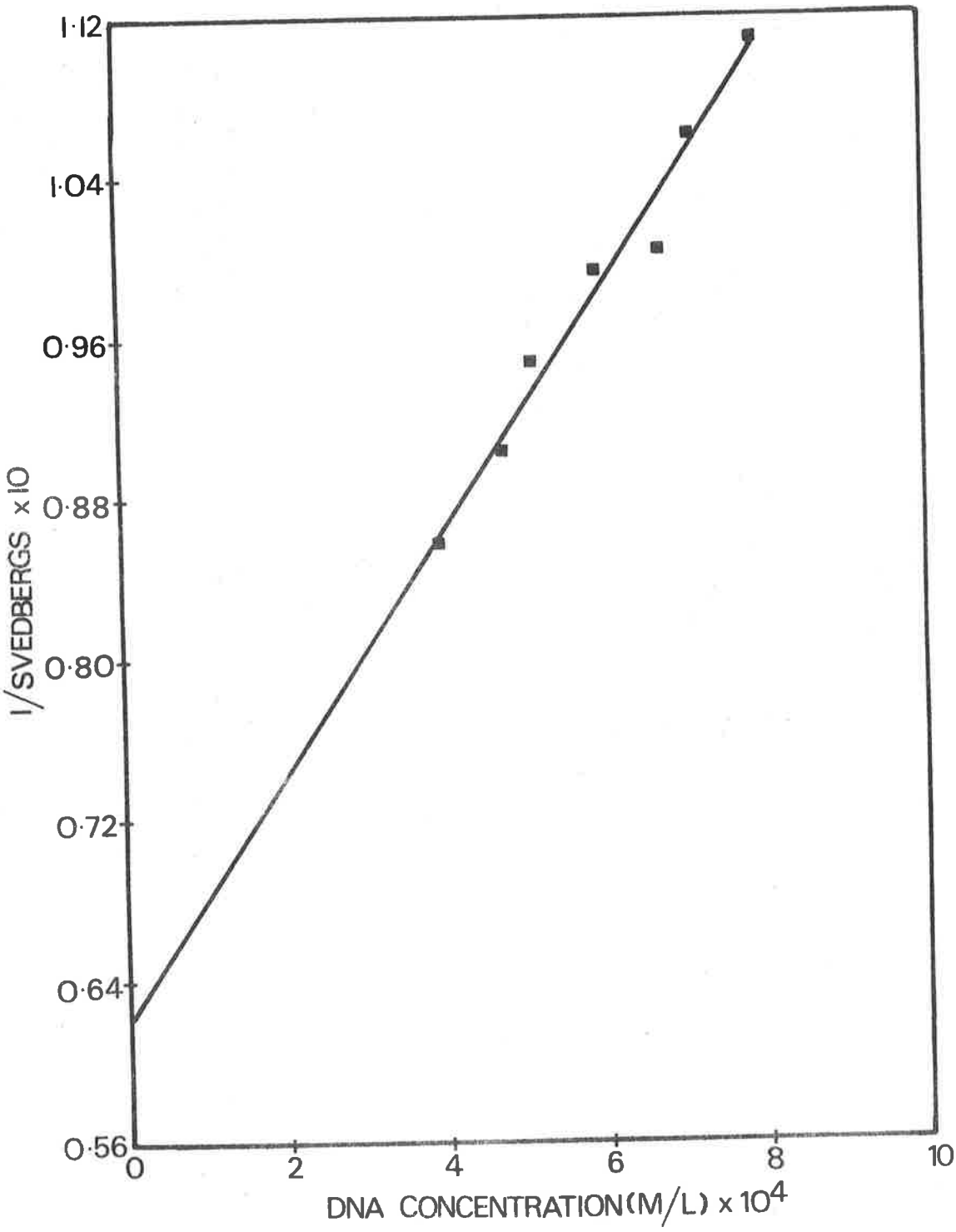

FIG. IV.16 : The concentration dependence of $^1/_S$ for the 9-aminoacridine-DNA complex at r = 0.110 in 0.1M NaCl at 25°C.

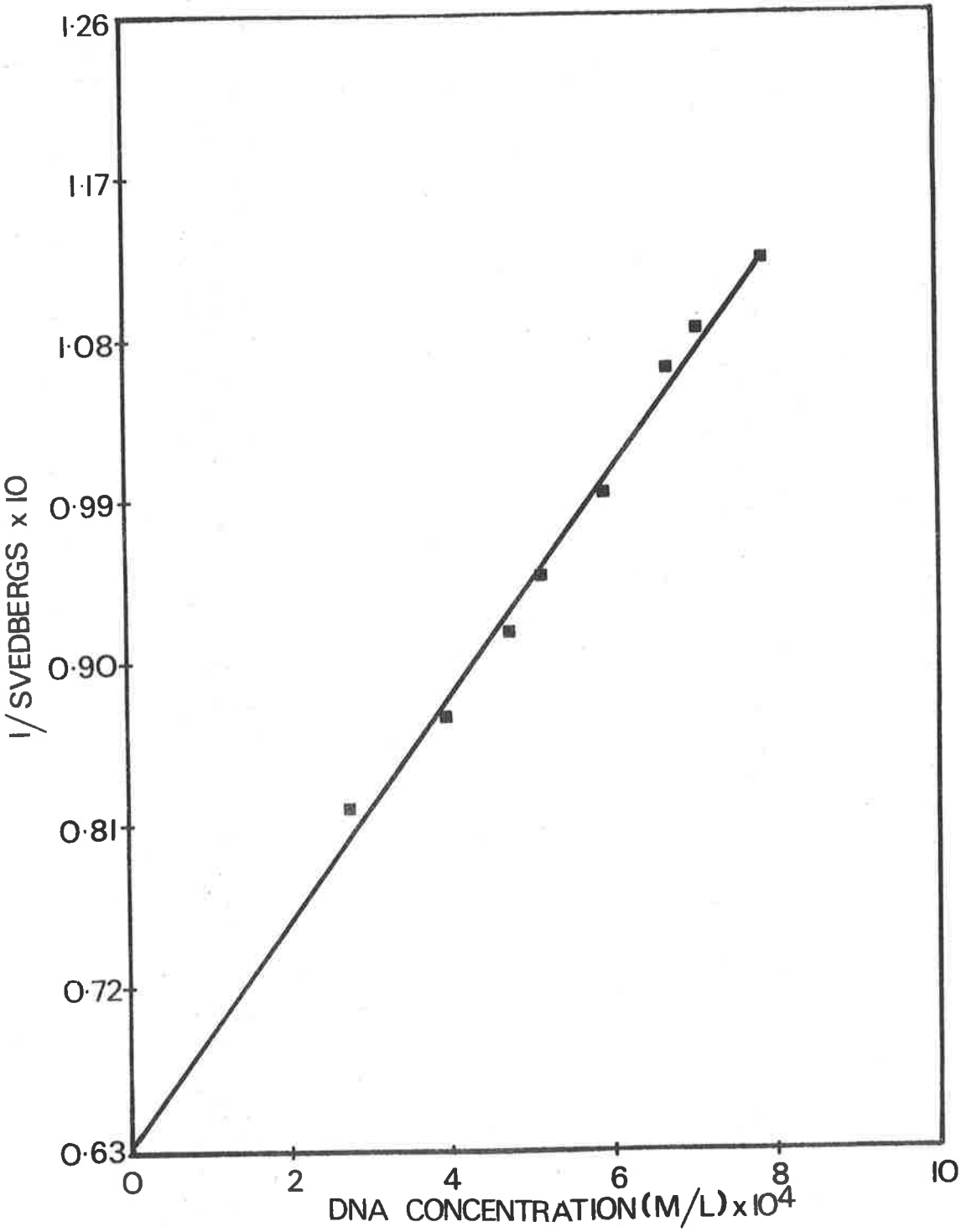

FIG. IV.17 : The concentration dependence of $^{1}/_{S}$ for the 9-aminoacridine-DNA complex at r = 0.125 in 0.1M NaCl at 25°C.

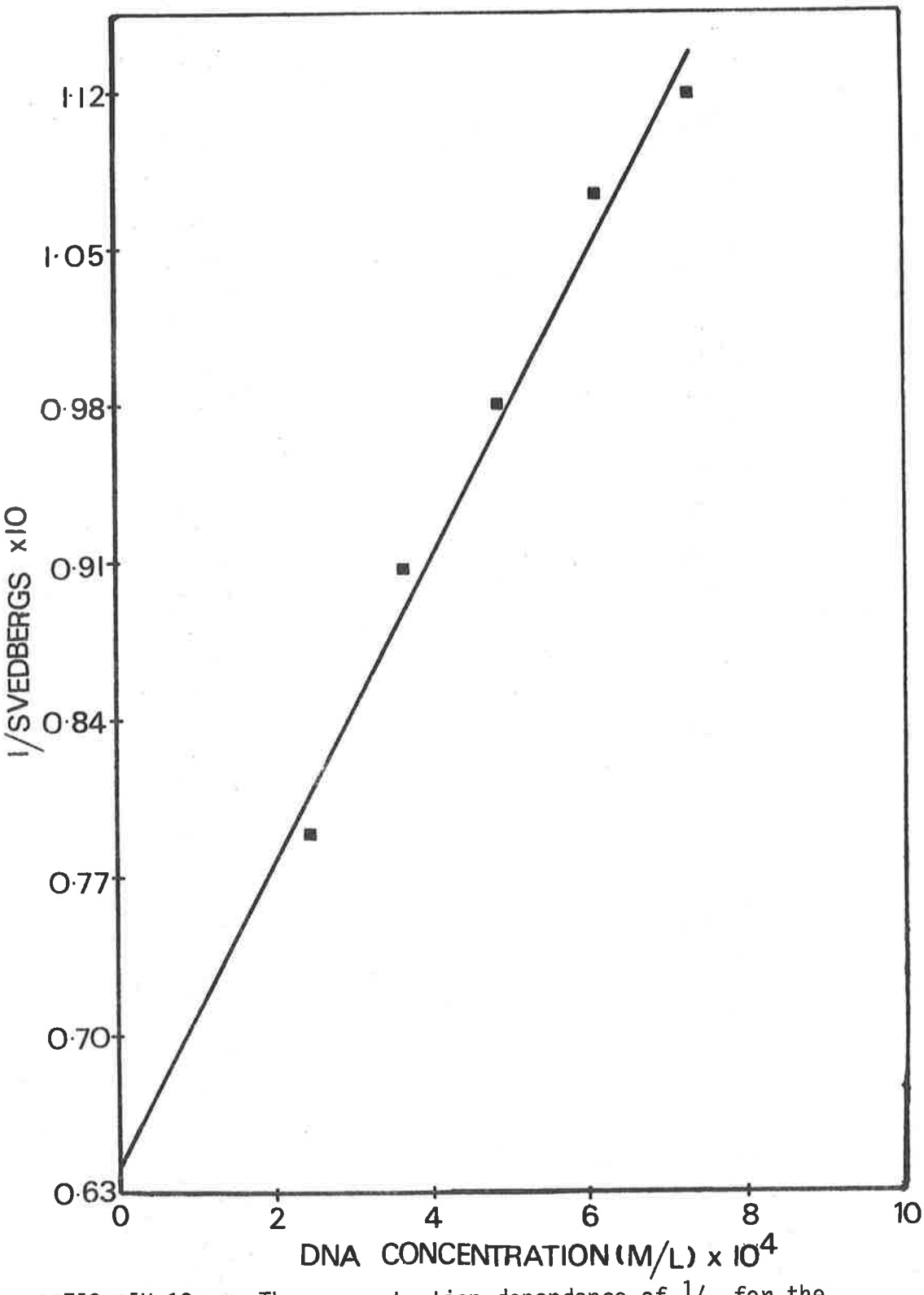

FIG. IV.18 : The concentration dependence of $^{1}/_{S}$ for the 9-aminoacridine-DNA complex at r = 0.150 in 0.1M NaCl at 25°C.

FIG. IV.19 : Variation of the sedimentation coefficient, $S^{o}_{25,r}$ with the extent of binding, r, for the 9-amino-acridine-DNA system in 0.1M NaCl.

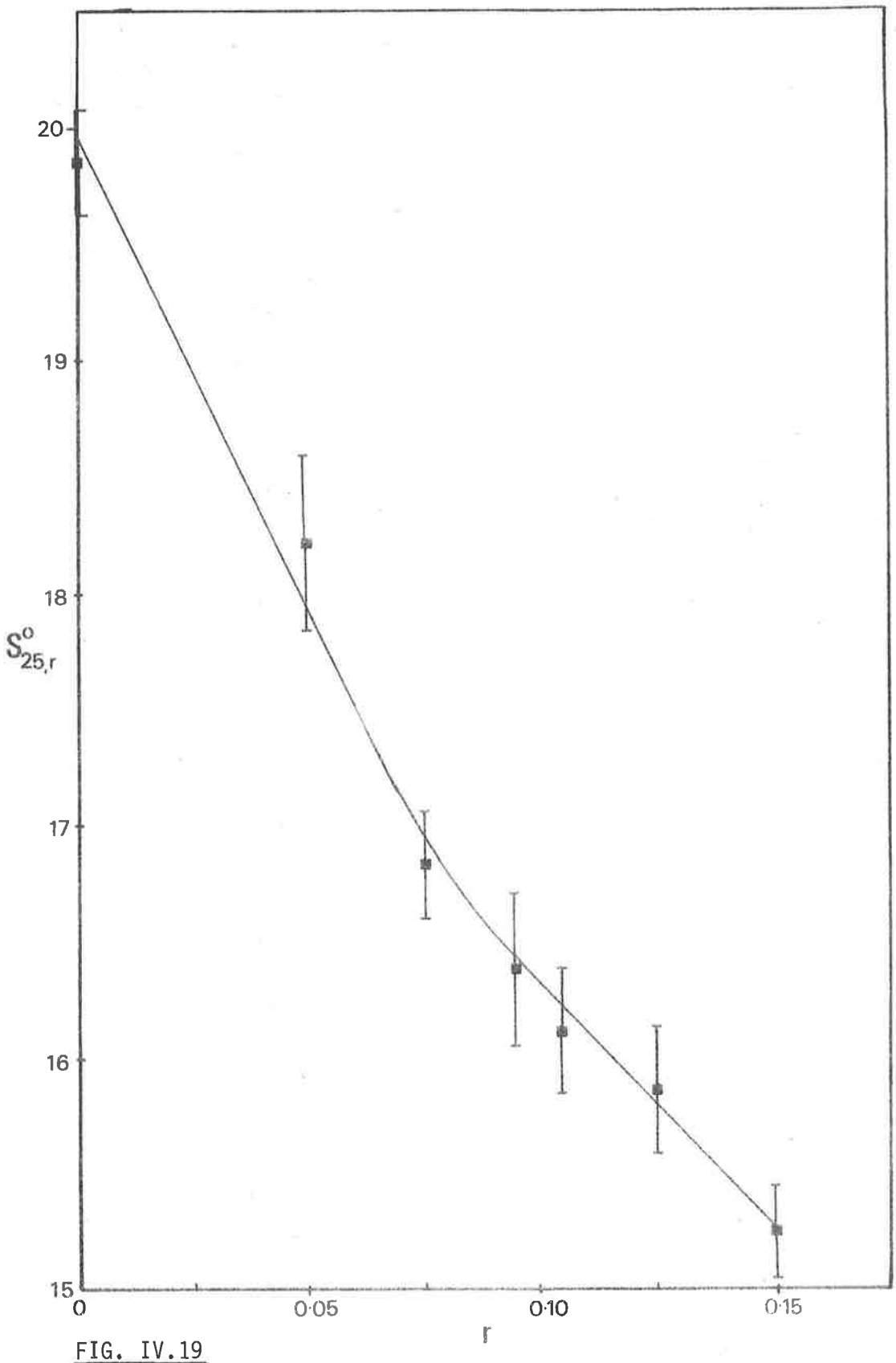

FIG. IV.19

The decrease in the sedimentation coefficient is much larger than that postulated by Lerman[1], who predicted that for intercalation up to r = 0.22 for proflavine, the sedimentation coefficient would be 90% of that for native DNA alone. Lloyd et al[4] have suggested that an increase in flexibility of the DNA molecule on intercalation by proflavine, may explain the greater decrease in sedimentation coefficient than predicted from mass per unit length calculations. A similar explanation may apply to 9-aminoacridine although intrinsic viscosity data would be required before any definite conclusion could be provided.

