

STUDIES ON NUCLEIC ACID METACHROMASY

I. The Effect of Certain Fixatives on the Dye Stacking Properties of Nucleic Acids in Solution

MICHAEL E. LAMM, LILLIAN CHILDERS, and
MERRILL K. WOLF

From the Section on Physical Chemistry, National Institute of Mental Health, Bethesda, Maryland, and the Laboratory of Cellular Neuropathology, Harvard Medical School, Boston, Massachusetts. Dr. Lamm's present address is the Department of Pathology, New York University School of Medicine, New York

ABSTRACT

The stacking coefficients (K 's) of nucleic acids have been thought to influence the color contrast between DNA and RNA in tissue sections stained with metachromatic dyes. This idea was tested by titrating toluidine blue (TB) and acridine orange (AO) in solution against DNA and RNA, native or treated with formaldehyde, acrolein, or Carnoy's fluid. Absorption spectra at varying polymer-dye ratios were used to compute K values by the methods of Bradley and colleagues. Results with both dyes fit Bradley's stacking equations. Fixatives did not block dye-binding sites but markedly altered K values. K of DNA was low, unaffected by aldehyde fixative, increased by Carnoy's fluid or heat denaturation. K of RNA was higher than that of DNA and was increased greatly by formaldehyde, almost as much by acrolein, considerably less by Carnoy's fluid. Aldehyde effects were partially reversed upon removal of aldehyde by dialysis. These observations accord with known effects of aldehydes and denaturation upon nucleic acid conformation. Differences between K 's of DNA and RNA were greater after aldehyde treatment than after Carnoy's, and were greater with AO than with TB. This is generally consistent with the magnitude of the color contrasts observed in tissues. Additional factors must contribute to the intense color contrast observed in acrolein-fixed tissues stained with TB.

INTRODUCTION

When certain cationic dyes are used to stain tissue sections, some tissue polyanions acquire the same color shown by the dye in dilute solution, *i.e.* are stained orthochromatically, while other tissue polyanions acquire a different color, *i.e.* are stained metachromatically. Most studies of this phenomenon have been concerned with the large metachromatic color changes produced by acid mucopolysaccharides. However, similar though smaller color changes are produced in many of the same dyes by nucleic acids. The metachromatic color change

has long been considered to result from reversible aggregation of dye molecules, which may occur when the concentration of dye is increased, the ionic strength is increased, the temperature is decreased, or when dye molecules are bound to neighboring sites on suitable polymers. For several representative metachromatic dyes, the formation of such aggregates has been proved (19, 30). The subject of metachromasy was comprehensively reviewed by Bergeron and Singer in 1958 (3).

Some metachromatic dyes can be used to pro-

duce a color contrast between DNA (deoxyribonucleic acid) and RNA (ribonucleic acid)¹ in tissue sections. Both nucleic acids will stain orthochromatically at one extreme and metachromatically at the opposite extreme of temperature, ionic strength, pH, or dye concentration, but intermediate conditions can be found under which DNA will stain orthochromatically, RNA metachromatically. This phenomenon is best recognized for the dye acridine orange (AO), which can be used to differentiate DNA from RNA in fluorescence microscopy (1, 2, 8, 9, 20, 28, 29). Analogous color contrasts have been observed with the more familiar thiazine dyes of ordinary light microscope histology (10, 11, and references cited there) but have received less attention because they are less striking to the eye and more difficult to obtain consistently. Flax and Himes (11) showed that a consistent color contrast between DNA and RNA could be produced with the thiazine dye azure B by staining at high temperature (40°C), followed by prolonged differentiation. They stained sections at several dye concentrations, and showed that at each concentration the spectral shapes of stained DNA and RNA in the section were different. They suggested that "the greater metachromasy that occurs with RNA may be due to closer spacings of phosphate groups available for dye attachment in the RNA molecule," and discussed this suggestion in terms of the hypotheses current at that time regarding nucleic acid structure.

In recent years, the usefulness of the aggregation concept has been extended by detailed studies (5-8, 25, 26) of the interaction of metachromatic dyes with polyelectrolytes in dilute solution at low ionic strength. Under these conditions, binding of dye to polymer is stoichiometrically complete, and the concentration of dye, the concentration of polymer, and the molar extinction of the dye can all be measured with sufficient precision to allow physico-chemical analysis of the metachromatic color change. It has been shown that a group of polymers, on which a dye has the same metachro-

matic spectrum when all binding sites are filled with dye molecules ($P/D = 1$) and the same orthochromatic spectrum when binding sites are present in great excess of the number of dye molecules ($P/D \rightarrow \infty$), may induce quite different proportions of the two spectra at intermediate values of P/D . For example, at $P/D = 5$, AO has less than 50 per cent of the metachromatic spectral component when bound to DNA, about 70 per cent when bound to RNA, and nearly 100 per cent when bound to polyadenylic acid. These differences result from a non-random distribution of dyes among available sites, caused by an attractive interaction between those dye molecules which are bound to neighboring sites and therefore form a metachromatic aggregate. The decrease in free energy resulting from this interaction weights the distribution of dye in favor of such aggregates and is greater for some polymers (*e.g.* polyadenylic acid) than for others (*e.g.* DNA). These phenomena are collectively termed stacking in recognition of the shape of the aggregates thought to be formed by the planar, aromatic dye molecules. The changes of spectral shape and molar extinction with changing P/D are used to compute a number called the stacking coefficient, K , which is a measure of the strength of the attractive interaction, and bears direct thermodynamic relationship (5) to the loss of free energy on stack formation. $K = 1$ corresponds to zero free energy loss on stack formation and consequent totally random distribution of dyes among available sites. The greater the value of K , the more stacking is obtained at a given P/D , or, conversely, the higher P/D must be to obtain a given ratio of unstacked to stacked dye. Observed K values with AO range from 1.2 with DNA, on which 50 per cent of the dye is unstacked at $P/D = 3.9$, to several thousand for certain acid mucopolysaccharides on which $P/D = 40,000$ is required to obtain the same percentage of unstacking (26). Since the stacking coefficient has been found to be quite sensitive to known alterations of the secondary structure of polymers, quantitative analysis of dye stacking provides a tool for the investigation of polymer structure.

The companion paper (10) describes a great enhancement of the color contrast between DNA and RNA in tissues fixed in acrolein, embedded in polyester wax, and stained with a metachromatic thiazine dye, toluidine blue (TB). If the action of acrolein on pure nucleic acids can be defined and

¹ Abbreviations employed: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; AO, acridine orange; TB, toluidine blue; P/D , number of polymer dye-binding sites divided by number of bound dye molecules; F_1 , that fraction of bound-dye molecules which are unstacked; E_m , molar extinction coefficient; λ_{max} , wavelength of the peak in the absorption spectrum; K , stacking coefficient.

compared with the action of other fixatives, it might be possible to offer a quantitative interpretation of this enhancement of the color contrast. In order to do this, the stacking experiments previously performed with AO must be matched by a parallel series of experiments using TB. We have therefore compared the stacking of AO and TB by DNA and RNA, both in the native state and after treatment with formaldehyde, acrolein, or Carnoy's fluid, the three fixatives whose effects at the histological level are compared in the companion paper. The two dyes show comparable, though not identical, stacking behavior. The effects of the fixatives can all be expressed as changes in the stacking coefficient, K , resulting from alterations of nucleic acid secondary structure. The differences between the K 's of similarly treated DNA and RNA with either dye are largely consistent, in direction and magnitude, with the color contrasts obtained histologically, suggesting that K is an important variable influencing the color of bound metachromatic dye in tissue sections.

