SYMPOSIUM ON MOLECULAR ACTION OF MUTAGENIC AND CARCINOGENIC AGENTS

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SYMPOSIUM ON

MOLECULAR ACTION OF MUTAGENIC AND CARCINOGENIC AGENTS

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Introduction

Mutagenesis has been one of the main problems that the Biology Division of the Oak Ridge National Laboratory has been working on since its initiation in 1946. Many interesting aspects have been developed using radiation as the primary agent that we are trying to understand. To this has been added in the last few years the problem of carcinogenesis, especially as initiated by radiation; and we are just now in the process of developing a cooperative study of co-carcinogenesis. This study involves the effect of chemicals on the initiation of carcinogenesis and mutagenesis, as well as the effect of radiation and chemicals in combination.

The seventeenth annual research conference, sponsored by the Biology Division of the Oak Ridge National Laboratory in cooperation with the Division of Biology and Medicine of the Atomic Energy Commission, deals with this timely topic, *i.e.*, the molecular aspects of mutagenesis and carcinogenesis. The discussion was extensive and most of it was recorded for publication in this report.

The following committee, under the chairmanship of Elliot Volkin of this Laboratory, helped to arrange the program as well as edit the papers: C. C Congdon, R. F. Kimball, G. D. Novelli, R. B. Setlow, and A. C. Upton.

Special thanks should go to the Biology Division Editorial Office for preparing the papers for publication. All the papers and a major part of the discussion are reproduced in this volume.

Previous symposia in this series are:

1948—Radiation Genetics

1949—Radiation Microbiology and Biochemistry

1950—Biochemistry of Nucleic Acids

1951—Physiological Effects of Radiation at the Cellular Level

1952—Some Aspects of Microbial Metabolism

1953—Effects of Radiation and Other Deleterious Agents on Embryonic Development

1954—Genetic Recombination

1955—Structure of Enzymes and Proteins

1956—Biocolloids

1957—Antibodies: Their Production and Mechanism of Action

1958—Genetic Approaches to Somatic Cell Variations

1959—Enzyme Reaction Mechanisms

1960—Mammalian Genetics and Reproduction

1961—Recovery of Cells from Injury

1962—Specificity of Cell Differentiation and Interaction

1963—Macromolecular Aspects of the Cell Cycle

ALEXANDER HOLLAENDER

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Acridine Mutagens and DNA Structure

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ABSTRACT A number of independent critical structural parameters, which have been determined by studies on sedimentation, low angle X-ray scattering, flow dichroism, flow-polarized fluorescence, and chemical reactivity, provide the evidence for intercalation of the acridines between two otherwise sequential base pairs, and require the rejection of various alternative hypotheses. The binding requires a local untwisting and extension of the double helix, which is found to be compatible with the normal structural and bonding parameters. The expectation of a substantial alteration in the chemical reactivity of intercalated molecules because of their inaccessibility to electrophilic attack has been verified by reaction rate studies. Study of the relation between viscosity enhancement in dilute DNA solutions and intercalation has been extended using very low shear viscometry. The results agree with the earlier measurements in that the enhancement is observed with the binding of intercalatable cations, while others yield a slight diminution. A pronounced stabilization of the double helix is found, corresponding to a substantially raised thermal transition temperature. Intercalation into polyribonucleotide complexes formed between poly A and poly U renders the double helix substantially more stable than the triple. While intercalation seems to be prerequisite for mutagenicity of the insertion-deletion type, the acridine structure is not essential, nor are all intercalating molecules mutagenic.

If DNA is regarded as a rouleau of base pairs, it is easy to wonder whether a false coin might be inserted into the stack; superficial considerations of the size, shape, and electronic structure of various of the polycyclic aromatic compounds suggest their appropriateness as the possible false coin. Because the biological activity of these substances with respect to mutagenicity, carcinogenicity, episome elimination, etc., may be derived by theory or hypothesis from an interaction with the cellular hereditary substance, it has been of interest to examine the structure of their complexes with DNA. In the following discussion, the evidence that binding to DNA obtains through intercalation into the rouleau will be reviewed, including some new data from reaction rate studies, and some other properties of the complex deriving from intercalation will also be presented. Although the structural investigations to the present have been restricted to complexes of DNA with the acridines and closely related aromatic cations, they provide a basis for speculation and further study with other classes of substances.

If it is supposed that the probable sites of acridine binding are related to the nucleotide sequence in DNA, the structure of the complex will be aperiodic, and hence, unlikely to provide an informative X-ray diffraction pattern. Our studies on DNA fibers containing about one proflavine molecule per three nucleotide pairs, examined over a considerable span of relative humidity, showed only a meridional spot, corresponding to a spacing of about 3.4 Å, and two or three equatorial spots, with none of the other features characteristic of DNA, even though the slight arcing of the spots indicated that a high degree of orientation had been attained. It was concluded that the pattern, or absence of pattern, was compatible with the supposition of an apparent randomness due to intercalation, but might also be explained in other ways. In the absence of a direct structural analysis, it becomes necessary to resort to a more conjectural method, in which a number of critical structural parameters that may be derived from distinctive features of competitive hypotheses can be compared with specific experimental results.