(ii) Denatured DNA

Solutions of mixtures of denatured DNA and 9-aminoacridine were prepared with varying values of r by reference to the binding curve shown in Fig. IV.6e. Because preliminary experiments on solutions of varying r but constant denatured DNA concentration indicated the absence of any change in sedimentation coefficient, extrapolation to infinite dilution was not considered necessary. Thus throughout this study the DNA concentration was maintained constant.

In Fig. IV.20 is shown the relative sedimentation coefficient, $(^{Sr}/_{Sr=o})$, obtained as a function of r for denatured DNA - 9-aminoacridine complexes. Each value is

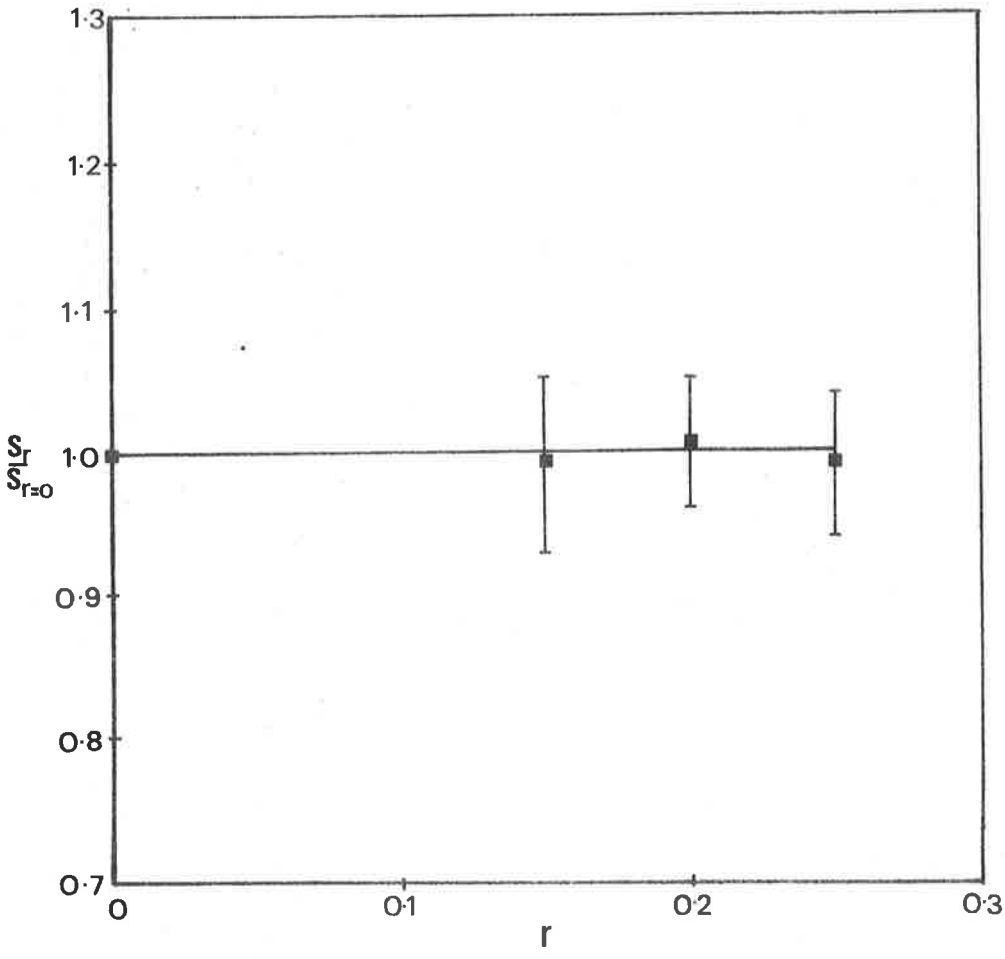

FIG. IV.20 : The variation of the ratio of the sedimentation coefficient of the 9-aminoacridine-denatured DNA complex to that of denatured DNA as a function of the extent of binding, r, at 25°C in 0.1M NaCl.

represented by a vertical line, representing the 95% confidence interval determined by a Student's t Distribution[39] analysis on at least four separate experiments. Within the experimental limit, no alteration in the sedimentation coefficient of denatured DNA was observed when complexed with 9-aminoacridine. This observation is in accord with viscosity data obtained by Drummond et al[40], who found that the intrinsic viscosity of denatured DNA at an ionic strength of 0.1, did not change on interaction with 9-aminoacridine.

Thus, in view of the lack of any structural change detected by either sedimentation or viscosity studies, it may be reasonably concluded that the structure of denatured DNA is such that the insertion of a dye cation between the bases does not cause any great change in the shape of the polynucleotide chains.

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CHAPTER V

THE INTERACTION OF 9-AMINOACRIDINE WITH SYNTHETIC POLYNUCLEOTIDES

1. Introduction

Studies on the interaction of acridine dyes with synthetic polynucleotides can be expected to yield information on the importance of base composition and base sequence in the interaction of acridines with the natural polynucleotides, DNA and RNA. Since the secondary structures of the synthetic polynucleotides have been found to include all gradations from completely random coil to highly helical structures, the examination of their interaction with acridines may also be useful in determining what effect structure may have on the interaction process. In view of the information likely to be obtained from such an examination, it is surprising that very few publications are available in this area. Steiner and Beers[1] have conducted a spectrophotometric study on the interaction of acridine orange with a number of synthetic polynucleotides under varying conditions of pH and temperature. Although their results were published prior to the detailed investigation of the binding of aminoacridines to nucleic acids which led to the intercalation hypothesis[2], it is interesting to note that many of their observations have subsequently been reported for nucleic acid-aminoacridine systems. Such phenomena as the change in absorbance at wavelength of the monomer peak of the dye and the quenching of fluorescence, are now observations frequently used in the determination of the extent of binding

of aminoacridines to DNA[3].

In order to examine the influence of major changes in base composition of DNA on the binding of aminoacridines, Chan and McCarter[4] determined the apparent binding constants for the interaction of proflavine and acriflavine with the double-stranded synthetic polynucleotides poly dAT, poly (dA). poly (dT), and poly (dG).poly (dC). The results indicated that the magnitude of the binding constant was insensitive to changes in base composition, which is in agreement with that previously found for samples of DNA with varying base content[5].

Using a relaxation kinetic technique, Schmechel and Crothers[6] have recently shown a similarity between the mechanism of binding of proflavine to poly (A).poly (U) and to DNA, although differences did occur in the rate constants and thermodynamic parameters for individual steps of their proposed mechanism. Sedimentation studies[6] showed that when proflavine was bound, an increase in the contour length of poly (A).poly (U) occurred of the same order as that obtained with DNA. This indicated that proflavine was intercalated between the bases in poly (A).poly (U).

Observations by Peacocke[7] on the interaction of proflavine with poly A, both in the stacked neutral form and the acid helical form, indicated that the binding of proflavine to poly A may be distinctly different from the

binding to DNA. He observed a greater extent of binding to the single-stranded species than to the helical form with the maximum extents of binding being approximately 0.7 and 0.5 moles of dye per mole of nucleotide respectively. These extents of binding are much higher than the value of 0.16 reported for both native and denatured DNA under similar conditions[8]. Moreover, it is difficult to reconcile the value of 0.5 for double helical poly A with the intercalation model for DNA proposed by Armstrong et al[9] in which 0.25 is the maximum binding attainable. Although the sugar moiety in poly A and DNA is different, ribose for poly A and deoxyribose for DNA, it is difficult to imagine this difference influencing the extent of binding to such a degree. It would thus appear that caution must be taken in extrapolating observations on the interaction of acridines with synthetic polynucleotides to interactions with DNA.

In this chapter the extent and temperature dependence of the interaction of 9-aminoacridine with several synthetic polynucleotides is discussed.

2. Spectrophotometric Study of the Interaction of 9-aminoacridine with poly A.

The structure of poly A has been shown to be markedly dependent upon pH and ionic strength[10,11]. In neutral solution at room temperature, it possesses a flexible

single-stranded conformation of a right-handed helix with the bases stacked perpendicular to the helix axis[12]. In hydrodynamic experiments this structure behaves as a random coil. At acid pH, poly A can exist in a rigid double-strand helical form with the adenine bases hydrogen bonded from the N^7 of the imidazole ring to the 6-amino group of the opposing adenine as shown in Fig. V.1.

a. Single-stranded poly A

In Fig. V.2 is shown the spectra of solutions of varying ratios of 9-aminoacridine and poly A at pH 7.2 in 0.1M NaCl. Under these conditions the poly A possesses a single-stranded conformation. A decrease in the absorbance and a slight red shift is observed at the wavelength of maximum absorbance of the dye, together with a clear isosbestic point at 429 nm. These spectra are similar to those presented in Chapter IV for denatured DNA - 9-aminoacridine complexes. The binding curve at pH 7.2 of the number of moles of dye bound per mole of nucleotide (r) plotted against the free dye concentration in moles/L (c) is shown in Fig. V.3. This binding curve was obtained using the spectrophotometric technique of Peacocke and Skerrett[13]. The non-sigmoidal shape of the curve is taken to indicate the lack of any cooperative phenomenon which Peacocke observed for the binding of poly A and proflavine at pH 6.2[7]. This observation perhaps further emphasizes the difference in the

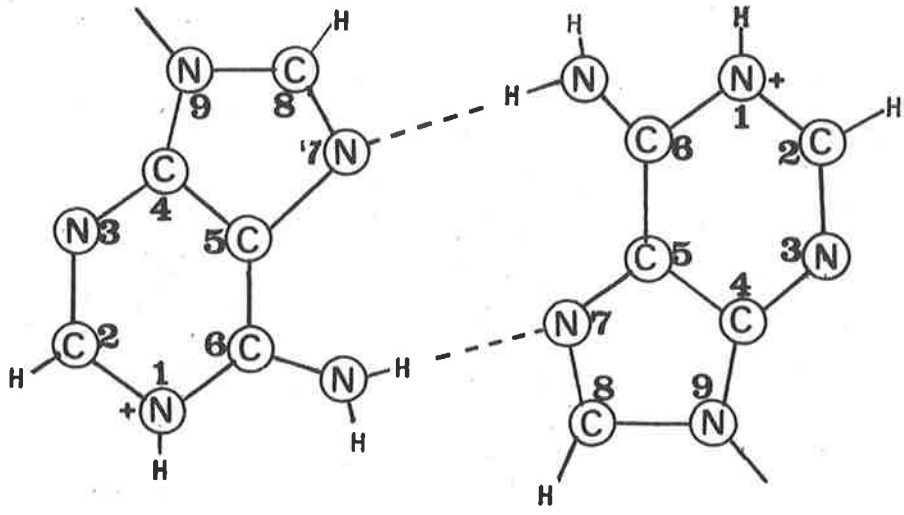


FIG. V.1 : Schematic representation of the hydrogen bonding in double helical poly A.

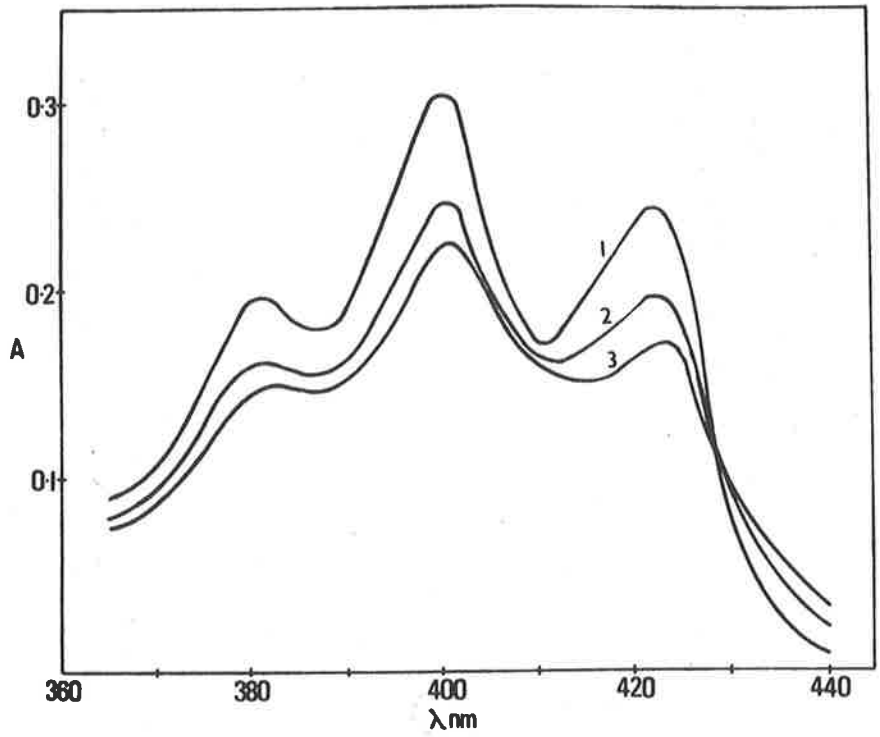


FIG. V.2 : Spectra at 22°C of 9-aminoacridine (curve 1) with Poly A $^{T_L}/_{T_A}$ = 2.25 (curve 2); $^{T_L}/_{T_A}$ = 1.12 (curve 3) in pH 7.2 in 10^{-1}M NaCl.
Constant dye concentration = 3.37×10^{-5}M.