MATERIALS

Calf thymus DNA was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Yeast RNA, prepared by a modification of the procedure of Crestfield, Smith, and Allen (16) and reported to be of molecular weight 50,000 to 80,000, was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Garden City, New York.

AO was taken from a purified, well characterized sample (25). Toluidine blue O chloride, "purified," was obtained from the National Aniline Division, Allied Chemical Corp., New York, New York. The commercial dye moved as a single band in paper chromatograms in eight different solvent systems (18, 27). Several distinct bands could be resolved, however, on alumina or hydroxyapatite columns. Therefore, the commercial dye was further purified by dissolving it in ethanol and passing the solution through an acid-washed alumina (Merck and Co., Inc., Rahway, New Jersey) column preequilibrated with ethanol. The main fraction was concentrated in a rotary evaporator, and the dye was then precipitated by addition of diethyl ether, collected, and dried first in an oven at 100°C for 2 hours and then *in vacuo* at 100°C overnight. The resulting sample moved as a single band when rerun on a small alumina column or in a thin layer silica gel chromatogram using a chloroform-methanol (2:1 v/v) solvent system. The nitrogen to sulfur ratio determined analytically (Pascher Microanalytical Laboratory, Bonn, Germany) agreed with the theoretical value to within

0.7 per cent (calculated, 1.311; observed, 1.302). Since the remainder of the elementary analysis suggested the presence of tightly bound solvent of crystallization, we used the nitrogen and sulfur content rather than the gross weight to compute the molarity of TB solutions. TB and AO were both used in solutions approximately 2×10^{-5} M. Nucleic acid solutions employed ranged from 0.1 to 1.0 mg/ml. All solutions were buffered with M/1000 sodium cacodylate/HCl to pH 6.7.

Formaldehyde solution (formalin), obtained from Fisher Scientific Co., Fair Lawn, New Jersey, was used without further purification. Acrolein, from Eastman Organic Chemicals, Rochester, New York, was redistilled, stabilized by the addition of 0.2 per cent hydroquinone, and stored in the cold. Carnoy's fluid was prepared by mixing absolute ethanol, chloroform, and glacial acetic acid (6:3:1 v/v).

METHODS

The procedures for executing polymer-dye titrations and for analyzing the raw titration data have been described in full detail elsewhere (6-8, 25). In brief, as polymer solution is added to dilute dye solution, dyes bind to and stack on the polymer and the molar extinction coefficient (E_m) at the absorption maximum of isolated dye in solution (λ_{max} of free dye) decreases linearly, reaches a minimum at the stoichiometric equivalence point ($P/D = 1$), and thereafter changes much more gradually and in the opposite direction. The initial region of the titration curve thus consists of two linear limbs, whose intersection defines the equivalence point (*cf.* Figs. 2 and 3). P/D for all other points on the titration curve are calculated by reference to this point. With further additions of polymer, as dyes distribute themselves among the excess sites, increasing numbers of dyes bind to sites where they are isolated, and E_m rises at λ_{max} of bound isolates, that is, the absorption maximum of those dye molecules which are bound to isolated sites and therefore unstacked. For most dye-polymer pairs, this peak is at a slightly longer wavelength than λ_{max} of free dye (17) as a result of binding of dye to polymer. (This *bathochromic* shift of λ_{max} is sometimes called "negative metachromasy." To avoid confusion, this nomenclature is not employed in the present paper.) Ultimately, a region of polymer excess is reached where further addition of polymer no longer significantly increases E_m at λ_{max} of bound isolates because most of the bound dye is unstacked (fraction of bound isolates, or F_1 , $\rightarrow 1$). Using E_m at λ_{max} of bound isolates in the region of ultimate polymer excess ($F_1 \rightarrow 1$) and at the equivalence point ($F = 0$) as reference standards, F_1 is calculated from E_m at λ_{max} of bound isolates for intermediate values of P/D . The stacking coefficient, K , may then be calculated by fitting the resulting series of values

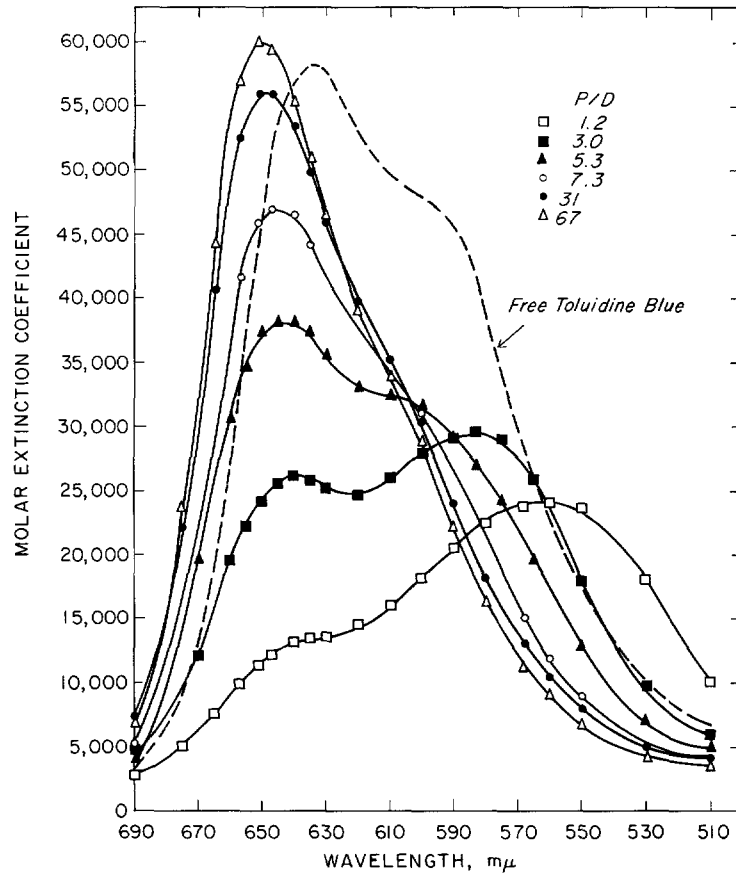


FIGURE 1 Spectra of free TB, 1.7×10^{-5} M, and of TB bound to native DNA at various P/D values. The nitrogen and sulfur content of samples of purified toluidine blue was used to determine E_m of the free dye at $635 \text{ m}\mu$ as a function of concentration. Thus, the number of moles of dye at the beginning of each titration was determined from the optical density at $635 \text{ m}\mu$, and was used to convert the spectrum of bound dye at any P/D from optical density to molar extinction.

of P/D and F_1 to any of several possible equations (5). The best equation is now considered to be:

$$P/D = (1 - F_1^{1/2})^{-1} + (K - 1) (1 - F_1^{1/2})^{-1} F_1 \quad 1$$

This is the exact solution of the statistical problem which was solved approximately (7) to give the originally published equation:

$$P/D = (1 - F_1^{1/2})^{-1} + (K - 1) (1 - F_1^{1/2}) (F_1 + F_1^{3/2} - F_1^{1/2}) \quad 2$$

In the present study, the mean and standard deviation of K over the range $F_1 = 0.2$ to $F_1 = 0.8$ were computed with both Equation 1 and Equation 2 for each titration. K values with the exact equation were about 30 per cent higher than with the approximate one. Goodness of fit, as estimated by the standard deviation, was comparable for the two equations.