Hypothetical structures

A set of conjectures for the binding of proflavine is shown in figure 1. DNA is sketched in the figure as viewed from a remote point, so that the base pairs appear only in edgewise projection, and the phosphate-deoxyribose backbone appears as a

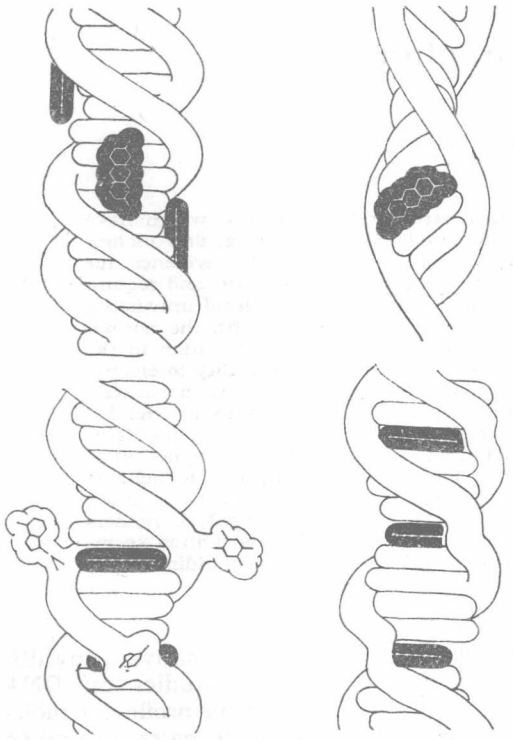

Fig. 1 Four conjectures for the structure of the complex of DNA with acridines. The compound represented in the sketches is proflavine.

smooth coil. (The sketch is based on the coordinates of Model 3 for the B configuration of DNA given by Langridge *et al.*, '60.) Proflavine is shown shaded either in edgewise or plan projection, according to its situation on the helix. In the sketch on the upper left, it is suggested that proflavine might bind externally, without any disturbance of the double helix, with the centers of positive charge brought relatively near to the phosphate groups; the plane of the acridine nuclei lies more or less parallel to the helix axis. Another arrangement with an undisturbed double helix could conceivably be included, having edgewise attachment of the acridine with its plane more nearly perpendicular to the helix axis, but this arrangement may be regarded as less favorable, since it requires a greater perturbation of the water structure (in the sense implied by hydrophobic bonding) and much less contact between the acridine and the bases. The sketch on the upper right again suggests external attachment, together with a structural perturbation of the DNA corresponding to a 50% extension of the double helix, which requires strong tilting of the base pairs to an angle of about 45° with the helix axis. The existence of a structure of this kind is suggested by the disappearance of dichroism in DNA fibers which have been stretched to half again their original length (Wilkins *et al.*, '51). The sketch on the lower left suggests that an acridine might enter the rouleau near the helix axis and perpendicular to it by displacing a base pair, so that each member of the disengaged pair is rotated to the outside of the helix, as proposed in the one nucleotide loop model of Fresco and Alberts ('60). In the sketch on the lower right, it is suggested that there is a local untwisting of the helical backbone, permitting a separation between previously adjacent base pairs without, however, disturbing their hydrogen bonding, and the acridine is intercalated into the space provided.

The hypothetical models can be grouped according to the physical properties that they might have in common. For any given length along the helix axis, both of the models on the left contain the mass or number of electrons of the bound acridine in addition to all of the mass or number of electrons in same length of uncomplexed DNA. In the models on the right, the DNA is sufficiently extended that even with the additional mass contribution of the bound acridine, the total mass per unit length of the complex may be lower than that of uncomplexed DNA. The bottom pair of models require that the plane of the acridine molecule is parallel to the plane of the base pair, while in the upper pair of models these planes must be more nearly perpendicular. In the upper left and lower right pairs, the bases remain nearly perpendicular to the helix axis, while in the others they are either strongly tilted or unconstrained. In the upper pair, it would appear that one face of the bound acridine is in contact with the DNA helix, and therefore out of contact with the solvent, while in the lower pair the solvent can touch only the edges, *i.e.*, the hydrogen atoms, of the acridine molecule.

The mass per unit length

A comparison of the mass per unit length of the DNA proflavine complex as compared with uncomplexed DNA based on the change in sedimentation coefficient has been presented; the same results have been found in a more extensive and detailed study by means of low angle X-ray scattering (Luzzati *et al.*, '61). The experimental data are in good agreement with the expression

$$\mu = 101 - 75\,\beta/\,(1 + \beta) \qquad (1)$$

where μ is the number of electrons per Angstrom unit along the helix axis, and β is the number of proflavine molecules bound per nucleotide pair. It will be seen that the pronounced decline in electrons per unit length as the number of bound proflavine molecules increases requires the rejection of both of the left-hand models of figure 1. The form of the equation above is based on the intercalation model, the lower right of figure 1, but an equally good fit to the experimental data is shown by the relation

$$\mu = 101 - 52\,\beta \qquad (2)$$

which corresponds to the sum of the decline in electrons per unit length resulting from extension of the DNA and the increase in electrons per unit length resulting from the attachment of proflavine, as in the upper right-hand model. An experimental discrimination between the two models can be made on the basis either of the orientation of the bases, or the orientation of the acridine, with respect to the helix axis.

Flow dichroism of the DNA-quinacrine complex

When a solution of a high polymer flows through a narrow channel, the flow gradient effects the partial orientation of the polymer, tending to align the polymer chain parallel to the flow lines. Since the probability that the purines and pyrimidines absorb light (in the 260 mμ band) is determined by $\cos^2\gamma$, where γ is the angle between the electric vector of the light wave and another vector characteristic of the chromophore lying in the plane of the purine or pyrimidine, oriented DNA in the B configuration should be expected to absorb light of this wave length poorly when it is polarized parallel to the direction of flow, and to absorb more strongly than usual when the plane of polarization is perpendicular to the direction of flow. Complete extension of DNA into a straight fully aligned rod is never achieved, and it is not possible to demonstrate a high degree of perpendicularity between the bases and the flow axis, even with uncomplexed DNA. However, we may assume (with adequate justification) that the bases are indeed perpendicular to the helix axis in uncomplexed DNA, and use its dichroism as a reference for the complex. If strong tilting of the bases occurs, the dichroism should disappear entirely, as it does in the stretched fibers. The experimental measurements (Lerman, '63) show that the dichroism, measured as the fractional decline in absorbance when the plane of polarization is parallel to the flow axis, is −0.19 for pure DNA and −0.24 for the DNA-quinacrine complex at 260 mμ; the complex is more, rather than less, dichroic. The measurements were carried out with sufficient bound quinacrine, a derivative of 9-aminoacridine, to have effected the tilting of 85% of the base pairs if equation 2 is to account for the mass per unit length. It is appropriate to attribute the increased dichroism of the complex to the increased straightening of the helix that must also be invoked to account for enhancement of the viscosity of DNA when acridines are bound.