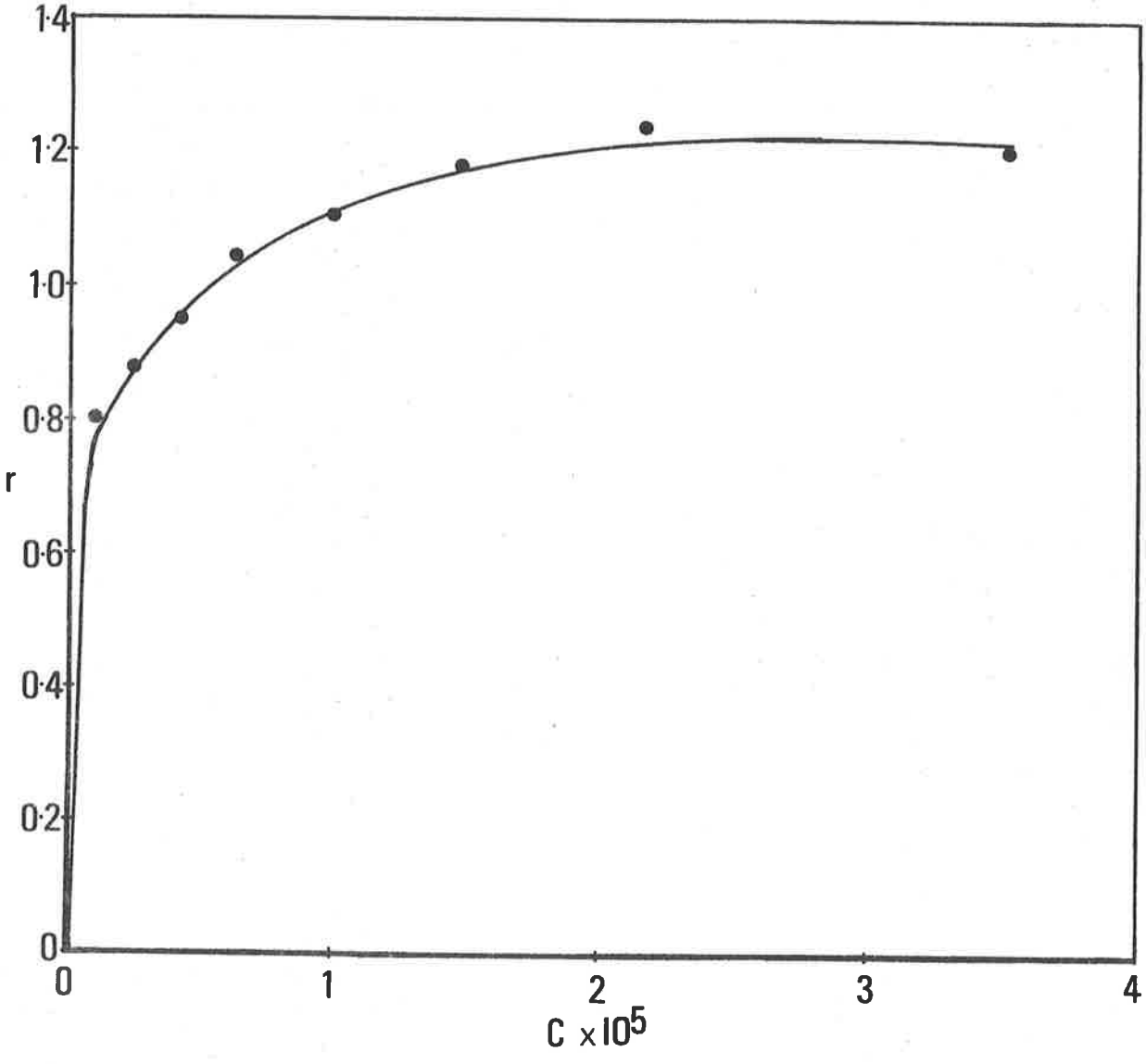


FIG. V.3 : Variation of r with the concentration (moles/l) of unbound 9-aminoacridine (c) for the interaction of 9-aminoacridine with Poly A at pH 7.2 in 0.1M NaCl. Temperature 22°C.

binding behaviour of proflavine and 9-aminoacridine to single-stranded polynucleotides which has previously been shown in their extents of binding to denatured DNA[3].

The significant observation from the binding curve is the large extent of binding attained. The value of r is seen to plateau at a value of 1.2, which is approximately five times that observed for denatured DNA under virtually identical conditions. These values indicate that the binding of aminoacridines to single-stranded poly A must be distinctly different from the binding to denatured DNA.

In Fig. V.4 is shown the Scatchard Plot of $r/_c$ versus r for the interaction of 9-aminoacridine and poly A at pH 7.2. The curvature of the graph indicates an overlapping of the binding to more than one type of binding site. Extrapolation to the abscissa axis yields an approximate value of 1.0 for the maximum site number of the stronger binding process. In view of the red spectral shift observed, it is likely that the strong binding involves interaction of the dye cations with the adenine bases, while the weaker process is probably the result of binding to the exterior of the macromolecule. To explain the maximum binding of 1.0 moles of dye bound per mole of nucleotide for the strong binding process, a model must be proposed in which the 9-aminoacridine cations are intercalated between every base of the poly A molecule. This model

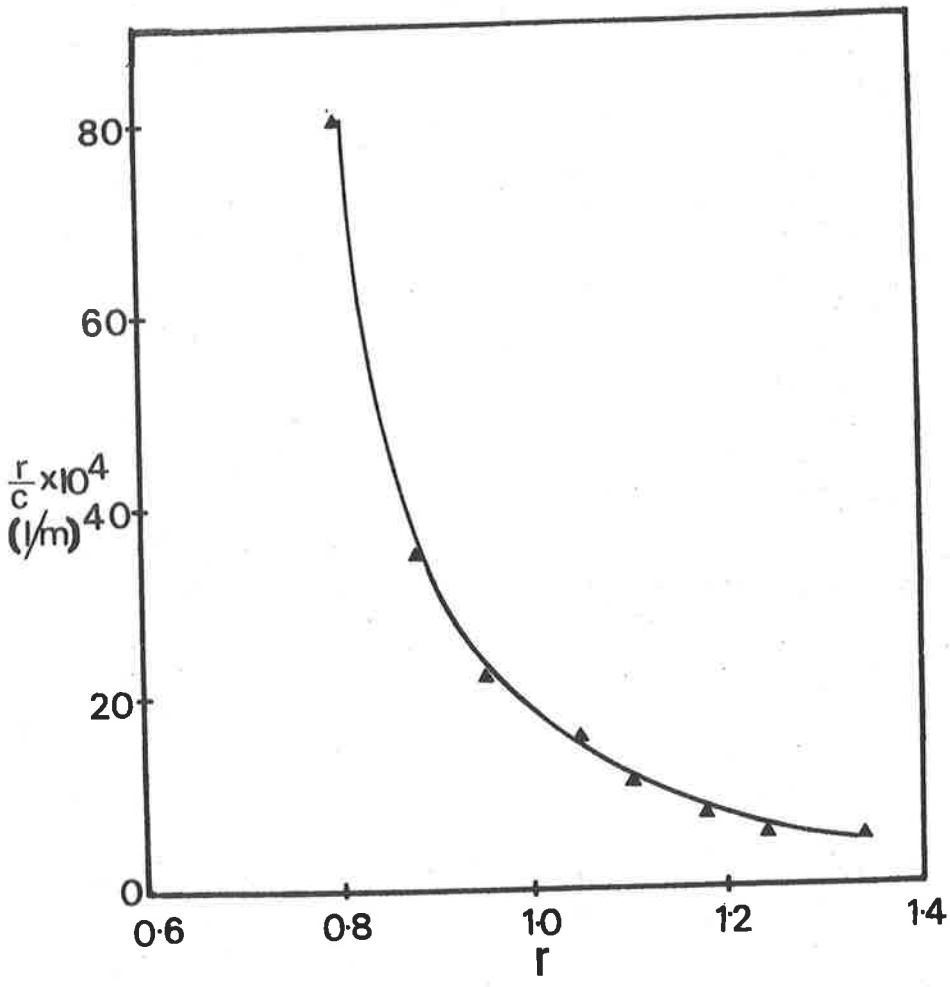


FIG. V.4 : The Scatchard Plot of $^{r}/_{c}$ against r for the binding of 9-aminoacridine to Poly A at pH 7.2 in 0.1M NaCl. Temperature 22°C.

differs from that postulated in Chapter IV for the binding of 9-aminoacridine to denatured DNA in which the maximum of 0.5 was proposed.

b. Double-helical Poly A

No change in the spectrum of 9-aminoacridine was observed for mixtures of varying ratios of poly A-9-aminoacridine at pH 4.5 in 0.1M NaCl, even at a T_L/T_A ratio of 1:7. While the absence of any spectral change by itself does not necessarily indicate the absence of any binding, the observation is surprising since complexes with single-stranded poly A give significant changes even at much higher T_L/T_A ratios. If binding of 9-aminoacridine to adenine bases of poly A is occurring at pH 4.5, then the orientation of the dye cation in the complex must be different from that occurring in single-stranded poly A.

3. Viscosity of 9-aminoacridine-poly A Complexes.

The intercalation of acridine dyes into the DNA double helix results in an enhanced viscosity of the DNA solutions as a result of chain lengthening and straightening[9,14,15]. This property of intercalation is in contrast to that observed when substances bind to the exterior of the helix, resulting in a diminution of the specific viscosity due to the reduction in electrostatic repulsion between neighbouring phosphate groups.

Studies on denatured DNA-aminoacridine complexes[15] failed to show any significant change in the viscosity of the denatured DNA solutions at an ionic strength of 0.1. However, at lower ionic strengths the intrinsic viscosity actually decreased when dye cations were bound. This decrease is exactly analogous to that expected for the influence of ionic strength on the viscosity of a flexible polyelectrolyte, and may be explained by the reduction in the mutual repulsions between phosphate groups. Because the observations reported in Chapter IV indicate an interaction between 9-aminoacridine and the bases of denatured DNA, the use of viscosity to determine whether such interaction occurs might therefore be suitable only for double-helical structures and to be unsuitable for the more flexible single-stranded polynucleotides. Thus, the absence of an increase in the viscosity of single-stranded polynucleotides when aminoacridines are bound cannot be taken as evidence against an intercalation mechanism.

Results

In Fig. V.5(a) and (b) are shown the graphs of specific viscosity versus T_L/T_A ratio for poly A - 9-aminoacridine at pH 4.5 and 7.0 respectively. In each case the concentration of poly A was maintained constant. At pH 4.5, poly A exists in the double-helical form and the absence of any increase in viscosity indicates that no

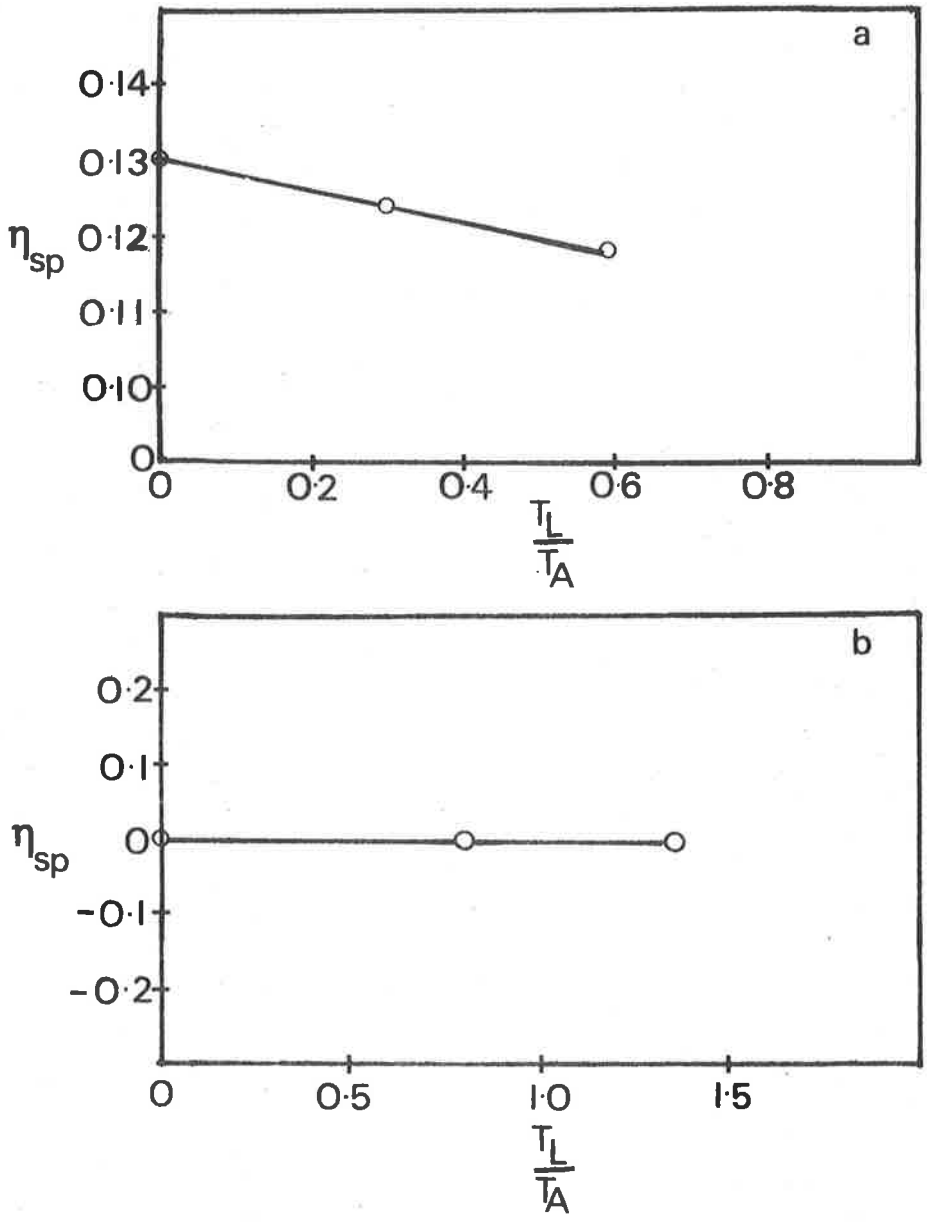


FIG. V.5 : Dependence of the specific viscosity on T_L/T_A ratio for the 9-aminoacridine-Poly A systems in 0.1M NaCl.

a. pH 4.5 : Constant poly A concentration 1.44×10^{-4} M_p

b. pH 7.0 : Constant poly A concentration 1.56×10^{-4} M_p

Temperature $20.2 \pm 0.1^{o}C$.

extension of the molecule occurs. This observation, together with the lack of any spectral change, indicates that intercalation of dye cations does not occur into double helical poly A. The observed decrease in the viscosity may be the result of external binding to the negatively charged phosphates, leading to a more compact structure due to reduced charge-repulsion.