For titrations in the presence of aldehyde, sufficient acrolein or formaldehyde was added to all solutions employed to produce a final concentration of 1 per cent aldehyde. One aliquot of nucleic acid solution containing aldehyde was immediately titrated against dye, while a second aliquot was allowed to stand for about 3 hours at room temperature and was then dialyzed against three successive 1-liter volumes of buffer for a total of 24 hours. After dialysis, no free fixative could be detected either spectrophotometrically or by odor. The dialyzed nucleic acid was then titrated against dye in aldehyde-free solutions. Thus, for both DNA and RNA, K 's were determined, with both AO and TB, for native polymer, polymer in the presence of aldehyde, and polymer after removal of aldehyde by dialysis. To expose nucleic acids to Carnoy's fluid, they were dissolved in buffer (about 4 mg/ml), and one volume of this solution added to 4 volumes of Carnoy's. When this was done, the

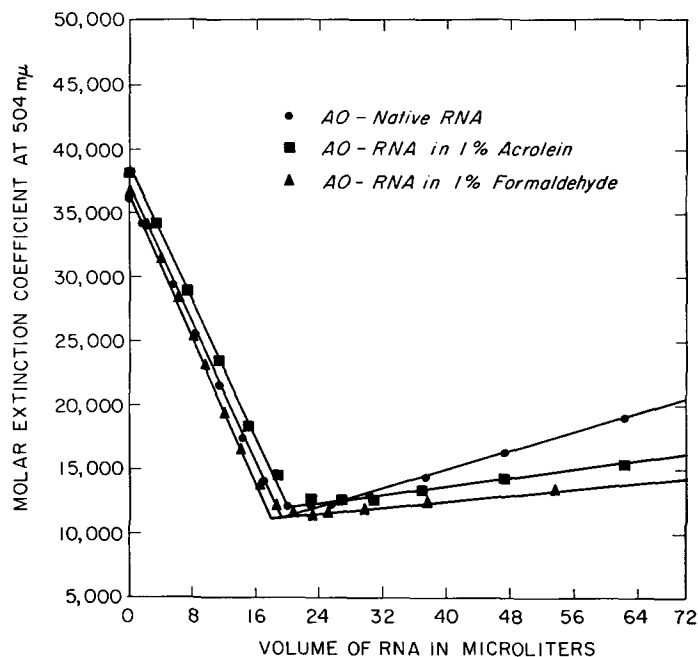


FIGURE 2 This is a plot of the initial region of the titration curve for three of the AO-RNA titrations. Ordinate, E_m of AO at 504 $m\mu$; abscissa, volume of RNA solution added to the cuvette containing a fixed amount of dye. The E_m at 504 $m\mu$ reaches a sharply defined minimum at the equivalence point, when the number of dye molecules equals the number of polymer binding sites ($P/D = 1$). From this equivalence point, P/D is calculated for all other points on the titration curve. Note that the equivalence point is not significantly altered in the presence of 1 per cent acrolein or formaldehyde, which indicates that these aldehydes do not block the dye-binding sites.

nucleic acids precipitated, as indicated by turbidity of the mixture, but buffer and Carnoy's were miscible in these proportions, so that the mixture formed a single fluid phase. This mixture was allowed to stand for 1 hour, and then excess buffer was added, breaking the solution into water- and chloroform-rich phases. The chloroform phase was removed, and the aqueous phase, containing the nucleic acid, was dialyzed against five successive 1-liter volumes of buffer, during which process the nucleic acids returned to solution. The resulting solutions were concentrated by ultrafiltration and titrated against dye. For titrations of heat-denatured DNA, a 1 mg/ml solution of DNA was kept at 100°C for 15 minutes, cooled to room temperature under running tap water, then titrated against dye.

In some titrations, precipitation occurred when polymer solution was added to dye solution. Such precipitation could often be prevented by using a more dilute polymer solution until the equivalence point had been reached. The titration could then be completed with more concentrated solution, since precipitation did not occur when polymer was present in excess. In the RNA/TB titrations, some precipitation occurred near the equivalence point even with

the lowest polymer concentrations employed. Such precipitates always redissolved when polymer was added in excess. The resulting titration curves had linear limbs, on either side of the zone of precipitation, sufficiently long to permit accurate interpolation of the equivalence point. We therefore believe that precipitation near the equivalence point did not materially affect the calculation of K values.

All spectra were measured in a Cary Model 14 spectrophotometer at room temperature (22°C).

RESULTS

DYE SPECTRA: Fig. 1 shows the spectrum of TB, free and bound to native DNA at varying P/D . Compared to the free dye, at $P/D = 1.2$ the spectrum is strongly hypochromic (at the λ_{max} , E_m 58,000 \rightarrow E_m 24,000) and the wavelength of λ_{max} much shorter (635 $m\mu$ \rightarrow 560 $m\mu$). As P/D increases, E_m increases at λ_{max} of bound isolates, which is at a slightly longer wavelength than λ_{max} of free dye (635 $m\mu$ \rightarrow 651 $m\mu$). At very high P/D the rate of increase of E_m with P/D declines: raising P/D from 31 to 67 raises E_m only from

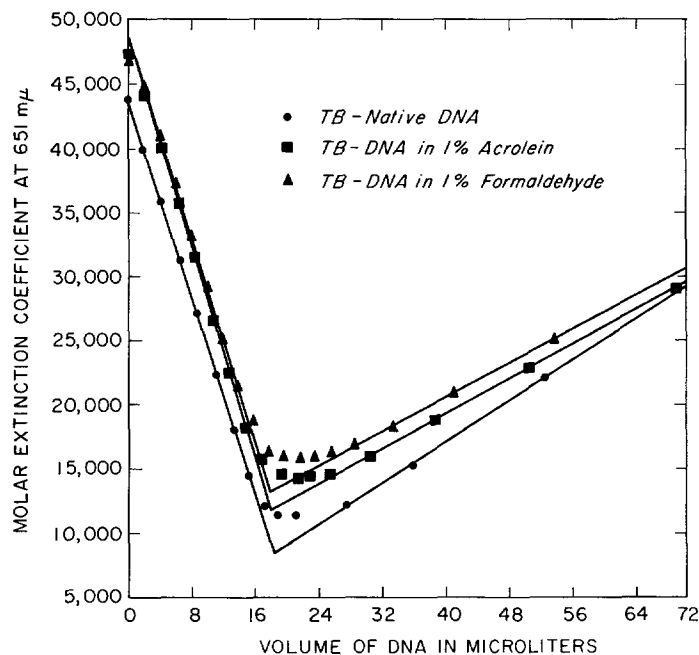


FIGURE 3 This is a plot, similar to Fig. 2, of three of the TB-DNA titrations. The ordinate in this plot is the E_m of TB at 651 $m\mu$. The TB-DNA equivalence point, like that of AO-RNA, is not significantly altered by the presence of 1 per cent acrolein or formaldehyde.