Flow-polarized fluorescence

The absorption of light by a molecule bound to DNA is regulated by the same considerations as those given above for absorption by the bases. In addition, the intensity of emitted light observed through a polarizing filter, if the bound molecule is fluorescent, depends again on $\cos^2$ of the angle between the polarization direction of the filter and the direction of a vector, the transition moment, in the plane of the fluorescent molecule, which may be the same or different from the vector representing the transition moment for light absorption. Since some of the final measurements depend on the orientations of two different vectors in the plane of an acridine bound to DNA with respect to the laboratory coordinates, as set by flow and

the polarizing filters, they can in principle define the plane of the acridine, rather than merely the direction of a vector in the plane, and limits on the possible deviation of this plane from perpendicularity to the flow axis can be specified from purely qualitative considerations of the effect of the flow on fluorescence intensities.

When fluorescence of the complex is observed with the polarization of the exciting light parallel to the flow axis and the emission perpendicular, or vice versa, the measured intensity will be given by the relations

$$I_{zx} = 1/2 \cos^2\gamma_1 \sin^2\gamma_2 \quad (3)$$

$$I_{yz} = 1/2 \sin^2\gamma_1 \cos^2\gamma_2 \quad (4)$$

where γ_1 is the angle between the absorbing transition and the flow axis, and γ_2 is the angle between the emitting transition and the flow axis. These equations correspond to the data presented in table 2 of our earlier paper (Lerman, '63) for measurements with the excitation and emission specified as ZX and YZ, respectively. When fluorescence is excited by the absorption of light of 4500 Å, both absorption and emission take place by means of the same transition moment, but with 3000 Å excitation absorption occurs along a different, approximately perpendicular transition moment (still in the plane of the rings) and emission is the same as from 4500 Å excitation. Thus, for 4500 Å excitation, γ_1 and γ_2 are identically the same, but for 3000 Å excitation they are nearly independent. The geometry of the latter case corresponds to the angles between a line in space, representing the flow axis, that intersects the corner of a rectangular box and two of the edges that meet at the corner, representing the directions of the transition moments. The third edge is then the vector normal to the plane of the dye. In the stationary solution, γ_1 and γ_2 will have random values which change to partially preferred values determined by the way the dye is attached to DNA in the flowing solution. Whether the difference in intensity between the flowing and stationary solution is positive, negative, or zero depends critically on the preferred values of γ_1 and γ_2. It will be seen from our data (Lerman, '63) on the flow of the DNA-quinacrine complex that the intensities during flow for both of these complementary configurations of the polarizers are less than the intensities from the stationary solution, and that both values are about the same. The similarity simplifies the problem in specifying that γ_1 must be nearly the same as γ_2. Comparison of the changes due to flow at 4500 Å with the intensity expected for a random orientation (derived by averaging equation 3 or 4) reveals that γ_2 cannot be less than 66½°, which would give zero change. If γ_1 is the same, the normal to the plane of the dye cannot have an angle of more than 34° to the flow axis. While this limit does not seem strongly restrictive (perpendicularity to the flow axis implies an angle of zero), it should be noted that we have not used the measurement of the amount of intensity decrease; we have calculated only the angle that would correspond to no change in intensity. The DNA is never completely, or even predominantly oriented in a shear gradient in dilute solution. Furthermore, the shear gradient is not uniform across the capillary tube, but has a large value only near the walls. Thus, interpretation of the limiting angle (see "Flow dichroism of the DNA-quinacrine complex," page 3) in view of the discrepancy between the measured dichroism values and the theoretical maximum dichroism, indicates that the actual deviation from the perpendicularity must be very much less than 34°.

Steric hindrance to electrophilic attack

If the upper and lower faces of the bound aromatic molecule are indeed out of contact with the solvent, as suggested by the lower pair of drawings in figure 1, the possibility of its reaction with an attacking reagent that requires an out-of-plane transition state, for example, through direct interaction with the π electrons, should be severely restricted. The way that proflavine might fit into the space between two base pairs is suggested in figure 2 where the backbone configuration is projected from a molecular model of the extended state. The second base pair that forms the top of the sandwich would lie almost directly above the one

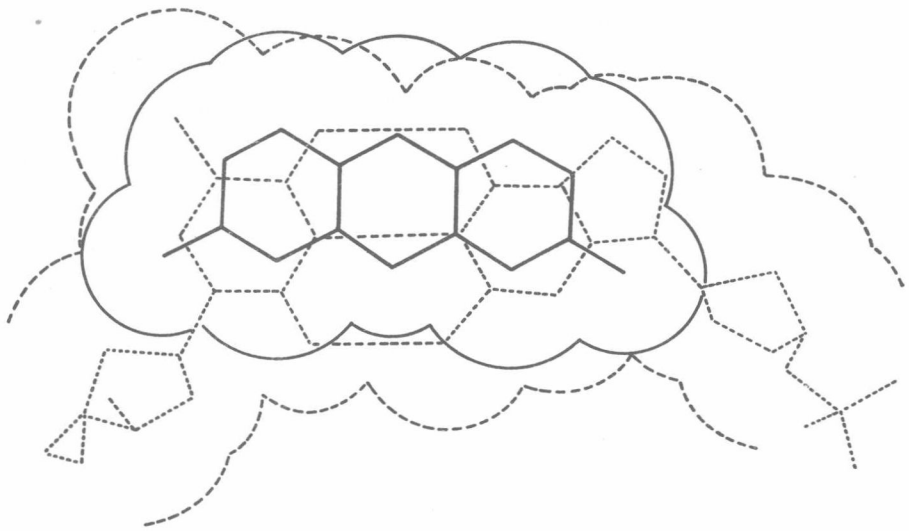

Fig. 2 A molecule of proflavine superposed over a nucleotide pair with the deoxyribose phosphate chain in the extended configuration.