The observation that the viscosity of the poly A solution does not change at pH 7.0 when 9-aminoacridine is added, is similar to that observed for denatured DNA[15]. This similarity is not unexpected since at pH 7.0 both polynucleotides exist as flexible single-stranded molecules.

4. Discussion of the Interaction of 9-aminoacridine with poly A.

a. Double Helical poly A

Spectral and viscosity measurements indicate that 9-aminoacridine does not interact with the adenine bases of poly A under those pH conditions where the polynucleotide possesses a double helical structure. From structural considerations the observation is unexpected, because the organised secondary structure of acid poly A is similar to that of native DNA[16]. The absence of any such interaction is explainable because at low pH values protonation occurs at N_1 of the adenine[11]. (Fig. V.1) Thus a positive charge

distribution exists between adjacent base pairs, which is the site of intercalation by the dye cations. Thus, an interaction between dye cations and the positively charged adenine bases would not be expected to occur because of the charge repulsion which would exist between them. This effect would not prevent dye cations binding to the exterior of the helix.

The difference between the lack of interaction observed in the present study with 9-aminoacridine and significant binding reported for proflavine by Peacocke[7] cannot be explained at this stage.

b. Single-stranded poly A

The interaction of 9-aminoacridine with single-stranded poly A has been demonstrated using a spectrophotometric technique. The extent of binding observed is much greater than that expected on the assumption that poly A at neutral pH behaves in a similar manner to denatured DNA. A model in which the dye cations intercalate into every space between adjacent adenine residues has been proposed, but substantiation of this hypothesis is not possible from the present data. The lack of any viscosity change is explainable on the basis of the flexible structure of compact volume possessed by poly A at neutral pH. If any change in the structure occurs due to the binding of dye cations, then this change must be too small to be detected

by viscosity measurements.

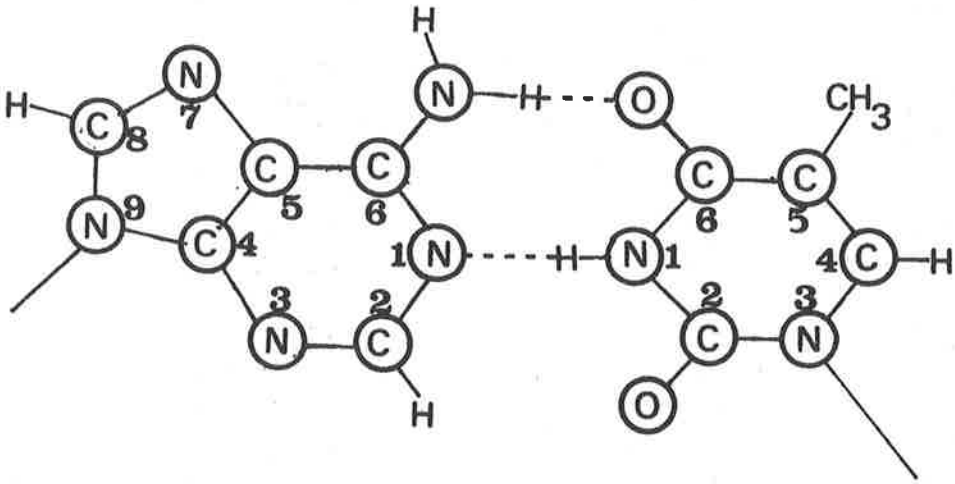
5. Interaction of 9-aminoacridine with poly dAT.

Poly dAT is a high molecular weight, double stranded copolymer composed of alternating dA and dT units (....$dA_p dT_p$....) with hydrogen bonding as shown in Fig. V.6. In view of the observations discussed in Section 4 indicating the absence of an interaction between 9-aminoacridine and poly A under acidic conditions, an examination of the binding behaviour of poly dAT under similar conditions might be expected to yield further information on the 9-aminoacridine-poly A system.

In Fig. V.7 is shown the spectra at 25^{o}C of mixtures of 9-aminoacridine and poly dAT in 0.1M NaCl at pH 4.7. A red shift of the wavelength of maximum absorbance of the dye is observed together with an isosbestic point at 429 nm.

The binding curve of r versus c, obtained by the spectrophotometric method[13] is shown in Fig. V.8, and the graph seen to plateau at an r value of approximately 0.25. Although evidence for intercalation is not available, the red shift in the dye spectrum is indicative of an interaction with the bases of the polynucleotides.

This observed binding of 9-aminoacridine to poly dAT under acidic conditions is to be compared with the apparent absence of binding to double-stranded poly A.



ADENINE THYMINE

FIG. V.6 : Schematic representation of the hydrogen bonding in poly dAT.

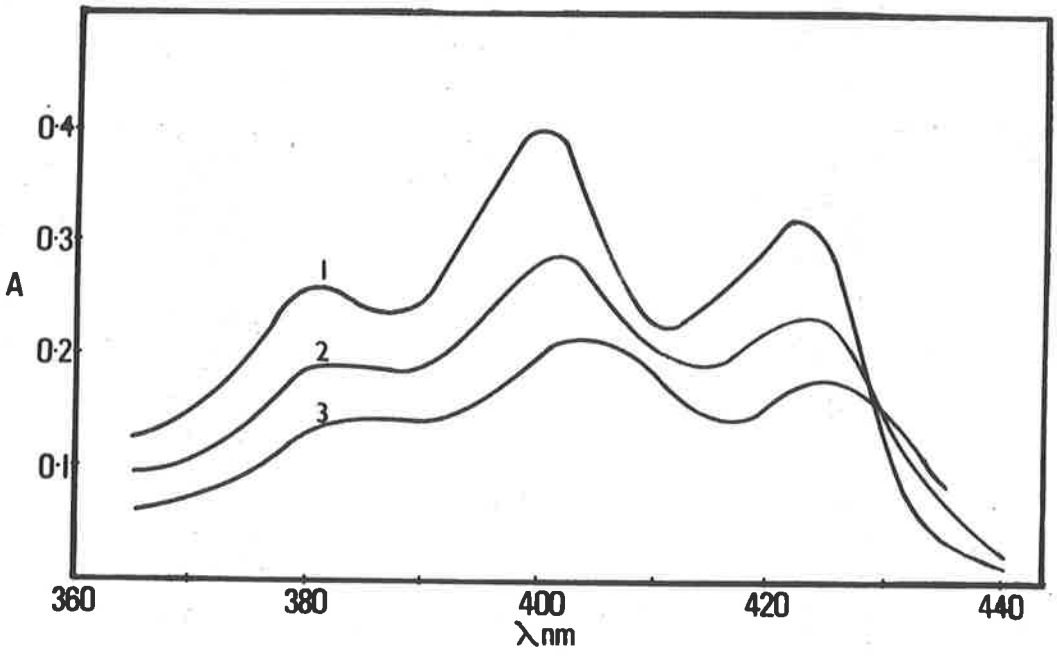


FIG. V.7 : Spectra at 25°C of 9-aminoacridine (curve 1) with poly dAT $^{T_L}/_{T_A}$ = 0.38 (curve 2); $^{T_L}/_{T_A}$ = 0.19 (curve 3) at pH 4.7 in 10^{-1}M NaCl. Constant dye concentration = 4.39 x 10^{-5}M.

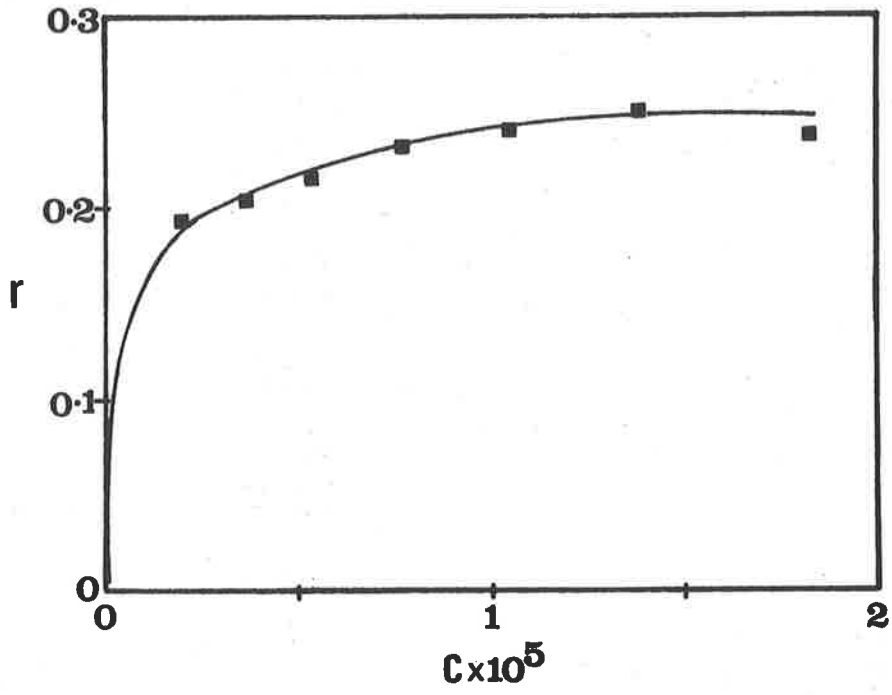


FIG. V.8 : Variation of r with the concentration of unbound 9-aminoacridine (c) in moles/l for the binding of 9-aminoacridine to poly dAT at pH 4.5 in 0.1M NaCl. Temperature 25°C.

However, as shown in Fig. V.6, the N_1 atom of adenine in poly dAT is involved in hydrogen bonding to a thymine residue, whereas the N_1 adenine in poly A is free (see Fig. V.1). The pKa of N_1 adenine in poly dAT is therefore expected to be less than that for the N_1 adenine in poly A. Thus, it is possible that at a pH of approximately 4.5 the N_1 of adenine is protonated in poly A, but not in poly dAT. Thus the difference in binding behaviour of 9-aminoacridine to poly A and poly dAT under acid conditions is explainable since no electrostatic barrier to binding exists in poly dAT, while the positive charge residing on the N_1 of adenine in poly A prevents interaction as previously discussed in Section 5.

6. Interaction of 9-aminoacridine with poly (A).poly (U).

The formation of a double helical structure when equimolar concentrations of poly (A) and poly (U) are combined at neutral pH is now well established[17]. The helical structure is stabilized by hydrogen bonding between adenine and uracil as shown in Fig. V.9.

The spectra at 25^{o}C of mixtures of 9-aminoacridine and poly (A).poly (U) in 0.1M NaCl at pH 6.7 are shown in Fig. V.10. A red shift of the wavelength of maximum absorbance of the dye is observed, together with a clear isosbestic point at 429 nm. These spectra are similar to those observed for native DNA - 9-aminoacridine complexes under similar conditions (refer Fig. III.1). The presence

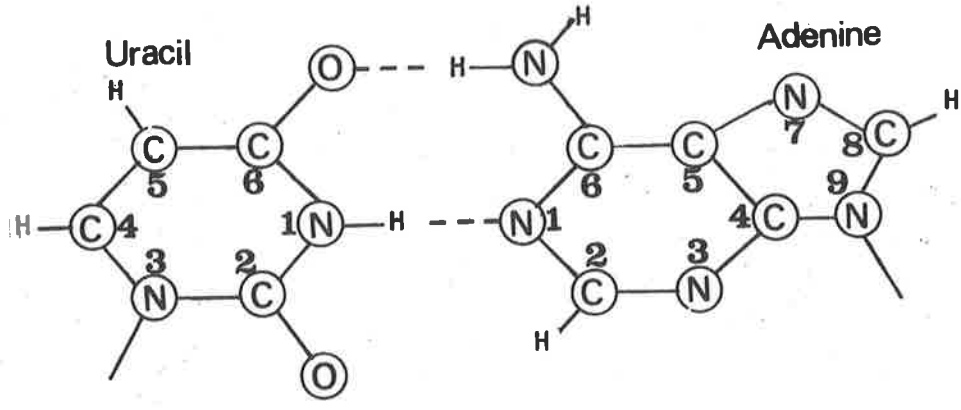


FIG. V.9 : Schematic representation of the hydrogen bonding in poly (A).poly (U).

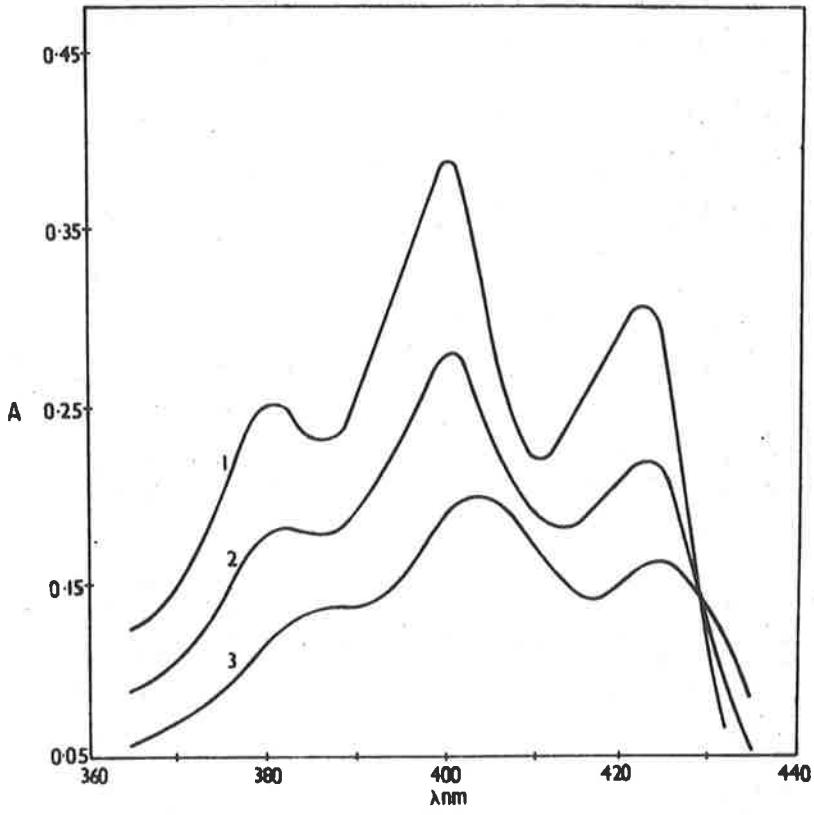


FIG. V.10 : Spectra at 25°C of 9-aminoacridine (curve 1) with poly (A).poly (U) $^{T_L}/_{T_A}$ = 0.241 (curve 2); $^{T_L}/_{T_A}$ = 0.067 (curve 3) at pH 6.7 in 0.1M NaCl. Constant dye concentration 4.39 x 10^{-5}M.

of an isosbestic point, unlike the observation of Blake and Peacocke for proflavine-poly (A).poly (U) complexes[18], allows the use of the spectrophotometric method for determination of the extent of binding[13].