56,000 to 59,000. The corresponding visible colors can best be described by naming the color of the free dye standard blue, the color at $P/D = 1.2$ standard purple; then as P/D was raised, the purple was rapidly replaced by increasingly pure standard blue so that the color at $P/D = 3.0$ was blue with only a slight tinge of purple which disappeared as P/D entered the range of 5 to 10. At $P/D = 31$ the color was just perceptibly more greenish than standard blue; this greenish hue became more distinct at much higher P/D . Except for minor differences of spectral shape at $P/D \sim 1$, which produced no difference in visible color (for any RNA, λ_{max} of bound stacks = 580 $m\mu$), a virtually identical series of spectra was obtained with DNA or RNA, native, heat-denatured or fixative-treated, with or without formaldehyde or acrolein present in solution. Differences between the polymers, or the effects of fixative, were shown only by the value of P/D necessary to produce a given spectrum.

The corresponding AO spectra, which have been previously published (6, 7, 8, 25), were confirmed in the present study. For AO, λ_{max} of free dye = 492 $m\mu$, λ_{max} of bound stacks = 464 $m\mu$, and λ_{max} of bound isolates = 504 $m\mu$. The AO

spectra, like the TB spectra, were virtually identical for DNA and RNA and were unaffected by treatment with, or presence of fixative, except that the value of P/D which produced a given spectrum was characteristically different for each system.

EQUIVALENCE POINTS: Figs. 2 and 3 are examples of the initial regions of titration curves, for the systems AO-RNA and TB-DNA, respectively, to illustrate the way in which the two linear limbs of the curve define an equivalence point and permit the calculation of P/D for all points. Figs. 2 and 3 also show that the equivalence point, and therefore the number of dye-binding sites on the polymer, is unaltered by the addition of formaldehyde or acrolein. The number of sites was found unaltered, by fixation or denaturation, in all titrations for which the equivalence point was compared with that of the native polymer.

STACKING CURVES AND K VALUES: Fig. 4 is a plot of F_1 as a function of P/D to illustrate the shapes of stacking curves and the quality of fit of titration data (symbols) to the family of curves generated by Equation 1 for various values of K (solid lines). The slope of the experimental stacking curves is in general slightly greater at the in-

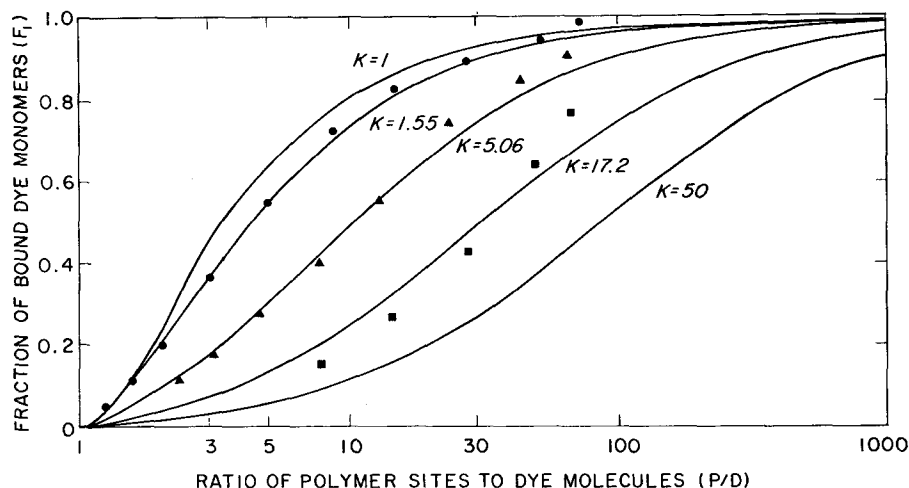


FIGURE 4 Semilogarithmic plot of the fraction of bound dye monomers (F_1) as a function of P/D . The points are taken from representative AO titrations. Circles, DNA after acrolein treatment and dialysis; triangles, native RNA; squares, RNA in the presence of formaldehyde. The solid lines are plots of Equation 1 for five values of K . $K = 1$ is the theoretical case of zero attractive interaction between adjacent dye molecules, resulting in a completely random distribution of dyes among available sites. $K = 1.55$, 5.06, and 17.2 are the mean K 's determined from the three AO titrations plotted. $K = 50$, a higher value than any encountered in the present study, is included to illustrate the progressive change in shape of the stacking curve with increasing K .

flection point than the slope of Equation 1 (faster unstacking as P/D increases). Except as noted below, the quality of fit was much the same for all titrations with both dyes, the standard deviation being larger for larger values of K . In Table I, the mean and standard deviation of K are listed for all titrations, as computed from both Equation 1 and Equation 2. To facilitate comparison and discussion, the mean K values obtained from Equation 1 are also summarized in pictorial form in Fig. 5. Attention is directed to the following points:

1. The K of our sample of native DNA with AO is near unity (1.46) and close to previously published values (25) for other native two-stranded DNAs with AO. The K of our sample of native RNA with AO is higher (5.06) than that of DNA, and slightly higher than previously published K 's for other native RNAs (7). Unpublished results (Bradley and Wolf, in preparation) indicate that native RNAs from various sources differ more from one another in K than do various DNAs.
2. The K of DNA with AO is virtually unaffected by acrolein or formaldehyde, while that of RNA is strikingly elevated by either aldehyde, returning, after 24 hours' dialysis, about halfway toward the original

value. To test the possibility that dialysis might act by removal of some component of the mixture other than fixative, a sample of unfixed RNA was dialyzed against buffer for 24 hours and then titrated: its K was unchanged.

3. A similar set of K values, demonstrating the same effect of aldehyde fixatives, is obtained when TB is used instead of AO. The K 's of native nucleic acids determined with TB are somewhat higher than with AO (DNA = 2.36, RNA = 6.00) while the K 's of fixative-altered RNA are somewhat lower: 9.72 in the presence of acrolein (13.0 with AO) and 13.4 in the presence of formaldehyde (17.2 with AO). Since the K of DNA with either dye is unaffected by aldehyde fixative, the final disparity between the K 's of aldehyde-treated DNA and RNA is greater with AO than with TB.
4. The K values for Carnoy's-treated nucleic acids differ greatly with AO (DNA = 4.68, RNA = 10.0) but are much closer with TB (DNA = 8.05, RNA = 10.7).
5. Heat denaturation raises the K of DNA with AO and TB to about the same extent (1.4 \rightarrow 5.6; 2.4 \rightarrow 6.0).