shown in the drawing, or perhaps rotated very slightly clockwise. Although there is no evidence bearing on the detailed positioning of the proflavine, it has been placed in the drawing to more or less maximize its contact with the aromatic regions of the purine and pyrimidine. With the three layers of the sandwich in van der Waals contact, it will be seen that out-of-plane approach to the electrons of the amino nitrogens is blocked. The rate of reaction of proflavine and some other aminoacridines with nitrous acid has been examined (Lerman, '64) (1) for the free dye in buffered aqueous solution, (2) with the dye bound to DNA, and (3) with the dye bound to one of several other polyanions.

In an aqueous solution containing sodium nitrite and acetate or phthalate buffer at pH 4.2 to 4.6, the principal reactive species is thought to be dinitrogen trioxide, N_2O_3, which reacts with an uncharged amino group to make $-\overset{+}{N}H_2-NO$, an unstable intermediate, that loses water to become the diazonium salt. In concentrated solutions the rate-limiting step is often the formation of N_2O_3, but in the present experiments where the amine is exceedingly dilute, the reaction rate is precisely pseudo-first order in the aminoacridine concentration. In the presence of DNA or other polymers the rate of disappearance of proflavine is also exponential, and the results may conveniently be described by a pseudo-first order rate constant. Where the rate constant for the reaction of that part of the proflavine that is actually bound to polymer can be calculated by means of independent binding measurements, or where only a negligible amount of proflavine is not bound, the data can be further simplified to give the ratio of the rate constant for the bound dye to the rate constant for reaction at the same pH in the absence of polymer. These relative values are presented in figure 3. It will be seen that binding to DNA diminishes the reaction rate as much as twentyfold, while binding to any of the synthetic polymers never results in as much as a 50% inhibition. Since proflavine in solution offers four modes of attack, from the top or bottom of either of two amino groups, inhibition by simple steric blockade cannot be more than 50% effective unless more than two modes are blocked. The effectiveness of the DNA inhibition is in good agreement with the hypothesis of nearly complete enclosure of the proflavine, while the other polymers can make at best an open-faced sandwich. Quantitatively similar results have been

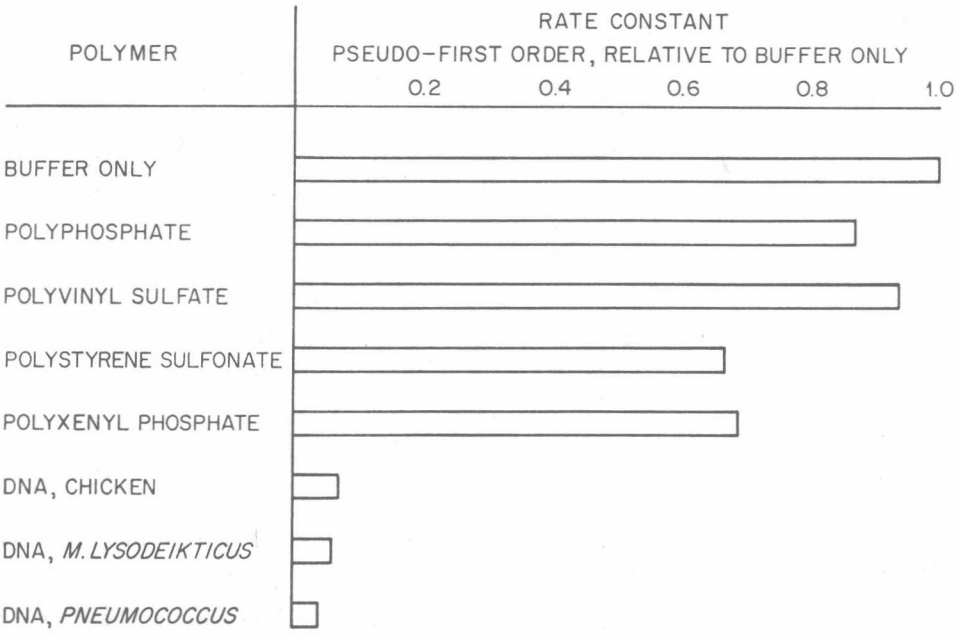

Fig. 3 The relative pseudo-first order rate constant for the diazotization of proflavine, pH about 4.4, 25.0°C, when bound to DNA and certain synthetic polymers.

obtained with two other compounds, 2-aminoacridine, and 6,9-diamino-2-ethoxyacridine, and 3,8-diamino-5-methyl-6-phenylphenanthridinium bromide. The similarity of the results with these acridines and proflavine is interesting because of the different distributions of positive charge as shown in figure 4, and because they have only one reactive amino group each. Because of the charge distribution, it may be expected that the 8-amino is rather more reactive than the 3-amino of the phenanthridinium compound.