The binding curve of r versus c for the interaction of 9-aminoacridine and poly (A).poly (U) is shown in Fig. V.11, and the corresponding Scatchard Plot in Fig. V.12. The Scatchard Plot may be partitioned into two distinct regions with the maximum extent of binding for the stronger binding process, obtained by extrapolation to the abscissa axis being 0.07 moles of dye per nucleotide, a value less than that observed for native DNA under identical conditions (0.17).

An explanation for this low degree of binding is not possible from the present data. The observation by Blake and Peacocke[18] that little or no interaction occurs between proflavine and poly (U) may be relevant to this system.

7. Influence of Temperature on the binding of 9-aminoacridine to Polynucleotides.

a. Poly (A) - 9-aminoacridine

In Fig. V.13, curve 4, is shown the increase in relative absorbance with temperature of 259 nm of a solution

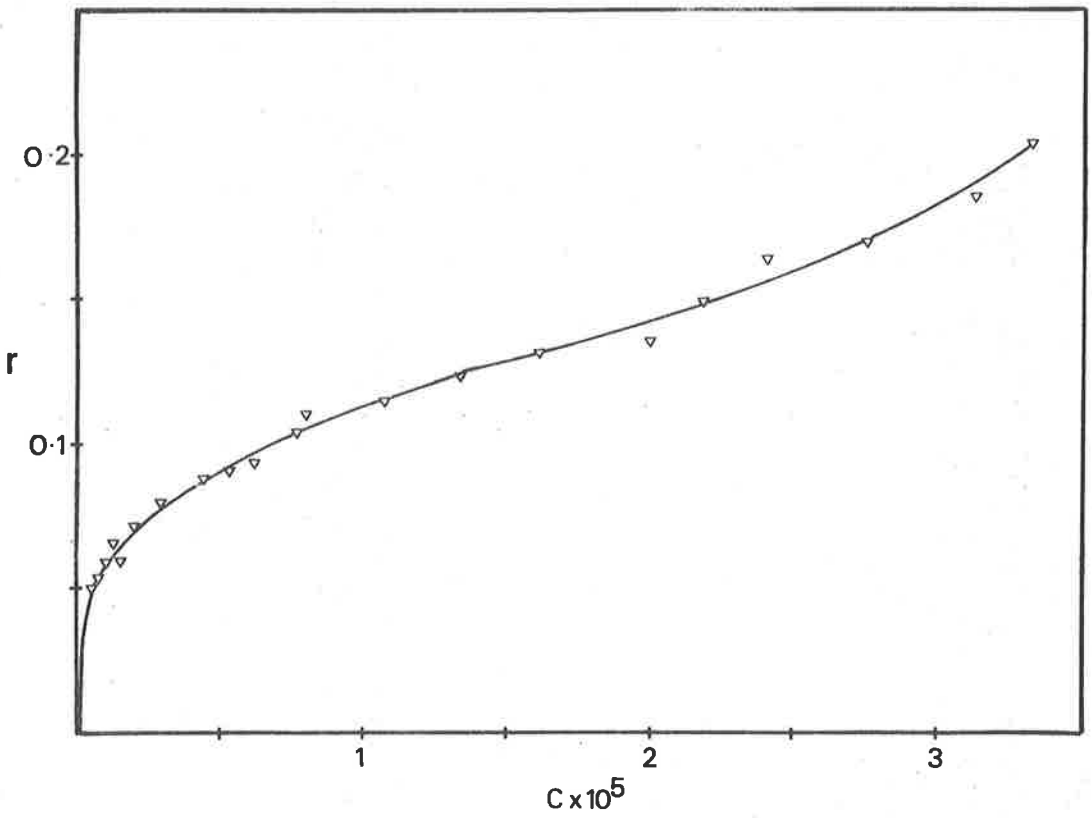


FIG. V.11 : Variation of r with the concentration of unbound 9-aminoacridine (c) in moles/l for the binding of 9-aminoacridine to poly (A).poly (U) at pH 6.7 in 0.1M NaCl. Temperature 25°C.

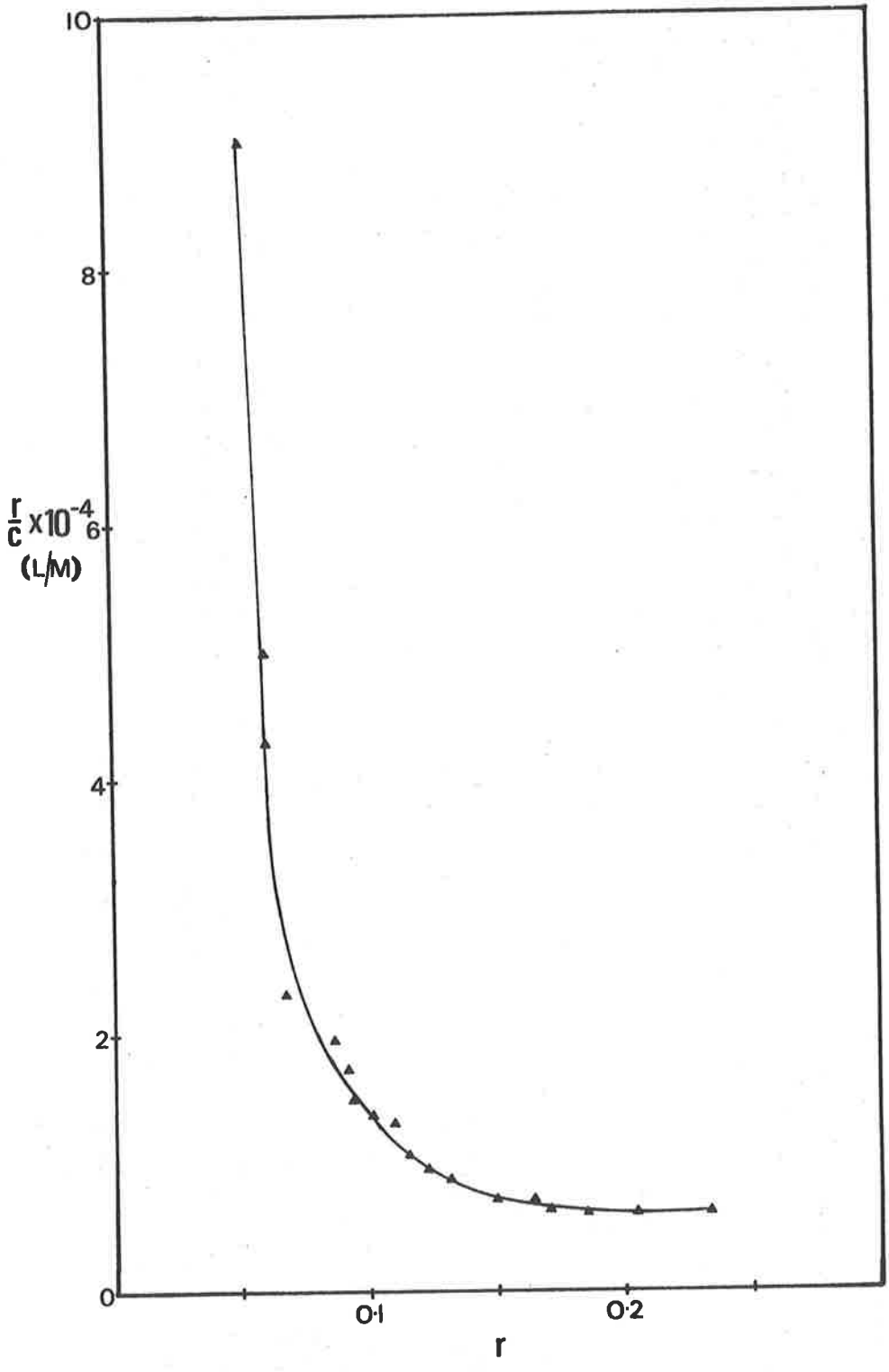


FIG. V.12 : The Scatchard Plot of $^{r}/_{c}$ against r for the binding of 9-aminoacridine to poly (A).poly (U) at pH 6.7 in 0.1M NaCl. Temperature 25°C.

of poly (A) at pH 6.8. The steady increase is similar to that observed for denatured DNA and illustrates the absence of any double-helical poly (A) structure. Fig. V.13, curves 1-3 shows the increase in absorbance at 400 nm observed when complexes of 9-aminoacridine and single-stranded poly (A) at pH 6.8 are heated. Dye release, indicated by the increase in absorbance, occurs over a wide temperature range, with the temperature at which 50% of the total increase is achieved being dependent on the T_L/T_A ratio. For the ratio of 2.39, this temperature is approximately 71^oC, while at a ratio of 1.08 it is only 61^oC. This observation is different from that obtained for denatured DNA - 9-aminoacridine complexes from which the dye is released at a lower temperature which is independent of the T_L/T_A ratio. This difference may be the result of the much greater extent of primary binding of 9-aminoacridine to poly (A) than to denatured DNA, and may be explained on the basis of the oscillating-base model proposed in Chapter IV. A mixture of 9-aminoacridine and poly (A) at a ratio of 2.39 would yield a value for the extent of binding (r) such that every space between adjacent adenine bases is occupied by a dye cation. The interaction between the intercalated dye cation and its neighbouring bases would be expected to restrict the relative oscillations of those bases. At lower extents of binding, as obtained in solutions of lower T_L/T_A ratios, there will be spaces between neighbouring

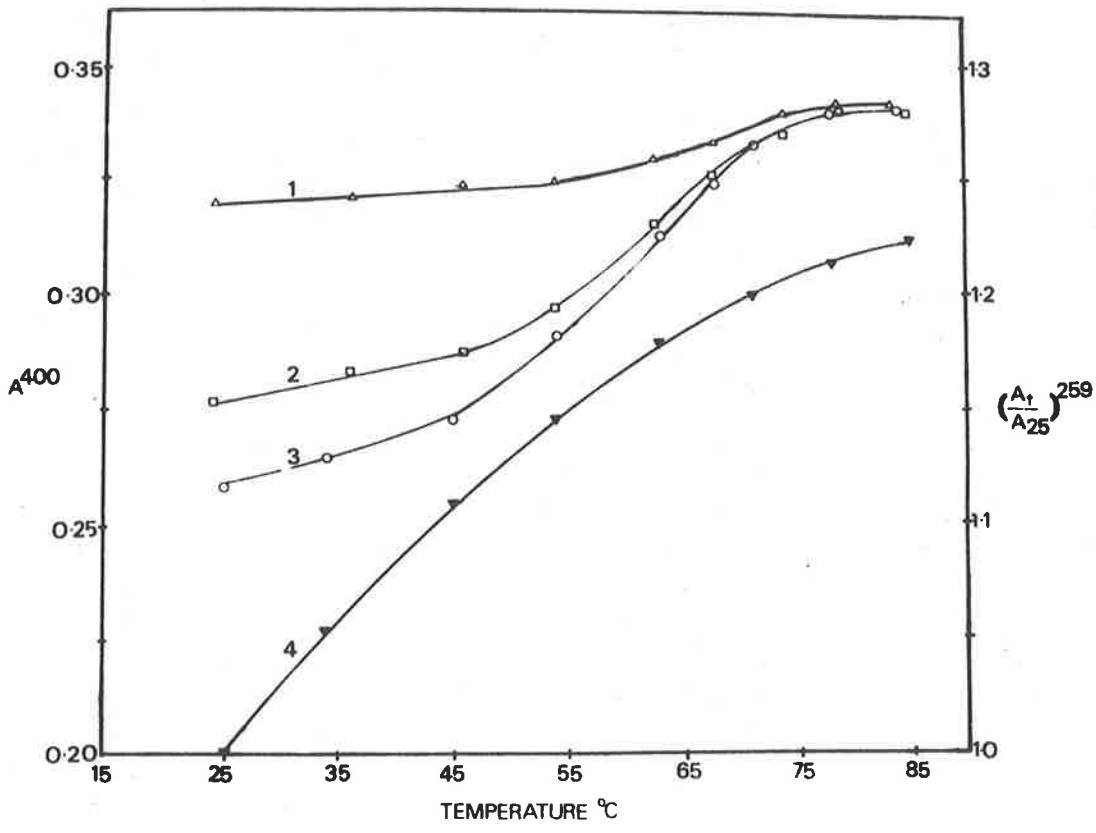


FIG. V.13 : Curves 1-3 : Variation with temperature of the absorbance at 400 nm of mixtures of 9-aminoacridine and poly A at pH 6.8 in 0.1M NaCl.
Curve 1, T_L/T_A = 2.39; curve 2, T_L/T_A = 1.19; curve 3, T_L/T_A = 1.08. Constant dye concentration 4.20×10^{-5}M.

Curve 4 : Variation with temperature of the relative absorbance at 259 nm of poly A at pH 6.8 in 0.1M NaCl.

bases which will not be occupied by dye cations, and thus no stabilization will occur in these regions. The oscillation of the bases which are not involved in an interaction with dye cations can be expected to become significant at lower temperatures than will the oscillation of the bases interacting with dye cations. Also it may be predicted that the oscillation of a base adjacent to an occupied intercalation site will decrease the stability of that site. The probability that an occupied site will be adjacent to an unoccupied site increases as the extent of binding decreases, because it can be assumed that sites are occupied in a random fashion. Thus the destabilizing effect of the oscillation of non-interacted adenine bases will be expected to result in the release of dye from intercalation sites at lower temperatures as the extent of binding decreases. It is to be anticipated that this effect will be observable only when the degree of binding is large since at low degrees of binding the destabilizing effect will be large enough to overcome the small amount of stabilization caused by intercalation. This hypothesis is supported by the observation that with denatured DNA - 9-aminoacridine complexes, for which the maximum binding by intercalation is 0.27 in 0.1M NaCl, the temperature of 50% dye release is independent of T_L/T_A ratio.

b. Poly (A).poly (U) - 9-aminoacridine

The increase in absorbance at 400 nm when complexes of poly (A).poly (U) and 9-aminoacridine are heated at pH 6.8 is shown in Fig. V.14, curves 1-4. The double helical structure of poly (A).poly (U) at neutral pH is demonstrated by the sharp hyperchromic effect observed at 260 nm when a solution of poly (A).poly (U) is heated in 0.1M NaCl at pH 6.8 (Fig. V.14, curve 5). The melting temperature of the sample is 55°C, which is in good agreement with the reported data[19,20].