TABLE I
K Values Obtained from all Dye-Nucleic Acid Titrations

Dye	Nucleic acid	Stacking coefficient (<i>K</i>)				
		Native	In 1 per cent aldehyde	Exposed to 1 per cent aldehyde, then dialyzed	Exposed to Carnoy's fluid, then dialyzed	Heated
AO	DNA	1.46 ± 0.26 (1.35 ± 0.17)	1.45 ± 0.17 [A] (1.39 ± 0.12)	— —		
		1.26 ± 0.19 (1.21 ± 0.14)	1.64 ± 0.30 [A] (1.49 ± 0.17)	1.55 ± 0.15 [A] (1.44 ± 0.04)		
		— —	1.51 ± 0.22 [F] (1.41 ± 0.13)	1.80 ± 0.21 [F] (1.65 ± 0.07)		
					5.57 ± 2.0 (5.22 ± 2.1)	
				4.68 ± 0.56 (3.94 ± 0.12)		
	RNA	5.06 ± 0.70 (4.42 ± 0.43)	14.0 ± 3.3 [A] (11.7 ± 1.7)	9.40 ± 1.79 [A] (8.06 ± 1.00)		
		— —	12.1 ± 2.8 [A] (9.95 ± 1.40)	— —		
		— —	17.2 ± 5.3 [F] (14.5 ± 3.0)	8.19 ± 1.56 [F] (6.62 ± 0.75)		
					10.0 ± 2.33 (8.19 ± 1.01)	

DISCUSSION

SIGNIFICANCE AND LIMITATIONS OF *K*: The stacking coefficient, as stated in the Introduction, is quite sensitive to certain known alterations of polymer secondary structure; however, it is independent of many other features of the polymer. For example, all native two-stranded DNAs have closely similar *K* values with AO, regardless of nucleotide sequence or molecular weight. Heat denaturation, which disrupts hydrogen bonds between base pairs and thus produces a helix → random coil transition, but which neither forms nor breaks any covalent bonds, raises the *K* of all DNAs to about the same extent without altering the amount of dye bound. From this sort of evi-

dence, the idea has been developed that *K* reflects the geometry of the run of dye-binding sites on the polymer surface. For any dye, there is an optimum intersite distance which will allow maximal attractive interaction between dyes bound to adjacent sites. If the intersite distance is not optimal, or if other surface features of the polymer molecule produce steric hindrance, the attractive interaction, and therefore the *K*, will be reduced. In the family of polynucleotides, the attractive interaction in general increases with increasing randomness of molecular conformation. DNA has the most perfect rigid helical conformation, and the lowest *K*; randomization of the DNA helix raises the *K*; polynucleotides with mixtures of ordered and random conformation have higher *K*'s; cer-

TABLE I—Continued

Dye	Nucleic acid	Stacking coefficient (K)				
		Native	In 1 per cent aldehyde	Exposed to 1 per cent aldehyde, then dialyzed	Exposed to Carnoy's fluid, then dialyzed	Heated
TB	DNA	2.36 ± 1.29 (1.85 ± 0.60)	2.50 ± 1.04 [A] (2.07 ± 0.63)	2.35 ± 0.52 [A] (2.16 ± 0.33)		
		2.36 ± 0.91 (1.97 ± 0.48)	2.24 ± 0.75 [A] (2.01 ± 0.50)	2.78 ± 0.83 [A] (2.37 ± 0.46)		
		—	2.80 ± 1.19 [F] (2.31 ± 0.65)	4.00 ± 0.91 [F] (3.22 ± 0.41)		
		—	2.95 ± 0.79 [F] (2.62 ± 0.49)	2.90 ± 0.61 [F] (2.53 ± 0.27)		
						5.99 ± 0.24 (5.48 ± 0.57)
					8.05 ± 0.99 (6.91 ± 0.25)	
		RNA	6.00 ± 1.17 (5.18 ± 0.57)	9.72 ± 2.33 [A] (7.92 ± 1.16)	7.33 ± 1.92 [A] (6.04 ± 0.94)	
			—	13.4 ± 2.9 [F] (11.4 ± 1.3)	7.83 ± 2.69 [F] (6.66 ± 1.36)	
					10.7 ± 3.02 (8.96 ± 1.53)	

This table presents the mean and standard deviation of K calculated for each titration. In each group the upper value is that calculated with Equation 1; the lower value, in parenthesis, is that calculated with Equation 2. All titrations in a given horizontal row were performed using the same stock solution of nucleic acid. [A] designates a titration in the presence of or after treatment with acrolein; [F] designates a titration in the presence of or after treatment with formaldehyde.

tain synthetic polynucleotides believed to have totally random conformation have the highest K 's. It appears that the surface of the rigid helix is in some way unfavorable for stacking, perhaps because the intersite distance is greater than optimum or perhaps for other reasons. As randomness of structure increases, the binding sites are increasingly free to approach more advantageous positions, and if dye molecules are bound to them, they will be stabilized in these improved positions by the loss of free energy on stack formation.

The algebraic expressions for K , such as Equation 1, are necessarily derived from an idealized physical model of the dye-polymer interaction.

For example, in the derivation of Equation 1, the polymer is assumed to be of infinite length, so that end-effects may be disregarded; the run of binding sites is assumed to have completely uniform conformation; all stacking interactions between any two nearest neighbor dye molecules are assumed to be exactly equivalent, regardless of the length of the stack and regardless of their position within or at the end of the stack; and the conformation of the polymer is assumed to be unaffected by the binding and stacking of dye on its surface. Since these specifications will be met only approximately by any real polymer-dye system, it is not surprising that experimental stacking curves show