A test of the steric hindrance requirements

It would appear that the completeness of the inclusion of an aminoacridine between base pairs is insensitive to the charge distribution, and the residual reaction rate is determined by some nonspecific property, as perhaps the flexing of the helix due to thermal agitation. To establish the dependence of this inhibition on direct steric obstruction, similar rate measurements have been carried out with a related series of compounds, the triphenylmethane dyes, carrying two or three amino groups on the three rings. Comparison of the size of these molecules with the size of the intercalation cavity shows that only two of the three rings can be included within it, so that when an amino group is present on the third ring it protrudes a little beyond the van der Waals limits of the nucleotides and should be in contact with the solvent above and below. Both of the triamino triphenylmethane dyes tested, pararosaniline and neofuchsin, were found to react when bound to DNA with very nearly one-third of the rate constant with which they react as free dye. On the other hand, the homologous diaminotriphenylmethane dye, Doebner's Violet, was as strongly inhibited as proflavine. We may conclude that the rate of reaction with nitrous acid is not sensitive to mere proximity to the DNA helix.

The structure of the extended backbone

The intercalation hypothesis requires that the phosphate deoxyribose backbone, which in the Watson-Crick-Wilkins structure for the B configuration connects two bases with a right-hand rotation of 36°

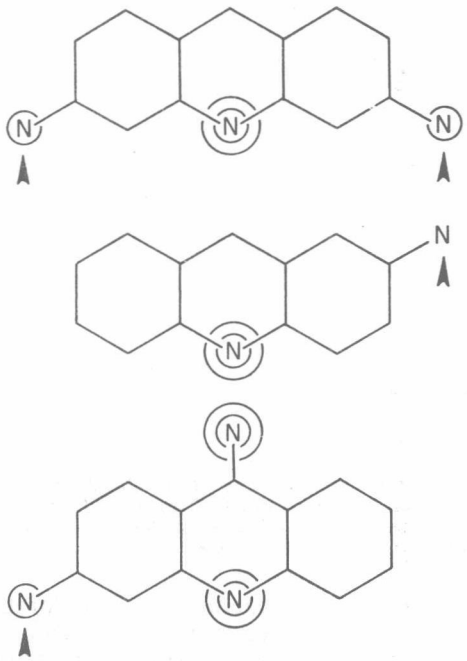

Fig. 4 A schematic representation showing the sites of reaction with nitrous acid (indicated by the arrows) and the relative distribution of positive charge for proflavine, 2-aminoacridine, and 3,9-diaminoacridine. In each case, there is only a single formal charge on each molecule, distributed roughly as shown by the circles.

and an advance along the helix axis of 3.36 Å, be rearranged to connect the same two bases with an advance of twice as far along the helix axis, in order to allow for the thickness of the intercalated acridine. The compatability of the required rearrangement with the bond length and angles proposed by Rich *et al.* ('61) for polyadenylic acid, together with other normal structural parameters including stagger and nonbonded contacts, was reported previously. We have made a brief examination of its compatability with the bond parameters of Langridge *et al.* ('60) and find no major difference. In the previous model, the best fit seemed to be obtained with a slight backward twist, about −9°, between the two separated nucleotides, but in the new model no rotation is needed — the bases are nearly superposed. In neither model has it been found possible to bring the phosphates any nearer to the helix axis.

Stabilization of the helix

The binding of the cationic acridines effects a substantial increase in the thermal denaturation temperature of DNA, as illustrated in figure 5, where the midpoint of the hyperchromicity function for chicken erythrocyte DNA is plotted against the total concentration of added cation, in the presence of 0.01 *M* tetraethylammonium, and data are given for 9-aminoacridine and streptomycin. It will be seen that at the lowest concentration of 9-aminoacridine, 1×10^{-6} *M* where there would be one acridine per 17 nucleotide pairs if all the added acridine were bound, the transition temperature is raised 13°. It will be more useful to know the dependence of the transition temperature on the amount of bound acridine; since suitable binding data are not yet available, the correction can only be estimated from reasonable values of the binding parameters, as suggested by other measurements. At the lowest point on the curve, it may then be reckoned that there is roughly one bound acridine per 25 or 30 base pairs. The somewhat different form of the data for streptomycin in the figure is compatible with the supposition that the intrinsic association constant per binding site is smaller for streptomycin than for 9-aminoacridine, but that the equilibrium can be driven, in the presence of more free streptomycin, to the binding of a larger number of molecules. It is plausible to suppose that the upper limit for intercalated acridine binding is effectively 0.2 per nucleotide pair at low salt concentrations when the extent of binding is small (Heilweil and Van Winkle, '55), rising more weakly to about 0.44 per nucleotide pair (Peacocke and Skerrett, '56), and that streptomycin may bind until charge neutrality is reached, about 0.67 per nucleotide pair. Since it is to be expected that the principal basis for the thermal stabilization is electrostatic, it is interesting to note that each acridine adds only a single positive charge to the helix while the binding of each streptomycin adds three positive charges.

The hyperchromicity of the dye spectrum, which can be followed independently of the DNA hyperchromicity but

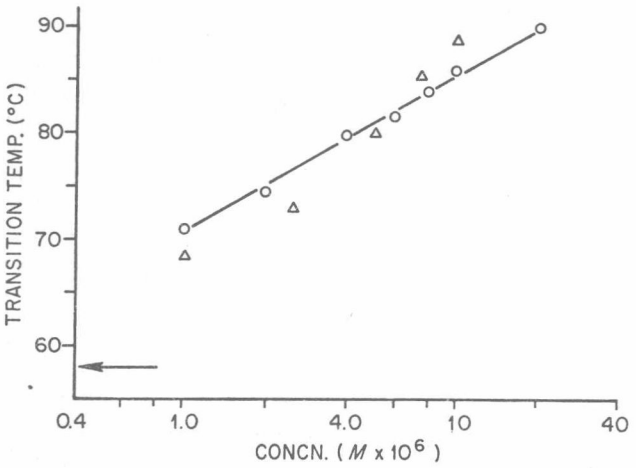

Fig. 5 The thermal transition temperature of chicken erythrocyte DNA is shown as a function of the concentration of 9-aminoacridine (○) and streptomycin (△) in 0.01 *M* tetraethylammonium bromide. The DNA concentration is about 3×10^{-5} *N* in nucleotides. The transition temperature of pure DNA in this solvent is shown by the arrow.

closely parallels it, together with independent estimates of the binding by fluorescence polarization, indicate that the binding of the acridine to random-coil DNA is very much weaker than the binding to helical DNA.