The absorbance increase at 400 nm, indicative of dye release, increases sharply at temperatures around 50°C and is complete at 60°C. This temperature range is coincident with the dissociation of the double helical structure of poly (A).poly (U) in the absence of dye. This suggests that the interaction with 9-aminoacridine does not increase the stability of the helix against thermal dissociation, at least at an ionic strength of 0.1. The absence of any stabilization by dye binding may be due to the low extent of intercalation observed under these conditions, because Lerman[15] has indicated that stabilization does occur at an ionic strength of 0.01, when the extent of binding is expected to be greater.

In view of the binding of 9-aminoacridine to poly (A) at elevated temperatures, it may have been anticipated

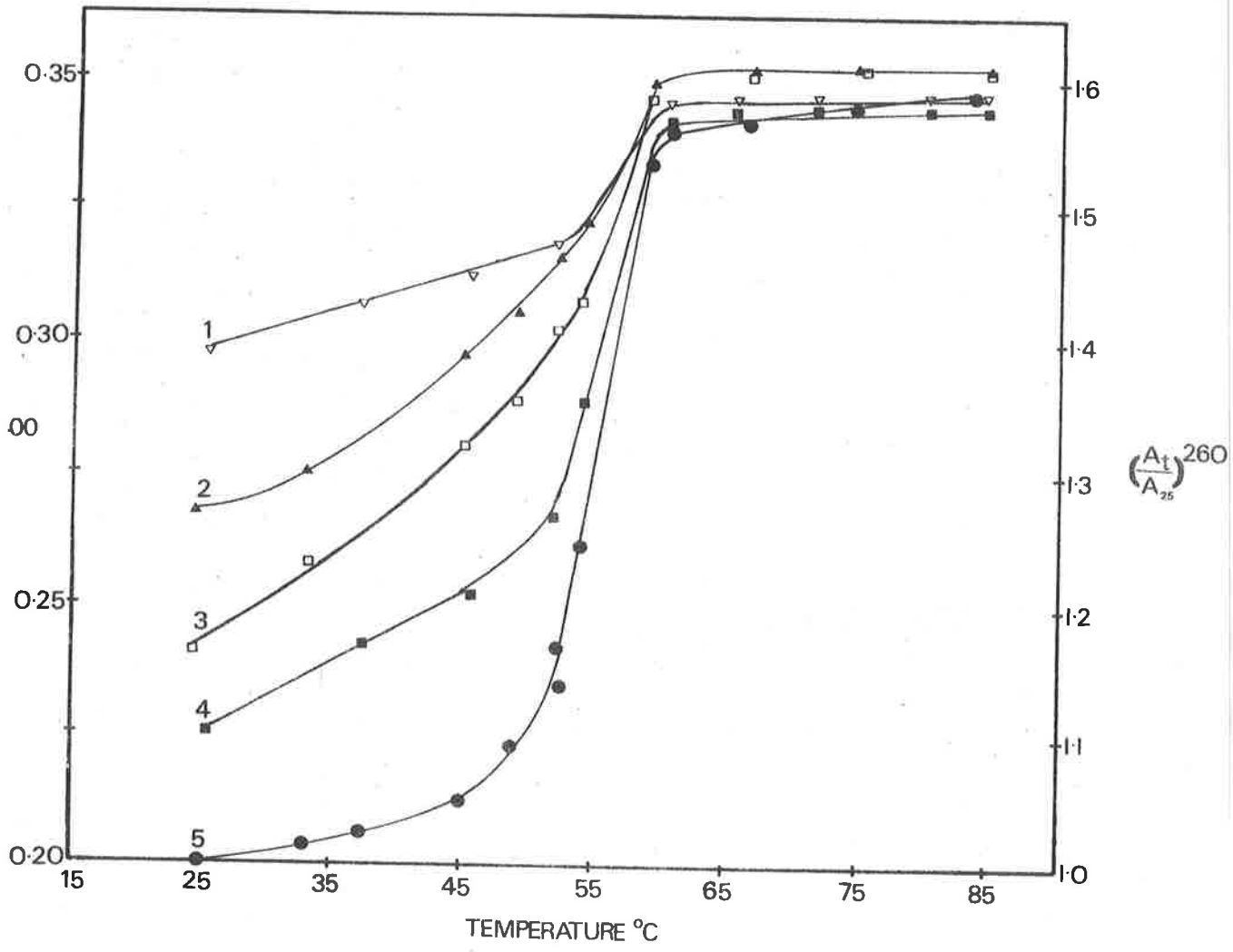


FIG. V.14 : Curves 1-4 : Variation with temperature of the absorbance at 400 nm of mixtures of 9-aminoacridine and poly (A). poly (U) at pH 6.8 in 0.1M NaCl.

Curve 1, $^{T}L/_{T_A}$ = 0.385; curve 2, $^{T}L/_{T_A}$ = 0.230; curve 3, $^{T}L/_{T_A}$ = 0.144; curve 4, $^{T}L/_{T_A}$ = 0.115.

Constant dye concentration 4.20 x 10^{-5}M.

Curve 5 : Variation with temperature of the relative absorbance at 260 nm of poly (A).poly (U) at pH 6.8 in 0.1M NaCl.

that the dye liberated when poly (A).poly (U) dissociates, may recombine with the poly (A) released from the polynucleotide system. That such an interaction does not occur can be concluded from the observation that no absorbance decrease was observed after 60°C. A possible explanation for this observation is that the T_L/T_A ratios used in the studies with poly (A).poly (U) are much lower than those used with poly (A). Since the temperature at which dye is released from poly (A) complexes is dependent upon the T_L/T_A ratio, any complex formed at the low ratios used in this study would be expected to dissociate at temperatures lower than those observed in Fig. V.13. Thus it is expected that for ratios of the order 0.1 to 0.4 used in this study with poly (A).poly (U), any complex formed between 9-aminoacridine and the poly (A) released would dissociate before the temperature is reached at which all the poly (A).poly (U) molecules have dissociated.

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CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

The primary binding of cationic aminoacridine dyes to native and to denatured DNA is generally regarded to involve an interaction between the aminoacridine and the purine and pyrimidine bases of the DNA. Although the interaction with single stranded denatured DNA indicates that the double helix is not a structural prerequisite for primary binding, this observation cannot be taken to imply that the mode of binding to both forms of DNA is identical. Also, the question remains of the exact location of the intercalated dye cation relative to the nucleotide bases. The current intercalation models for native DNA differ in respect to the positioning of the intercalated aminoacridine with respect to the helix axis (Refer Figs. 11-2 and 3). Lerman[1] suggests that the dye is centrally located over the base pairs, while the model of Pritchard et al[2] proposes an insertion between the bases on one of the polynucleotides chains. In view of the conflicting reports concerning the influence of aminoacridine structure on the binding to DNA (Refer Section 11-5), the possibility of other models must be considered.

As discussed in Chapter IV, the precise location of an intercalated dye cation will be that for which the highest binding energy occurs as a result of molecular interactions and steric factors. Since the nature and position of attached groups can alter the electron distribution with the aminoacridine nucleus,

quite apart from any steric considerations, it seems logical to suggest that the exact location of an intercalated aminoacridine cation will be different for each individual aminoacridine.

Information on the location of 9-aminoacridine with respect to the helix axis of DNA can be obtained from binding studies for atebrin and 9-amino-1,2,3,4 Tetrahydroacridine (T.H.A.)[3]. The bulky side chain attached to the 9-amino group (as in atebrin) has no influence on the binding, suggesting that atebrin is not completely enclosed within the double helix as in the Lerman model, since steric effects would make this model unlikely. Pritchard et al[2] used this observation as evidence for their modified intercalation model. However, if this model is correct for 9-aminoacridine then the T.H.A.-DNA complex would be expected to have the same structure as the complex with 9-aminoacridine, since the buckled nature of one ring should not hinder the insertion of the other two planar rings between the bases on one polynucleotide chain. The observations of a decreased extent of binding and the absence of a red spectral shift when DNA interacts with T.H.A.[3], indicate that hydrogenation of one of the rings of 9-aminoacridine decreases the extent of coupling between the orbitals of the acridine and those of the nucleotide bases. The increased thickness across the buckled ring must be the reason for the decrease in the interaction between T.H.A. and DNA, since the reduction in resonance energy is not sufficient to account for the decreased interaction.

Therefore the location of 9-aminoacridine within DNA must be such that the major part of the dye structure is enclosed within the helix. A possible model is one in which the dye is located at a position intermediate between that proposed in the Lerman or Pritchard models. This hypothesis therefore predicts that the mode of binding of 9-aminoacridine to native and to denatured DNA is different, with the binding to denatured DNA being similar to that proposed in the Pritchard model for native DNA. Assuming this 'intermediate' model for the location of 9-aminoacridine in native DNA, the increase in intercalation site number on denaturation (Refer Section IV-4b), is explainable without the necessity of introducing the concept of potential sites, since the dye cation effectively occupies the complete space between adjacent base pairs.

Although the question of base site-specificity remains unanswered (Refer Section II-6a), it can be anticipated that the exact location of an intercalated aminoacridine will be influenced by the nature of its adjacent bases. While the interaction energy for the intercalation of an aminoacridine may be virtually identical for any base sequence, the position of the dye may be different to allow the maximum interaction between the delocalized π orbital systems of both the dye and the bases. This π-π interaction is expected to be greater for purine bases (adenine and guanine) than for the pyrimidine bases (cytosine and thymine). Using two base pair sequences of

guanine and cytosine as an example, namely GC:GC and GC:CG, the location of the aminoacridine might be expected to be between the two guanine residues in GC:GC (i.e. to one side of the helix axis), while for the GC:CG sequence to be situated in a position allowing some overlap between the two guanine residues on the complementary strands. This second position would require the dye to be centrally located about the helix axis. Thus it is possible that base sequence may play a role in determining the exact location of intercalated aminoacridines even within a given DNA of fixed base composition. The use of synthetic polynucleotides with known base sequences may prove useful in the examination of this problem.

A technique which may be extremely useful in deciding the exact location of intercalated aminoacridines is electron-spin resonance spectroscopy (E.S.R.) using spin-labelled amino-acridines.

While it has been shown that the double helical structure of DNA is not essential for intercalation to occur, the DNA secondary structure does have a pronounced effect on the stability of the DNA-aminoacridine complexes. As mentioned in Chapters III and IV, the thermal stability of native DNA-9-aminoacridine complexes is much greater than for complexes with denatured DNA. This difference in stability is not unexpected because of the larger overlap between an intercalated dye and its neighbouring bases in native DNA allowing a greater interaction

than is possible in denatured DNA. Therefore the free energy difference between native DNA and its complex with 9-aminoacridine is expected to be greater than that between denatured DNA and its complex with 9-aminoacridine. However, the observation of the considerable release of dye from native DNA complexes at temperatures coincident with strand separation, suggests that the rigid double-helical structure of native DNA also contributes to the greater stability of native DNA-9-aminoacridine complexes. The hypothesis proposed in Chapter IV involving the concept of 'lifetimes of suitable overlap conditions', is based on the assumption that the nucleotide bases in denatured DNA have a greater degree of rotational freedom than the hydrogen-bonded bases in native DNA. This oscillating base model satisfactorily explains the temperature effects on both native and denatured DNA complexes. The determination of rate constants for dye release at elevated temperatures and the activation energies involved may provide further information on the validity of this model. A temperature-jump technique similar to that used by Li and Crothers[4] for their study of the kinetics of the intercalation of proflavine would be suitable.

The results obtained for the binding of 9-aminoacridine to synthetic polynucleotides suggest that while these molecules are structurally less complex than DNA, their interaction with aminoacridines may in fact be much more complex

than with DNA. Therefore, their use as model systems in the study of aminoacridine-DNA interactions must await more detailed investigations.

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CHAPTER VII

MATERIALS AND METHODS

1. Cleaning of Apparatus.
2. Preparation of Samples
   - a. DNA Solutions
   - b. Denatured DNA Solutions
   - c. Aminoacridine Solutions
   - d. Synthetic Polynucleotides
     - (i) Poly (A)
     - (ii) Poly (U)
     - (iii) Poly (A).Poly (U)
     - (iv) Poly dAT.
3. Spectra and Spectrophotometric Titrations.
4. pH Measurement.
5. Viscosity.
6. Sedimentation Velocity.

References

1. Cleaning of Apparatus.

All glassware was cleaned by standing it in a mixture of 3% w/v $NaNO_3$ and 3% w/v $NaClO_4$ in concentrated sulphuric acid for 24 hours. The apparatus was then rinsed many times with deionised water and then leached for 48 hours in deionised water. The silica cuvettes and the viscometer were cleaned in the same manner as above. With the exception of the cuvettes, viscometer and volumetric glassware, all apparatus was dried at 160^{o}C before use. The other items were rinsed with AR acetone and allowed to dry at room temperature.

The centrepieces and windows of the cells used in ultracentrifuge studies were cleaned by rinsing with a dilute solution of "Teepol" detergent (Shell Chemical (Aust.) Pty. Ltd.), and rinsed many times with deionised water. The centrepieces were dried in a stream of hot air and the windows were rinsed with AR acetone and allowed to dry at room temperature.

2. Preparation of Solutions

a. DNA Solutions

The DNA used throughout this work was isolated from E.Coli K12 using the method of Marmur[1] with an additional phenol extraction as recommended by Hirschman and Felsenfeld[2]. Stock solutions of DNA (0.15%) in 10^{-3}M NaCl were prepared by

gentle shaking at $4^{o}C$ for 5 days, followed by centrifugation at 16,000g for 30 minutes. Chloroform A.R. was then added to give a final concentration of approximately 0.3%. This stock solution was stored at $4^{o}C$ and diluted as needed with $10^{-3}M$ NaCl or 1.0M NaCl solutions to give the required sodium chloride concentrations in the range 10^{-3} to $10^{-1}M$. Spectral analysis using the method of Hirschman and Felsenfeld[2] gave a G+C content of 54% and a Δ value of -0.005. The value of T_m in $10^{-3}M$ NaCl was $59^{o}C$ in agreement with previously reported values. Concentrations of the solutions were calculated from the absorbance measurements at 260 nm taking $\varepsilon_p = 6500$ at this wavelength.

b. Preparation of solutions of Denatured DNA

Solutions of dilution-denatured DNA (approximately 0.005%) were prepared by dissolving 10 mgm. of E.Coli DNA in 200 ml of double distilled water, (specific conductivity less than 2×10^{-6} ohm^{-1} cm^{-1}), with gentle agitation at $4^{o}C$. Chloroform A.R. was added to give a final concentration of 0.3% and the solutions were stored at $4^{o}C$. In order to obtain higher concentrations of DNA for use in the sedimentation studies, aliquots of this stock solution were freeze dried and then reconstituted immediately before use. Solutions were then heated at $100^{o}C$ for 10 minutes and shock cooled to $0^{o}C$. Concentrated sodium chloride solution was added to give the required final NaCl concentration. The

concentration of the denatured DNA solutions were determined by the method of Hirschman and Felsenfeld[2]. The spectral measurements as required by this method were conducted on solutions prior to the addition of sodium chloride. The concentration of the final solutions was then calculated allowing for the volume of NaCl solution added.

c. Solutions of Aminoacridines

9-Aminoacridine hydrochloride (A.G.Fluka) was recrystallised twice from ethanol and dried under vacuum. λ_{max} = 400 nm and $\epsilon_M^{400} = 8.97 \times 10^3$. Stock solutions were stored in pyrex glass containers.