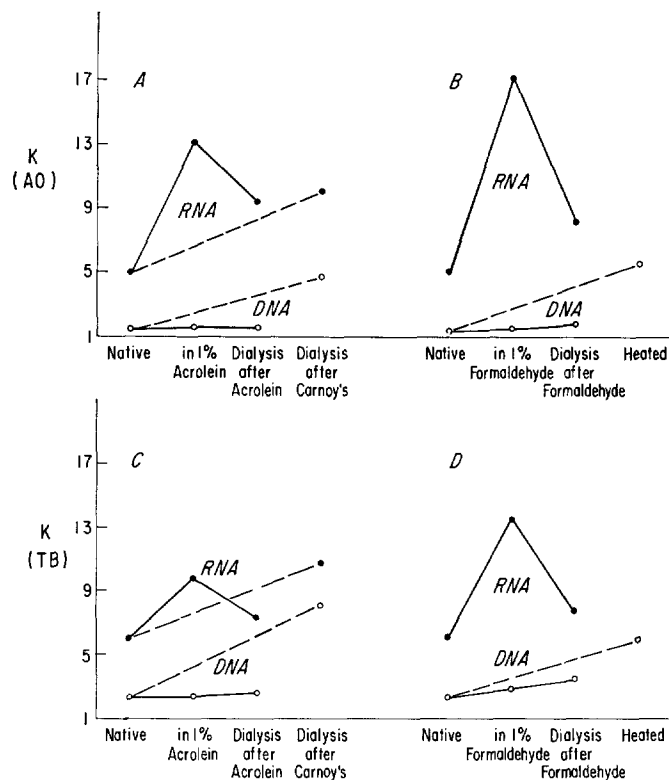


FIGURE 5 The mean K values (Equation 1) listed in Table I are here represented in quasi-graphic form. Upper brace, AO titrations; lower brace, TB titrations. Ordinate of each brace, value of K ; abscissa, condition of nucleic acid; filled circles, RNA; open circles, DNA. For ease of visual reference, the points for native DNA and RNA are repeated from panel A to panel B, and from panel C to panel D, and all the RNA points and DNA points in each panel are connected by lines.

some imperfection of fit to equation 1 (*cf.* Fig. 4), which is reflected in minor fluctuations of the value of K as P/D and F_1 change. A given stacking curve may represent the behavior of a mixture of different polymer molecules or of regions of varying conformation within the same polymer molecule, all with different stacking tendencies. Nevertheless, the mean value of K is still a powerful tool for the characterization of such a mixture, and may provide clues regarding the identity and behavior of its components (6, 25).

EFFECT OF FIXATIVES ON NUCLEIC ACIDS: The great elevation of the K of RNA by formaldehyde (with either dye), while the K of DNA remains unaffected, is consistent with previous studies of the reaction of formaldehyde with polynucleotides (4, 12-15, 23), which suggest that formaldehyde binds to available NH_2 - groups on the nitrogenous bases, and thus disrupts hydrogen bonds between base pairs and produces a helix \rightarrow

random coil structural transition. The perfectly paired helical structure of native two-stranded DNA tends to protect the amino groups involved in hydrogen bonds from reacting with formaldehyde in low concentration, but imperfections in the helix provide points at which the reaction is readily initiated. Thus, RNA, which has a more random structure, and a higher K , than DNA, undergoes further randomization of structure, with further elevation of K , when treated with formaldehyde. Semmel and Huppert (21, 22) have already described an increase in the metachromasy of RNA following formaldehyde treatment. The absence of alteration of the stoichiometric equivalence point when the bases of RNA are saturated with formaldehyde provides further evidence for the previous contention (8) that the dyes are bound to the anionic phosphate groups rather than to the nitrogenous bases, as suggested elsewhere (24). The reaction of formaldehyde with

RNA and denatured DNA has been reported to be reversible; that of formaldehyde with RNA was at least partially reversed by dialysis in this study. The difference in susceptibility to formaldehyde between DNA and RNA must be considered relative. Native DNA is attacked by formaldehyde if concentration and exposure time are sufficient, and experiments now in progress suggest that RNAs from various sources differ in their susceptibility to formaldehyde (Furano and Bradley, in preparation).

The close parallel between the behavior of acrolein and that of formaldehyde, namely: elevation of the K of RNA, reversal of this effect by dialysis, and no effect on DNA, suggests that acrolein may react, as formaldehyde does, with exposed amino groups of nucleotide bases, disrupting hydrogen bonds and producing a helix \rightarrow coil transition. Acrolein may perhaps be somewhat less reactive than formaldehyde, as suggested by a smaller elevation of the K of RNA.

In contrast with the two aldehyde fixatives, treatment with Carnoy's fluid raises the K of both DNA and RNA, from which it is inferred that this strongly acid mixture denatures native DNA. Heat denaturation also raises the K of native DNA with either dye. Comparison of the TB and AO titrations of Carnoy's-denatured and heat-denatured DNA, however, reveals an interesting and probably significant discrepancy between the behavior of the two dyes. With AO, heat-denatured DNA has a somewhat higher K , with poorer fit to Equation 1, than Carnoy's-denatured DNA. With TB, heat-denatured DNA has a somewhat lower K than Carnoy's-denatured DNA, with much better fit to Equation 1; in fact, heat-denatured DNA gives a better fit to Equation 1 with TB than native DNA in spite of having a K three times as high. This suggests that the alterations of the DNA structure produced by heat and by Carnoy's fixative are different not only in degree, but in kind, and illustrates how additional information may be obtained from stacking studies by the use of more than one dye.

CONTRIBUTION OF K TO METACHROMATIC COLOR CONTRASTS: The experiments described above were carried out with pure nucleic acid solutions under conditions permitting complete binding of dye when $P/D \geq 1$. The color of such nucleic acid-dye systems was shown to be determined by two variables, P/D and K . In tissue sections, nucleic acids are fixed in the

solid phase together with other polymers, notably proteins, which might produce chemical blockage or steric hindrance of dye-binding sites, and are exposed to an excess of dye in a bath which may contain other cations competing for binding sites. Some of the bound dye may then be removed during differentiation or dehydration. These maneuvers will influence the same two variables, in the tissue section, which determine the dye spectrum in solution: the amount of dye bound to the tissue components (P/D) and the molecular architecture of the tissue components (K). P/D in solution can be manipulated precisely and at will, but P/D in stained tissue components cannot be directly measured at present. However, by comparing the results of various staining procedures with the results of the titrations, it may be possible to infer the relative contribution of the two variables to the metachromatic color contrast in tissues.

Both DNA and RNA run the complete gamut of color from metachromasy to orthochromasy as P/D is progressively increased, either by direct manipulation, in the cuvette, or by progressive alteration of staining conditions, in the section. One might therefore suppose that a color contrast between the two nucleic acids is produced simply by local variations in P/D , more dye being bound to RNA in the section than to DNA. Fixation, dehydration, embedding, and staining procedures which promote a color contrast would be considered to act by promoting point-to-point variations in the amount of dye bound within the same section. Certain experiments (Bradley and Wolf, in preparation), showing that the binding of dye by DNA is more sensitive to ionic strength than the binding of dye by RNA, suggest one possible mechanism for the production of such point-to-point variations in P/D . This result, however, must be interpreted with great caution, since other experiments suggest that, when DNA and RNA are mixed in the same cuvette, DNA tends to preempt bound dye from RNA. Furthermore, if P/D is assumed to be the only effective variable, then extremely large differences in P/D must be postulated to explain the observed color contrasts. For example, Fig. 1 shows that for DNA-TB the value of P/D must be increased from about 1 to over 30 to go from the pure metachromatic spectrum to the pure orthochromatic spectrum. If a tissue section stained with TB shows the maximum obtainable color con-

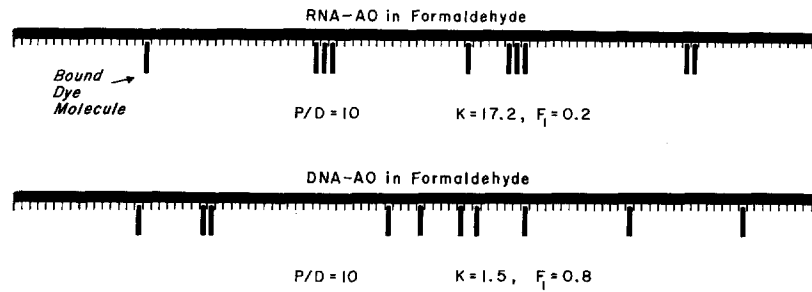


FIGURE 6 This illustrates the possible distribution of 10 dye molecules among a run of 100 binding sites for two different values of K . Upper diagram, RNA-AO in the presence of formaldehyde; lower one, DNA-AO in the presence of formaldehyde. The positions shown for individual dyes are of course schematic, but the values of P/D and F_1 represent actual points, slightly rounded off, taken from the two respective titrations.