Thus, the increased transition temperature corresponds to the additional energy needed to dissociate the acridine from the helix in addition to separating the strands. As the complex passes through the transition temperature, the nearly complete dissociation of the denatured portion provides a higher effective total dye to helical nucleotide ratio, on which the new equilibrium of the undenatured portion will be based. It may be expected that an assymmetrical broadening of the transition zone will be observed when the acridine concentration is low and the DNA is not too low.

Elevation of the denaturation temperature of DNA in the presence of acridine orange has been noted by Freifelder *et al.* ('61), and the effect of proflavine has been studied by J. A. McCarter (personal communication).

EXCLUSION OF TRIPLE HELIX

The snug fit of an acridine into the intercalation cavity as shown in figure 2 indicates that only part of an analogous cavity in a triple helix could be occupied. A hypothetical structure with so large a cavity would be regarded as energetically less favorable than a structure in which the third strand of the triple helix is not extended, but allows a nucleotide to occupy the neighboring position in the layer containing the intercalated acridine. However, the helical configuration of the nucleotide at the intercalated site will itself be unstable with respect to water bonding, since the acridine can provide, in general, only one hydrogen bond, or none at all. We are led to suspect that the three-stranded helix will be unstable relative to a two-stranded helix with intercalated acridine. The results of mixing polyadenylic acid, polyuridylic acid, and various cations, including 9-aminoacridine and 10-methylacridinium, are shown in figure 6. It will be convenient to examine the curves on the left-hand part of the figure first. The experimental measurement is the ultraviolet absorption of mixtures containing an approximately constant total nucleotide concentration, but differing ratios of adenine to uracil in the presence of 0.2 *M* tetraethylammonium bromide. When no other cation is added, the mixtures show a vaguely defined minimum, but in the presence of a low concentration of magnesium ion, as is well known, there is a distinct minimum at the mole fraction corresponding to two uracils for each adenine, pre-

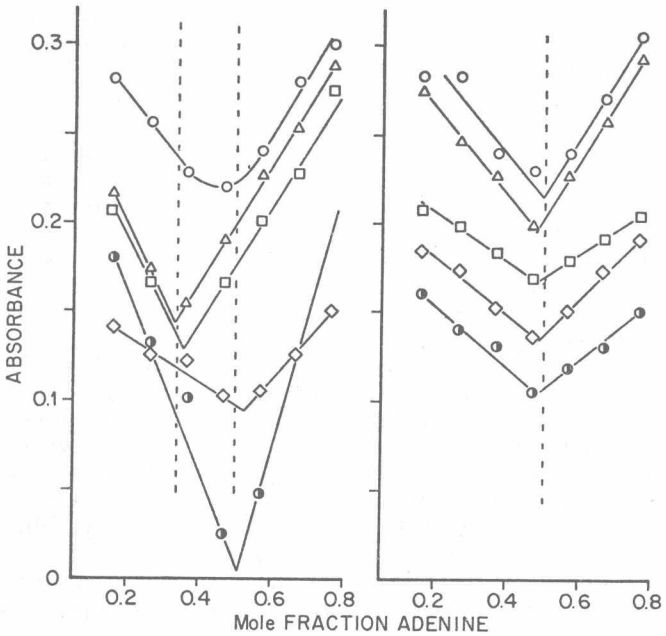

Fig. 6 The formation of complexes between polyadenylic acid and polyuridylic acid in 0.2 *M* tetraethylammonium bromide together with various cations at 25.0°C. The added cation and its concentration are indicated in the left panel by the relevant curve for the following: At 270 mμ, ○, 0.2 *M* tetraethylammonium only; △, 5×10^{-3} *M* diaminobutane; □, 10^{-3} *M* magnesium; ◊, 10^{-5} *M* 9-aminoacridine; and at 259 mμ, ◑, 10^{-5} *M* 9-aminoacridine.

All of the mixtures in the right-hand panel contain 10-methylacridinium iodide measured at the following wavelength and concentration: At 259 mμ ○, 5×10^{-5} *M*, and △, 2.5×10^{-6} *M*; and at 270 mμ, □, 5×10^{-5} *M*, ◊, 1×10^{-5} *M*, and ◑, 2.5×10^{-6} *M*. For clarity most of the curves have been arbitrarily displaced along the ordinate. The dotted lines indicate the expected positions of the minima for the formation of three-stranded and two-stranded helices.

sumably signifying a three-stranded helix. Fresco ('63) has shown that only at very low ionic concentrations is the double-stranded helix more stable than the triple. The addition of diaminobutane, which will be a doubly charged cation at neutral pH, has the same effect as magnesium — a distinct stabilization of the triple helix. The lowest pair of curves on the left represent a single set of mixtures containing 9-aminoacridine examined at two different wave lengths; at 270 mμ the 9-aminoacridine makes no contribution to the observed hypochromicity, while at 259 mμ the hypochromicity of the dye is superposed on the hypochromicity of the nucleotides. The concentration of 9-aminoacridine corresponds, if it were all bound, to one dye per 2.3 nucleotide pairs. It will be seen that the curves at both wave lengths clearly indicate the stabilization of a double helix containing equivalent amounts of adenine and uracil. The curves on the right are similar, and show the effect of three concentrations of 10-methylacridinium iodide, observed at two wave lengths (except for the highest concentration). The lowest concentration of 10-methylacridinium corresponds to a total of bound and free dye of slightly more than one per ten nucleotide pairs; at the highest concentration there is roughly one dye for every nucleotide. It will be seen that the double helix is stabilized in all three mixtures. The conspicuous feature of these results is a clear distinction between the effects of the nonintercalating cations, such as magnesium and diaminobutane, and that of the intercalating ions, 9-aminoacridine and 10-methylacridinium.