Proflavine hemisulphate (B.D.H.) was recrystallised twice from double distilled water and dried under vacuum. λ_{max} = 444 nm and $\epsilon_M^{400} = 3.94 \times 10^4$. All solutions of proflavine were protected from light since proflavine in solution undergoes rapid photoreduction[3].

The stock solutions of these two compounds were prepared in 10^{-3}M NaCl, and diluted when necessary with 10^{-3} or 1.0M NaCl to give the required sodium chloride concentration in the range 10^{-3} to 10^{-1}M.

d. Synthetic Polynucleotides

(i) Poly (A)

Polyadenylate (poly A), as the potassium salt, was the product of Miles Laboratories Inc., Indiana, U.S.A. (Control No. 11-54-304) and was used without further

purification. Solutions (0.04%) were prepared in 10^{-3}M NaCl by shaking at 4^{o}C for 18 hours, and then filtered through a Gelman Filter, type AM-5. Concentrated NaCl solution was then slowly added to give a final neutral salt concentration of 0.1M. Sufficient 0.05M sodium hydroxide or 0.05M hydrochloric acid in 0.1M NaCl was then added to give the required pH. Solutions were stored at 4^{o}C. The concentrations of the solutions were determined from the absorbance values at 257 nm

$$\left(\varepsilon_p^{257} \text{ pH 7.0} = 9.9 \times 10^3 \text{ and } \varepsilon_p^{257} \text{ pH 4.6} = 8.4 \times 10^3 \right)^4$$

In Fig. VII.1 is shown the variation with temperature of the relative absorbance at 257 nm for poly A solutions at pH 4.7 (curve 1), and pH 6.7 (curve 2) in 0.1M NaCl. The rapid increase in absorbance observed at pH 4.7 confirms the presence of the double helical structure of poly A under these conditions. The melting temperature (T_m) obtained is 67^{o}C which is in good agreement with reported values. The steady increase in absorbance observed at pH 6.7 is typical of the profile observed for single stranded polynucleotides (refer Section IV.2).

(ii) Poly (U)

Polyuridylic acid (poly U), as the ammonium salt, was the product of Miles Laboratories Inc., Indiana, U.S.A. (Control No. 45751) and was used without further

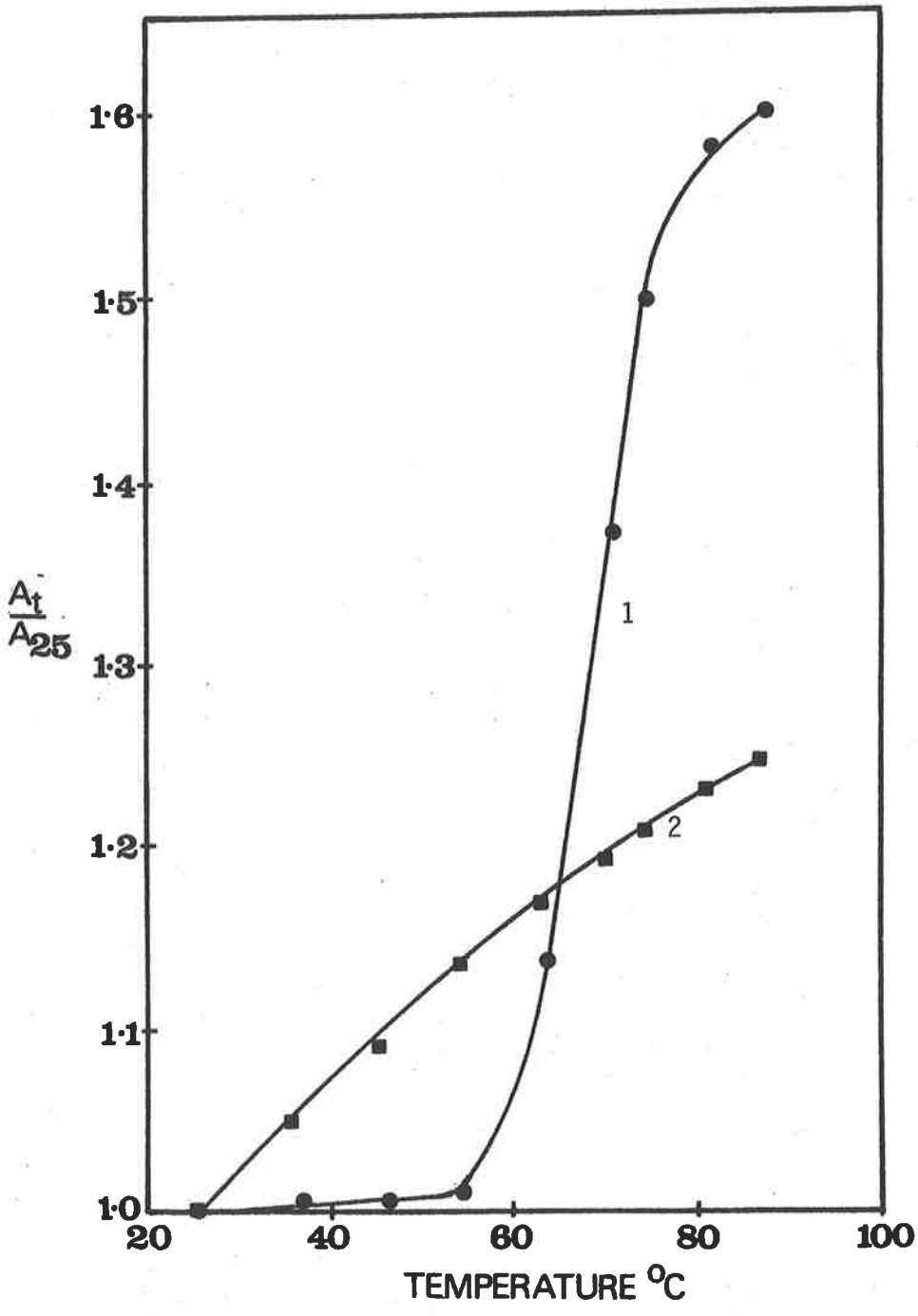


FIG. VII.1 : Variation with temperature of the relative absorbance at 257 nm for poly A in 0.1M NaCl.
Curve 1, pH 4.7, poly A concentration 7.86 x $10^{-5}M_p$;
Curve 2, pH 6.7, poly A concentration 7.32 x $10^{-5}M_p$.

purification. A stock solution (0.04%) was prepared in 0.1M NaCl and stored at 4°C. The concentration of this solution was determined from the absorbance value at 261 nm.

$$\left(\varepsilon_p^{261} = 9.5 \times 10^3 \right)^5$$

(iii) Poly (A).Poly (U)

A stock solution of Poly (A).poly (U) was prepared by mixing equimolar proportions of poly (A) and poly (U) in solution in 0.1M NaCl.at pH 7.0. The mixed solution was then left for 15 hours at room temperature before storage at 4°C. The concentration was determined from the absorbance measurements at 260 nm

$$\left(\varepsilon_p^{260} = 7.14 \times 10^3 \right)^5$$

(iv) Poly dAT

Polydeoxyadenylate - Thymidylate, sodium salt was the product of Miles Laboratories, Inc., Indiana, U.S.A. (Control No. 26) and was used without further purification. A stock solution was prepared in 0.1M NaCl and stored at 4°C. The concentration of the solution was determined from the absorbance value at 262 nm

$$\left(\varepsilon_p^{262} = 6.65 \times 10^3 \right)^6$$

3. Spectra and Spectrophotometric Titrations

All spectral measurements were made using a

Gilford model 2000 spectrophotometer. Solutions of polynucleotide, aminoacridine or of aminoacridine and polynucleotide in the required sodium chloride concentration were contained in 10 mm silica cells and solutions of identical sodium chloride concentration were used as references.

For spectral measurements at temperatures above 25°C, evaporation was prevented by using a seal of Teflon tape (Permacel, Johnson and Johnson) and a latex-rubber gasket with a Teflon cap held in position by a brass screw clamp. Loss of solution through evaporation using this method was always less than 1%. Solutions were saturated with helium before heating. Cells were placed in the constant temperature cell compartment of the Gilford spectrophotometer and the temperature near the cell was measured by a platinum resistance probe embedded in the cell carrier.

A period of about 45 minutes was allowed for the system to reach thermal equilibrium before absorbance measurements were taken. Optical densities were then corrected for the thermal expansion of the solution.

Spectra of solutions of 9-aminoacridine and of varying 9-aminoacridine/polynucleotide ratios were determined between the wavelength values of 365 and 435 nm. The range

was covered in 2.5 nm increments except in the region of maxima and minima and isosbestic points, where readings were taken every 1 nm. After measurement the solution cell was washed repeatedly with solvent and cell corrections determined.

Binding curves were determined using the method of Peacocke and Skerrett[7] by use of the formula

$$r = \{(D_1 - D) / (D_1 - D_2)\} (^{T_L}/_{T_A})$$

where r is the number of moles of dye bound per nucleotide; D_1 is the absorbance at λ_{max} of the aminoacridine in the absence of polynucleotide; D_2 is the absorbance at λ_{max} of the aminoacridine of a solution of the aminoacridine containing an excess polynucleotide; D is the absorbance at λ_{max} of the aminoacridine for the polynucleotide-aminoacridine solution; T_L is the total concentration of the aminoacridine and T_A is the total concentration of polynucleotide. Binding curves were obtained from the volume corrected optical densities of solutions prepared by the addition (by weight) of small volumes of the polynucleotide solution to dilute dye solutions contained in 10 mm silica cells.

The binding of 9-aminoacridine and of proflavine to native DNA at various temperatures was studied by preparing solutions by two different methods - (a) solutions

of aminoacridine and DNA were prepared at 20°C and then heated to the required temperature, (b) the DNA solution was heated to the required temperature and aminoacridine solution was then added to the cell using a Agla Micrometer all-glass syringe (Burroughs-Wellcome). For the significance of this technique refer to Section III.2.

4. pH Measurement

All pH measurements were made using a Metrohm pH meter employing a combined glass-calomel electrode. The pH values of all stock dye and DNA solutions and dye-DNA mixtures were in the range of 5.8 to 6.5. Since the degree of binding to DNA is independent of pH within this range[6], the addition of buffering agents was considered unnecessary. For the examination of the interaction of 9-aminoacridine with the synthetic polynucleotides the solutions were adjusted to the required pH by the addition of dilute NaOH and HCl solutions before use.

At the completion of an experiment, if the pH of the final mixture of dye and polynucleotide varied by more than 0.2 pH units from the pH of the original solutions, the results obtained were disregarded.

5. Viscosity

The viscosity of solutions of poly (A) and

poly (A)-aminoacridine complexes was determined at 20.2 ± 0.1°C using a low shear suspended level viscometer with an average rate of shear of about 7 $sec.^{-1}$.

The flow times were found to the nearest 1/100th second using a "Hanhart" stopwatch (Dobbie Bros. Pty. Ltd., Melbourne, Australia). Flow times were repeated until three times agreed to ± 0.05 second.

6. Sedimentation Velocity

A Beckman analytical ultracentrifuge equipped with monochromator, photoelectric scanner and multiplexer attachment was used in this study.

The sedimentation experiments were run at 25°C and 30,000 rpm for studies with native DNA or 36,000 rpm for denatured DNA studies. Scans were performed at 290 nm for DNA solutions and at 410 nm for DNA - 9-aminoacridine systems. Double-sector centrepieces of carbon-filled epon, having an optical path of 12 mm, were used together with sapphire windows. Solutions were added slowly using a 22 gauge needle. Control experiments indicated that the amount of dye or DNA adsorbed onto the surfaces of the centrepiece and windows was insignificant and no correction was needed. The average rotor speed was determined from the odometer readings and the RTIC unit was used for temperature control.

Solutions of native DNA - 9-aminoacridine complexes

in 0.1M NaCl were prepared such that the value of r for a given series remained constant with varying DNA concentrations. The method used is discussed in Chapter IV.6. For denatured DNA-dye systems the DNA concentration was constant at 1.46×10^{-4} M_p.

The distance of the midpoint of the boundary from the centre of rotation in cms. (x) was calculated using the following expression

$$x = 6.5 - (X_B - X_{LR}) / 10\ C\ E$$

where 6.5 is the mean of the distance in cms. of the inner and outer reference holes from the centre of rotation; X_B is the chart reading in mms. of the distance between the midpoint of the boundary and the outer reference; X_{LR} is the mean of the distance in mms. between the inner and outer reference holes as read from the chart and C E is the magnification factor for the instrument, which under the conditions used was 5.06.

The sedimentation coefficient was then determined using the expression

$$S = \left(\frac{d \ln x}{dt}\right) / \omega^2$$

where ω is the rotor speed in radian per second.

The sedimentation coefficient at infinite dilution was obtained by extrapolation of the graph of $^1/_S$ versus DNA

concentration to zero concentration.

All calculations were carried out using the Fortran (IV) computer programme as shown in Appendix II, run on a CDC 6400 computer.

References

1. Marmur, J., J. Mol. Biol., 3, 208 (1961).

2. Hirschman, S. and Felsenfeld, G., J. Mol. Biol., 16, 347 (1966).

3. Millich, F. and Oster, G., J. Am. Chem. Soc., 81, 1357 (1959).

4. Weigold, J., Ph.D. Thesis, University of Adelaide, (1965).

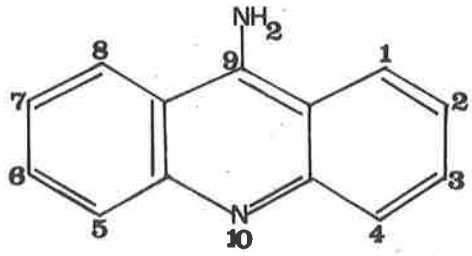
5. Schmechel, D.E.V. and Crothers, D.M., Biopolymers, 10, 465 (1971).