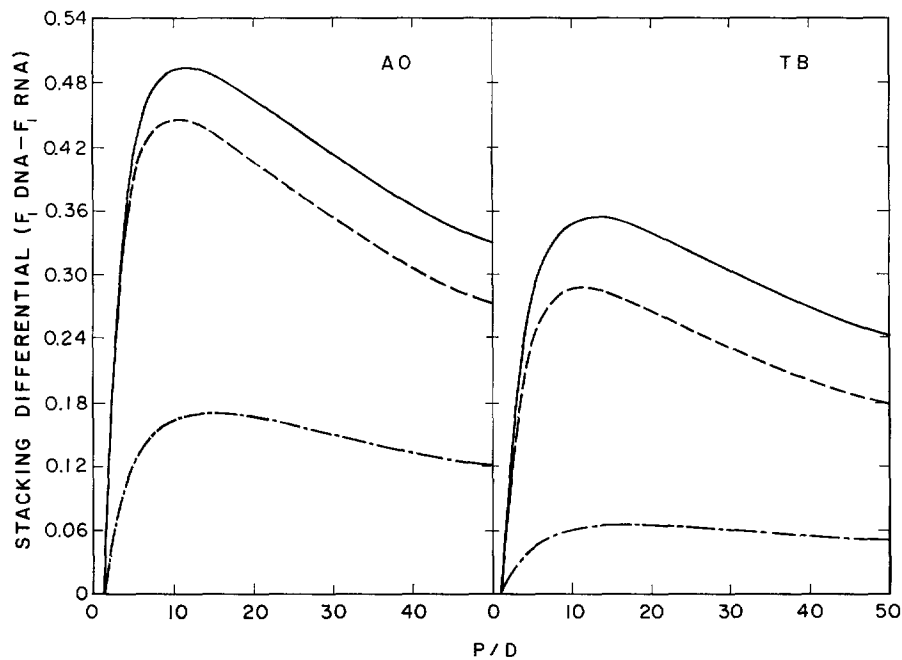


FIGURE 7 This diagram represents the difference between the F_1 of dye bound to DNA and the F_1 of dye bound to RNA, taken as one measure of the stacking differential between the two polymers after treatment with fixative. Each line is the arithmetic difference between the stacking curve (such as shown in Fig. 4) of a fixative-treated DNA and the corresponding stacking curve of the similarly treated RNA. —, in the presence of formaldehyde; ---, in the presence of acrolein; - · - · - ·, after treatment with Carnoy's fluid followed by dialysis.

trast, and this contrast is to be explained by P/D alone, it must then be postulated that less than 1/30 as much dye is bound to DNA as to RNA. This is not only inherently improbable, but carries the further implication that as the color contrast is increased, the staining of DNA ought always to become much weaker, as well as more ortho-

chromatic; an expectation which is not consistently borne out.

The stacking coefficient, however, is a second effective variable influencing the amount of metachromasy. For any P/D greater than 1, the higher K is, the greater the amount of stacking of bound dye will be. If the K values of two poly-

mers are sufficiently far apart, there will be some value or range of values for P/D at which the difference in the stacking of dye bound to the two polymers is perceptible to the eye as a color contrast. For example, Fig. 6 is a diagram of the stacking of AO on DNA and RNA at $P/D = 10$ in the presence of formaldehyde. AO is 8/10 stacked on RNA, but only 2/10 stacked on DNA. This stacking differential between DNA and RNA is sufficiently large to produce a visible color contrast between them at the same P/D . Smaller differences in K , which are not sufficient to produce a color contrast at the same P/D for DNA and RNA, may nevertheless make it possible to obtain a color contrast with less difference in P/D than would be required if K were not an effective contributing variable. The magnitude of the stacking differential between DNA and RNA might therefore influence the magnitude of the color contrast and the ease and reproducibility with which it can be obtained.

In Fig. 7, the stacking data taken from Fig. 5 and Table I have been used to compute the stacking differential between DNA and RNA with each of the dyes and each of the fixatives in the present study. Each line in Fig. 7 is the arithmetic difference between the stacking curve of a fixative-treated DNA and that of the correspondingly treated RNA. These curves represent the potential contribution of differences in K to the metachromatic color contrast between DNA and RNA. It will first be noted that the stacking differential is in each instance in the correct direction (RNA more metachromatic) to contribute to the color contrast. The stacking differentials are larger with AO than with TB; AO produces a color contrast under a wider range of conditions than TB. For TB, there is a considerable stacking differential with aldehyde fixatives, but hardly any with Carnoy's fluid; with this dye, color contrasts have been observed by Feder and Wolf (10) in aldehyde-fixed but not in Carnoy's-fixed tissues. AO, however, gives a larger stacking differential with Carnoy's fluid, and can be used to produce color contrasts in Carnoy's-fixed tissue (20). The stacking differential is slightly larger with formaldehyde than with acrolein; however, Feder and Wolf (10) have found acrolein superior to formaldehyde in producing a color contrast. Acrolein-fixed, polyester wax-embedded tissues stained with TB show a larger color contrast than can be explained by the stacking differential shown in Fig. 7 for TB

and acrolein. This superiority of acrolein must therefore be attributed to properties other than its action on pure nucleic acids. Recent experiments (Furano and Bradley, in preparation) show that RNA in the ribosome has a configuration radically different from its configuration free in solution. It is possible that acrolein provides superior preservation of this or other nucleoprotein configurations which differ from the configurations of free nucleic acids. This matter is discussed in detail in the companion paper (10).

The curves in Fig. 7 must not be taken as direct statements concerning the amount of visible color contrast. The quantitative treatment of spectral data which gives rise to the stacking curves and stacking coefficients is based on only one component of the dye-polymer system: the fraction of dye molecules which are bound but unstacked, or monomeric. However, the absence of an isobestic point from the nests of AO and TB spectra (see Fig. 1) proves that the system must contain at least three or more components. In this case, the components presumably are bound-dye monomers, dimers, and longer stacks, all contributing to the total absorption spectrum. In order to make direct predictions of visible color in a tissue section, it would be necessary to know the fractions of each of these components present, and weight this information with a series of factors, such as the total optical density, the amount of non-specific absorption, the retinal sensitivity of the individual observer, the emission spectrum of the microscope lamp, etc., which influence the way in which the eye of the histologist will perceive color but which are irrelevant for a physico-chemical analysis. Microspectrophotometry offers a more exact and powerful way to determine the spectral shape, and hence the state of stacking of bound dye, in stained tissue components. If methods could be found for independent measurement of P/D in the tissue components, microspectrophotometric observations on metachromatically stained tissue sections could then be used for direct investigation of the molecular architecture of polyelectrolytic tissue components *in situ*.