The thermal transition temperature of the poly A-poly U double helix is also elevated by combination with the acridines to an extent that it is roughly similar to the effect on DNA as presented in figure 5.

Viscosity enhancement

It will be of interest to establish more clearly the relation between intercalation and the effect on the viscosity of DNA solutions (Lerman, '61). The intercalated complex can be expected to exhibit a higher intrinsic viscosity (based on its DNA concentration) than DNA only, through the lengthening and straightening of the helix, while the binding of other cations can be expected to effect a slight decrease in the viscosity due to a relaxation of the electrostatic repulsions between nearby segments of the helix. Viscometric results, similar to those reported previously for the acridines, are presented in table 1 for a number of organic cations. None of these substances alone has an appreciable contribution to the viscosity of water or a buffer solution at these concentrations. The compounds represented fall into five groups: (1) small cations with not more than one ring, (2) basic dyes with three or four fused rings, (3) methyl derivatives of two benzacridine isomers, (4) actinomycin D, a neutral oxazine molecule with three fused rings, and (5) the triphenylmethane dye, fuchsin. All of the substances in group 1, when present in sufficient concentration, effect a diminution in specific viscosity because of the increased ionic strength and simple external binding of the cation. All the substances in group 2 are essentially flat polycyclic molecules, and like the acridines, they considerably enhance the DNA viscosity. The two oxazine dyes, brilliant cresyl blue and Meldola's blue, are of particular interest because one of the aminonitrogens of the former carries two ethyl groups, which may be too

TABLE 1

The effect of some acridines, related compounds, and some other organic cations on the specific viscosity of DNA

Compound added to DNA solution	Concentration	DNA concentration	Viscosity ratio at zero shear
		$\mu g/ml$	
4-Aminopyridine-HCl	0.001 M	29.7	0.97
4-Aminopyridine-HCl	0.01 M	29.7	0.70
Benzyltrimethylammonium Chloride	0.01 M	29.7	0.78
Tetramethylammonium Iodide	0.01 M	29.7	0.71
Sodium Chloride	0.01 M	29.7	0.67
Spermine	4.5 $\mu g/ml$	23.2	0.67
Neutral Red	6 $\mu g/ml$	23.2	1.47
Thionine	2×10^{-5} M	20	1.78
Brilliant Cresyl Blue	6 $\mu g/ml$	23.2	1.56
Meldola's Blue	9 $\mu g/ml$	29.7	1.49
Acriflavine	$\sim 2 \times 10^{-5}$ M	20	1.71
Benz[a]acridine	saturated [a]	46	1.17
8,9-Dimethylbenz[a]acridine	saturated [a]	46	1.11
10,12-Dimethylbenz[a]acridine	saturated [a]	46	1.52
10,12-Dimethylbenz[a]acridine	saturated [a]	23	1.45
7,9-Dimethylbenz[c]acridine	saturated [a]	46	1.14
Actinomycin D	125 $\mu g/ml$	20	1.26
Fuchsin	10^{-5} M	32	1.98

All measurements were carried out with chicken erythrocyte DNA in a glass capillary shear viscometer at 25.0°C as described previously (Lerman, '61). Data taken at shear rates near 50, 100, and 160 sec^{-1} were extrapolated to zero shear assuming continuation of the linear relation between the square root of the shear rate and the log of the specific vicsosity. The zero-shear reduced specific viscosity of the DNA in buffer only was 160 dl/g at 23 $\mu g/ml$ and 173 at 46 $\mu g/ml$. The viscosity changes are tabulated as the ratio of the zero shear specific viscosity in the presence of the test substance to that of DNA alone at the same concentration.

The buffer contained phosphate, EDTA, and chloride pH 6.9, with a total sodium concentration of 0.003 M and ionic strength of about 0.006. Dye concentrations are given in terms of the commercial material, uncorrected for actual dye content; they are in the vicinity of 2×10^{-5} M. Since only meager precautions against photodynamic oxidation were taken, it is possible that the viscosities for the red and blue dyes include the effect of slight degradation, and should be taken to represent minimum values.

[a] The DNA solutions were agitated with crystals of the specified substance at room temperature in darkness for two days and filtered through Whatman no. 50 (very fine) paper before viscometry.

bulky to intercalate, and the latter is comprised of four, rather than three, fused rings. One may surmise that the disposition of brilliant cresyl blue between nucleotide pairs permits the ethyl groups to protrude beyond the edge of the sandwich. Although the viscosity increments given by the benzacridines (also four rings) are smaller, they are nevertheless distinct, and correspond roughly to the amount of each compound bound by DNA, as determined by extraction of the complex with benzene. The data indicate that the potent carcinogen, 7,9-dimethylbenz [c] acridine, intercalates, but not more, nor necessarily as much, as the less active or inactive compounds.

The data on actinomycin (undertaken at the suggestion of Dr. Julia Kirk, personal communication) argue in favor of attachment by intercalation to DNA, but in view of the complexity of the molecule, other evidence would seem essential to a firm conclusion.

In the results discussed above, the viscosity at zero shear was estimated by an extrapolation based on an arbitrary empirical relation that fits the data at the shear values of the measurements. It has seemed reliable for comparative values since all of the data correspond to a uniform shear dependence that varies only slightly, and monotonically, with the intercept value. Thus, all of the extrapolated values are in the same sequence as the measured specific viscosities at a particular shear.