6. Miles Laboratories Inc., Indiana, U.S.A. Control Sheet for Poly dAT - Lot No. 26.

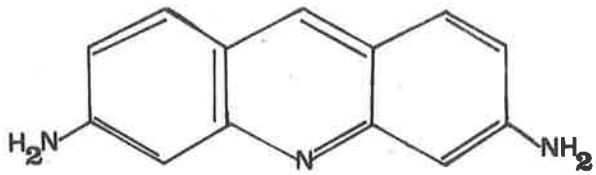
7. Peacocke, A.R. and Skerrett, J.N.H., Trans. Faraday Soc., 52, 261 (1956).

APPENDIX I

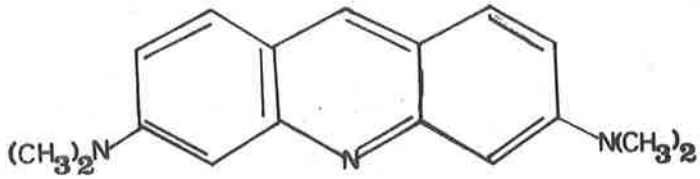
STRUCTURE OF AMINOACRIDINES MENTIONED IN THE TEXT.



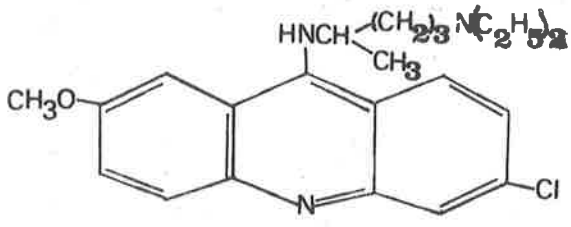
9-aminoacridine



Proflavine



Acridine Orange



Atebrin

APPENDIX II

COMPUTER PROGRAMME FOR THE ANALYSIS OF SEDIMENTATION DATA, AND THE CALCULATION OF SEDIMENTATION COEFFICIENTS

```
      PROGRAM SEDMNA(INPUT,TAPE1=INPUT,OUTPUT)
C
C THIS PROGRAM CALCULATES SEDIMENTATION DATA FROM EXPERIMENTAL READINGS
C OBTAINED ON AN ANALYTICAL ULTRACENTRIFUGE. AS SET UP HERE IT IS CAPABLE OF
C PROCESSING 50 DATA SETS IN ANY PREDETERMINED GROUPING,EACH DATA SET HAVING
C UP TO 50 DATA POINTS. FOR FURTHER INFORMATION ON THE METHOD SEE TEXT.
C IT REQUIRES ONE EXTERNAL SUBROUTINE (QIKPLT) A LINE PRINTER PLOTTING ROUTINE.
C
C INDEX OF VARIABLES. NB ANY VARIABLES NOT MENTIONED IN THIS LIST ARE DEFINED
C AS THEY OCCUR.
C A AND B--DUMMIES  IN THE CALCULATION OF LN X.  DL--DEVIATION OF CORRECTED Y
C FROM DATA Y. DY--CORRECTED Y FROM LEAST SQUARES FIT. PE--DL AS A PERCENTAGE
C S--SEDIMENTATION COEFFICIENT. SB--SEDMN COEFFICIENT EXPRESSED IN SVEDBERGS.
C SDF--STANDARD MEAN DEVIATION OF FIT. SPS--STANDARD ERROR OF SEDMN COEFFICIENT
C AS A PERCENTAGE. SXS--STANDARD ERROR OF SEDMN COEFFICIENT. ZE--ABSOLUTE
C VALUE OF PE.
C
      DIMENSION A(50),B(50),CD(50),R(50),RN(50),S(50),SB(50),SPS(50),SXS
     1(50),T(50),TITLE( 8),W(50),WR(50),XB(50),XL(50),ZE(50)
      COMMON SDF,PE(50),DY(50),DL(50)
C
C CE IS THE MAGNIFICATION FACTOR OF THE ULTRACENTRIFUGE,XLR IS THE MEAN OF THE
C DISTANCE IN M.M. BETWEEN THE INNER AND OUTER REFERENCE MARKS AS READ FROM
C THE CHART.
C
      DATA CE,XLR/5.06,38.00/
C
C NF IS NO. OF MAJOR DATA SETS.
C
   41 READ3,NF
C
C IF NO MORE DATA,GO TO END.
C
      IF(EOF,1)40,50
   50 DO16L=1,NF
C
C TITLE IS ONE CARD OF TITLE INFORMATION.
C
      READ2,TITLE
C
C N IS NO. OF POINTS IN THIS DATA SET.
C
      READ3,N
```

```
C
C XB IS THE DISTANCE OF THE BOUNDARY FROM THE OUTER REFERENCE IN M.M. AS READ
C FROM THE CHART
C T IS TIME IN SECS FROM START   PUNCHED 1 PAIR PER CARD
C
      READ4,(XB(I),T(I),I=1,N)
C
C CALCULATE LN X
C
      DO5I=1,N
      A(I)=(XB(I)-XLR)/CE
      A(I)=A(I)/10.0
      B(I)=6.5-A(I)
  5   XL(I)=ALOG(B(I))
C
C W IS THE ROTOR SPEED IN R.P.M.  R IS THE EXTENT OF DYE BINDING(SEE TEXT)
C CD IS CONC. OF DNA IN MOLES OF DNA PHOSPHORUS PER LITRE  RN IS RUN NO.
C
      READ6,W(L),R(L),CD(L),RN(L)
C
C CONVERT ROTOR VELOCITY TO RADIANS PER SEC SQUARED.
C
      WR(L)=(W(L)**2)*0.010974
C
C LEAST SQUARES FIT OF LN X VERSUS T.
C
      CALL LSQ(T,XL,N,Z,E)
C
C PRINT OUT TABLE OF DATA FOR THIS DATA SET.
C
      PRINT8,TITLE
      PRINT9
      PRINT10,(XB(I),T(I),A(I),B(I),XL(I),I=1,N)
      PRINT13
      PRINT14,(XL(I),T(I),DL(I),DY(I),I=1,N)
C
C CALCULATE ERRORS
C
      S(L)=E/WR(L)
      SXS(L)=(((DL(N)+(2.0*SDF))-DL(1))/(T(N)*WR(L)))-S(L)
      SXS(L)=SXS(L)*1.0E13
      WR(L)=SQRT(WR(L))
      SB(L)=S(L)*1.0E13
      SPS(L)=(SXS(L)*100.0)/SB(L)
C
C PRINT OUT TABLE OF RUN AND ERROR INFORMATION.
C
      PRINT15,RN(L),W(L),WR(L),E,Z,SDF,S(L),SB(L),SXS(L),SPS(L)
  16  CONTINUE
C
C BEGIN CALCULATING INFORMATION FOR THIS MAJOR DATA SET.
C
```

```
      DO24I=1,NF
   24 SB(I)=1.0/SB(I)
C
C LEAST SQUARES OF 1/SVEDBERGS AGAINST DNA CONC.
C
      CALL LSQ(CD,SB,NF,Z,E)
      CALL QIKPLT(CD,SB,-NF,1,21H*CONCN DNA (MOLES/L)*,13H*1/SVEDBERGS*)
      PRINT27
C
C CALCULATE ERRORS.
C
      KN=0
      DO30I=1,NF
      ZE(I)=ABS(PE(I))
C
C REJECT VALUE FROM FINAL FIT IF ERROR EXCEEDS 5 PERCENT.
C
      IF(ZE(I).GE.5.0)31,32
   31 PRINT33
      PRINT34,(CD(I),SB(I),DL(I),DY(I),PE(I))
      GO TO 30
   32 PRINT51,(CD(I),SB(I),DL(I),DY(I),PE(I))
      CD(KN+1)=CD(I) $ SB(KN+1)=SB(I) $ DL(KN+1)=DL(I) $ W(KN+1)=W(I)
      DY(KN+1)=DY(I) $ PE(KN+1)=PE(I) $ R(KN+1)=R(I)
      SXS(KN+1)=SXS(I) $ SPS(KN+1)=SPS(I)
      KN=KN+1
   30 CONTINUE
C
C CALCULATE FINAL FIT.
C
      CALL LSQ(CD,SB,KN,Z,E)
C
C PRINT TABLE OF FINAL FIT.
C
      PRINT35
      PRINT26,(CD(I),SB(I),DL(I),DY(I),PE(I),I=1,KN)
      PRINT18,KN
      DO25I=1,KN
   25 SB(I)=1.0/SB(I)
C
C PRINT SUMMARY FOR THIS MAJOR DATA SET INCLUDING FINAL ERROR CALCULATION.
C
      PRINT19,(RN(I),CD(I),W(I),R(I),SB(I),SXS(I),SPS(I),I=1,KN)
      ZZ=1.0/Z
      PRINT22,E,Z
      PRINT29,ZZ
      GK=FLOAT(KN)
      GY=GX=0.0
      DO53I=1,KN
      GX=GX+CD(I)
```

```
53 GY=GY+CK(I)**2
   GA=GY-(GX**2/GK)
   GC=(GX/GK)**2
   SEI=1.0/GK+GC/GA
   SEI=SQRT(SEI)
   SEI=SEI*SDF
   PRINT55,SEI
   DDD=Z+SEI
   DDD=1.0/DDD
   DDD=ZZ-DDD
   PRINT61,DDD
   GO TO 41
 2 FORMAT(8A10)
 3 FORMAT(I2)
 4 FORMAT(2F10.0)
 6 FORMAT(3F10.0,A5)
 8 FORMAT(1H1,////,40X,*SEDIMENTATION PROGRAM A*,//,30X,8A10)
 9 FORMAT(///,20X,*X BOUNDARY*,10X,*TIME (SECS)*,10X,*C VALUE*,10X,*X
  * (=6.5-C)*,10X,*LN X*,//)
10 FORMAT(22X,F6.2,13X,F7.1,13X,F8.5,10X,F7.4,11X,F7.5)
13 FORMAT(    ////,20X,*LN X*,10X,*TIME (SECS)*,10X,*CORRECTED LN X*,10X,*
  * 10X,*RESIDUAL LN X*//)
14 FORMAT(20X,F7.5,10X,F7.1,12X,F7.5,17X,F8.5)
15 FORMAT(///,20X,*RUN NO.*, A5 ,//,20X,*ROTOR SPEED WAS *,F10.1,*(RP
  1M)        OR *,F10.1,* RADIANS/SEC*,//,20X,*SLOPE IS *,E12.5,10X,*
  2INTERCEPT IS *,F10.5,//,20X,*S. M. D. OF FIT IS *,E12.5,//,20X,*SE
  3DIMENTATION COEFFICIENT IS *,E12.5,*            OR *,F12.7,* SVEDBERGS*
  4ERGS*,//,20X,*STANDARD ERROR OF SEDMN COEFFICIENT IS + OR -  *,E12
  5.5,*     SVEDBERGS    OR *,F7.3,* P/C*)
18 FORMAT(*1*,////,30X,*SUMMARY OF RESULTS*,//,30X,*NUMBER OF RUNS IS
  1*, I3,////,10X,*RUN NO*,10X,*CONCN DNA*,10X,*ROTOR SPEED*,10X,*R VA
  3LUE*,10X,*SVEDBERGS*,10X,*STANDARD ERROR*,/,26X,*(MOLES/L*,12X,*(
  4RPM)*,29X,*(10-13 SECS)*,14X,*OF S*,/)
19 FORMAT(//,10X, A5 ,11X,F9.7,11X,F10.1,11X,F7.4,10X,F8.5,12X,F10.7,
  1*  OR*,F6.3,*  P/C*)
22 FORMAT(//,20X,*SLOPE IS *,E12.5,*         INTERCEPT IS *,F10.5)
26 FORMAT(/,20X,E11.4,9X,F10.7,13X,F10.7,12X,F10.7,17X,F7.3)
27 FORMAT(*1*,////,20X,*DNA CONCN*,10X,*(SVEDBERGS)-1*,10X,*CORRECTED
  1 1/S*,10X,*RESIDUAL 1/S*,10X,*PERCENT ERROR*,//)
29 FORMAT(//,20X,*INTERCEPT IN SVEDBERGS IS *,F10.6)
33 FORMAT(/,20X,*THE BELOW VALUE IS REJECTED FROM THE FINAL FIT*)
34 FORMAT(  20X,E11.4,9X,F10.7,13X,F10.7,12X,F10.7,17X,F7.3)
35 FORMAT(////,30X,*FINAL FIT OF SELECTED DATA*)
51 FORMAT(/,20X,E11.4,9X,F10.7,13X,F10.7,12X,F10.7,17X,F7.3)
55 FORMAT(//,20X,*STANDARD ERROR OF INTERCEPT IS *,E12.5)
61 FORMAT(//,20X,*S + OR - *,E12.5)
40 STOP $ END
   SUBROUTINE LSQ(X,Y,N,Z,E)
```

```
C
C THIS SUBROUTINE CALCULATES A LINEAR LEAST SQUARES FIT AND RETURNS TO THE
C CALL STATEMENT,SLOPE AND INTERCEPT,(E,Z).  STANDARD DEVIATIONS AND ERRORS
C OF FIT TOGETHER WITH CORRECTED Y VALUES ARE HELD IN COMMON STORAGE.
C
      DIMENSION X(50),Y(50),SE(50)
      COMMON SDF,PE(50),DY(50),DL(50)
      SES=0.0
      Q=FLOAT(N) $ SX=SXY=SXX=SY=0.0
      DO1I=1,N
      SX=X(I)+SX
      SXY=X(I)*Y(I)+SXY
      SXX=X(I)*X(I)+SXX
    1 SY=Y(I)+SY
      E=(SXY-SX*SY/Q)/(SXX-SX*SX/Q)
      Z=(SY-E*SX)/Q
      DO2I=1,N
      DL(I)=E*X(I)+Z
      DY(I)=Y(I)-DL(I)
      PE(I)=(DY(I)*100.0)/DL(I)
      SE(I)=DY(I)**2
    2 SES=SES+SE(I)
      SDF=SES/(Q-1.0) $ SDF=SQRT(SDF)
      RETURN$END
```

APPENDIX III

PUBLICATIONS

1. "Interaction of Proflavine and 9-aminoacridine with DNA at Temperatures below and above the Melting Temperature."

   Jordan, D.O. and Sansom, L.N., Biopolymers, 10, 399 (1971).

2. "The Interaction of 9-Aminoacridine with DNA"

   Jordan, D.O. and Sansom, L.N., Studia Biophysica, 24/25, 225 (1970).

Jordan, D. O. and Sansom, L. N. (1971), Interaction of proflavine and 9-aminoacridine with DNA at temperatures below and above the melting temperature. *Biopolymers, 10*(2): 399–410.

Jordan, D. O. and Sansom, L. N. (1970), Interaction of 9-aminoacridine with DNA. *Studia Biophsica, 24/25,* 225-232