The authors thank Dr. D. F. Bradley and Dr. Ned Feder for stimulating and critical discussions. Dr. Merrill K. Wolf was supported by Special Fellowship #2F11 NB 978-02 and Grant #B-1938 from the National Institute of Neurological Diseases and Blindness, Bethesda, Maryland.

Received for publication, June 8, 1965.

REFERENCES

1. ARMSTRONG, J. A., Histochemical differentiation of nucleic acids by means of induced fluorescence, *Exp. Cell Research*, 1956, **11**, 640.
2. ARMSTRONG, J. A., and NIVEN, J. S. F., Fluorescence microscopy in the study of nucleic acids—Histochemical observations on cellular and virus nucleic acids, *Nature*, 1957, **170**, 1335.
3. BERGERON, J. A., and SINGER, M., Metachromasy: an experimental and theoretical re-evaluation, *J. Biophysic. and Biochem. Cytol.* 1958, **4**, 433.
4. BERNIS, K. I., and THOMAS, C. A., JR., A study of single polynucleotide chains derived from T2 and T4 bacteriophage, *J. Mol. Biol.*, 1961, **3**, 289.
5. BRADLEY, D. F., Molecular biophysics of dye-polymer complexes, *Tr. New York Acad. Sc.*, Series 2, 1961, **24**, 64.
6. BRADLEY, D. F., and FELSENFELD, G., Aggregation of an acridine dye on native and denatured deoxyribonucleates, *Nature*, 1959, **185**, 1920.
7. BRADLEY, D. F., and WOLF, M. K., Aggregation of dyes bound to polyanions, *Proc. Nat. Acad. Sc.*, 1959, **45**, 944.
8. BRADLEY, D. F., and WOLF, M. K., The neurochemistry of polynucleotides, in *The Neurochemistry of Nucleotides and Amino Acids*, (R. O. Brady and D. B. Tower, editors), John Wiley and Sons, Inc., New York, 1960.
9. DART, L. H., and TURNER, T. R., Fluorescence microscopy in exfoliative cytology: Report of acridine orange examination of 5491 cases, with comparison by the papanicolaou technique, *Lab. Inv.*, 1959, **8**, 1513.
10. FEDER, N., and WOLF, M. K., Studies on nucleic acid metachromasy. II. Metachromatic and orthochromatic staining by toluidine blue of nucleic acids in tissue sections, *J. Cell Biol.*, 1965, **27**, 327.
11. FLAX, M. H., and HIMES, M. H., Microspectrophotometric analysis of metachromatic staining of nucleic acids, *Physiol. Zool.*, 1952, **25**, 297.
12. FRAENKEL-CONRAT, H., Reaction of nucleic acid with formaldehyde, *Biochim. et Biophysica Acta*, 1954, **15**, 307.
13. FREIDFELDER, D., and DAVISON, P. F., Hyperchromicity and strand separation in bacterial DNA, *Biophysic. J.*, 1962, **2**, 249.
14. GROSSMAN, L., LEVINE, S. S., and ALLISON, W. S., The reaction of formaldehyde with nucleotides and T2 bacteriophage DNA, *J. Mol. Biol.*, 1961, **3**, 47.
15. HASELKORN, R., and DOTY, P., The reaction of formaldehyde with polynucleotides, *J. Biol. Chem.*, 1961, **236**, 2738.
16. KAWADE, Y., Physicochemical study of ribonucleic acid, *Ann. Rep. Inst. Virus Research*, Kyoto Univ., series B, 1959, **2**, 219.
17. MORTHLAND, F. W., DEBRUYN, P. P. H., and SMITH, N. H., Spectrophotometric studies on the interaction of nucleic acids with amino acridines and other basic dyes, *Exp. Cell Research*, 1954, **7**, 201.
18. PERSIJN, J. P., Improved solvents for paper chromatography of thiazine stains, *Stain Technol.*, 1961, **36**, 27.
19. RABINOWITCH, E., and EPSTEIN, L., Polymerization of dyestuffs in solution. Thionine and methylene blue, *J. Am. Chem. Soc.*, 1941, **63**, 69.
20. SCHÜMMELFEDER, N., KROGH, R. E., and EBSCHNER, K. J., Farbungsanalysen zur Akridinorange-fluorochromierung, 1958, *Histochemie*, **1**, 1.
21. SEMMEL, M., and HUPPERT, J., Le rôle des liaisons H dans l'interaction du RNA et des colorants basiques *in vitro*, *Compt. rend. Acad. Sc.*, 1963, **256**, 5649.
22. SEMMEL, M., and HUPPERT, J., The interaction of basic dyes with ribonucleic acid, *Arch. Biochem. and Biophysic.*, 1964, **108**, 158.
23. STAHELIN, M., Reaction of tobacco mosaic virus nucleic acid with formaldehyde, *Biochim. et Biophysica Acta*, 1958, **29**, 410.
24. STEINER, R. F., and BEERS, R. F., Polynucleotides. V. Titration and spectrophotometric studies upon the interaction of synthetic polynucleotides with various dyes, *Arch. Biochem. and Biophysic.*, 1959, **81**, 75.
25. STONE, A. L., and BRADLEY, D. F., Aggregation of acridine orange bound to polyanions: the stacking tendency of deoxyribonucleic acids, *J. Am. Chem. Soc.*, 1961, **83**, 3627.
26. STONE, A. L., CHILDERS, L. D., and BRADLEY, D. F., Investigation of structural aspects and classification of plant sulfated polysaccharides on the basis of the optical properties of their complexes with metachromatic dyes, *Biopolymers*, 1963, **1**, 111.
27. TAYLOR, K. B., Chromatographic separation and isolation of metachromatic thiazine dyes, *J. Histochem. and Cytochem.*, 1960, **8**, 248.
28. VON BERTALANFFY, L., MASIN, M., and MASIN, F., A new and rapid method for diagnosis of vaginal and cervical cancer by fluorescence microscopy, *Cancer*, 1958, **11**, 873.
29. WOLF, M. K., and ARONSON, S. B., Growth, fluorescence and metachromasy of cells cultured in the presence of acridine orange, *J. Histochem. and Cytochem.*, 1961, **9**, 22.
30. ZANKER, V., Über den Nachweis definierter reversibler Assoziate ("reversible Polymerisate") des Acridinorange durch Absorptions- und Fluoreszenzmessungen in wasriger Lösung, *Z. physikal. Chem.*, 1952, **199**, 225.