Preliminary experiments with the very low shear concentric cylinder viscometer of Zimm and Crothers ('62) confirm the qualitative features of these observations at 100-fold lower shear rates. Some measurements with *E. coli* DNA are shown in figure 7. The specific viscosity of DNA alone is indicated on the ordinate by an arrow. The abscissa represents the total concentration of added cation in a solvent consisting of 0.02 *M* NaCl and 10^{-3} *M* EDTA. A small diminution in viscosity

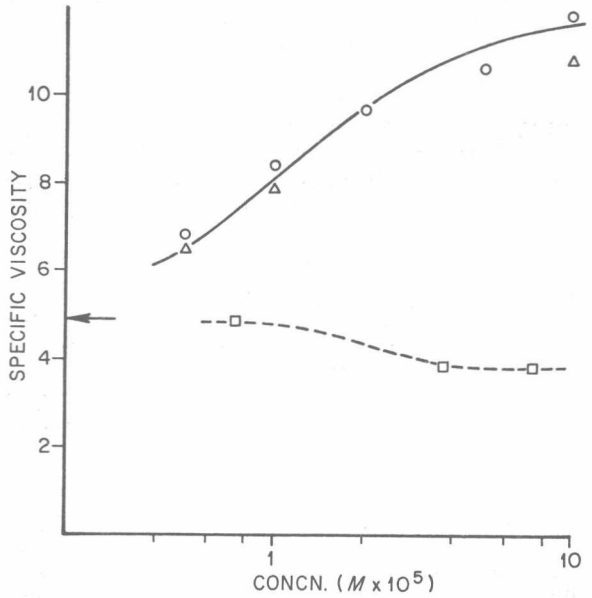

Fig. 7 The specific viscosity of *Escherichia coli* DNA, 21 μg/ml in 0.02 *M* NaCl, 10^{-3} *M* EDTA, is shown as a function of the total added concentration of one of the following: 9-aminoacridine (○), streptomycin (□), or 10-methylacridinium iodide (△). The specific viscosity of DNA alone at the same concentration is shown by the position of the arrow. The solid line is a hypothetical simple binding curve, where the increment of the ordinate over the value at the arrow is proportional to the fractional saturation of binding sites; the assumed value of the binding constant is $2 \times 10^5 M^{-1}$, and the concentration of binding sites is taken to be 0.22 of the nucleotide concentration.

(0.77 of the control) due to the presence of streptomycin will be noted, while both 9-aminoacridine and 10-methylacridinium effect about a twofold increase. The solid curve drawn alongside the acridine points represents a simplified guess at the relation between the amount of added acridine and the amount bound to DNA; a Langmuir binding isotherm is assumed with a unique, plausible value of the binding constant, and a number of binding sites corresponding to 0.44 of the concentration of nucleotide pairs; zero binding is set at the position of the arrow on the figure. While a direct proportionality between viscosity increment and binding cannot be claimed, it will be seen that there is at least qualitative correspondence.

Mutagenicity and structure

A convenient selective technique for determining mutagenicity is based on the induction of apparent back-mutants in the *r*II genes of T4. While a test stock of phage carrying an *r*II mutation cannot yield plaques on a K-12 (λ) strain of *E. coli*, the action of a mutagen during infection may result in the appearance of many plaque-forming phage in the lysate, Crick *et al.* ('61) have shown that these are usually the consequence of a second mutation, closely linked to the original acridine-induced marker in the test stock, which suppresses the *r*II phenotype. A number of aminoacridines which were examined for mutagenicity with this system (Orgel and Brenner, '61) were found to vary in efficacy, with the more basic compounds showing greater mutagenicity. One surmises that the differences may be explicable principally in terms of the differences in binding to DNA.

To study the relation between mutagenesis and the structural changes in DNA, a somewhat more diverse set of compounds has been examined; some results are persented in table 2. The inefficacy of spermine, diaminobutane, and magnesium show that affinity for DNA is not itself an adequate qualification for mutagenicity. The activity of the intercalating compounds, phenanthridine and 6-aminophenanthridine, indicates that the process does not specifically require the acridine structure. Several strongly basic substituted acridines are considerably less active than the simpler compounds. The most drastic effect is given by methylation of the ring nitrogen; 10-methylacridinium is nonmutagenic, although its properties toward intercalation resemble 9-aminoacridine.

It may be concluded tentatively that intercalation is a necessary, but not a sufficient, condition for the generation of insertion-deletion mutations.

Certain other distinctive features of the *in vivo* interaction with acridines have

TABLE 2

Mutagenicity with respect to the induction of back-mutants and/or suppressors of the rII mutant, P3 (except 10-methylacridinium, measured with P43). Activity is expressed as the ratio of the increment due to induced mutations to the control

Compound	Maximum concentration	
	Tested, *M*	Activity
9-Aminoacridine	3×10^{-5}	100
Proflavine	2×10^{-5}	430
Acridine	3×10^{-4}	7
9-(N-piperidino)-Acridine	1×10^{-3}	25
10-Methylacridinium	1×10^{-3}	0
9-Amino-10-Methylacridinium	1×10^{-3}	0
Phenanthridine	2.2×10^{-4}	64
6-Aminophenanthridine	2.6×10^{-4}	63
1-Aminophenanthridine (crude)	5×10^{-3}	3
3,8-Diamino-5-Methyl-6-Phenylphenanthridinium Bromide	10^{-3}	0
1,6-Hexanediamine	4.2×10^{-2}	0
Spermine	1×10^{-3}	0
Spermidine	1×10^{-2}	0
Magnesium	8×10^{-3}	0