

THERMAL DEPOLARIZATION OF FLUORESCENCE FROM POLYTENE CHROMOSOMES STAINED WITH ACRIDINE ORANGE

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ABSTRACT

The degree of polarization of fluorescence from stretched *Chironomus thummi* polytene chromosomes, stained with low concentrations of acridine orange (AO), decreases with increasing temperature. The "half temperature" of this decrease ($T_{1/2R}$) is lower than the expected DNA thermal denaturation temperature (T_m) by about 20°C. $T_{1/2R}$ is lowered as histone is removed from chromosomes. Balbiani ring regions of the fourth chromosome have $T_{1/2R}$'s much lower than other regions, and nearly as low as chromosomes which had been extensively pretreated with trypsin to remove histone and other proteins. Measurements of the thermal change in the rotational diffusion rate of AO in solution with DNA indicate that the temperature at which the DNA-AO bonding changes from a "rigid" to a "loose" mode varies with the GC percentage of the DNA, and in the same fashion as T_m , although 20°C lower.

INTRODUCTION

In vitro experiments on the thermal denaturation of DNA have shown that T_m (the temperature of half-denaturation) is dependent upon a number of variables. Among these are the pH and ionic strength of the buffer (1) and added substances such as ribonuclease (2), actinomycin D (3), certain metal ions (1) and various organic solvents (1). In addition, T_m is affected by per cent GC content (4) and the presence of nuclear histones (5). Under conditions in which a standard buffer is employed, T_m can be used as a fairly sensitive measure of either GC content or histone association. It would, consequently, be of value to have a reliable method for detecting DNA denaturation in situ, that is, in microscopic preparations, rather than after the extensive DNA extraction procedures that are necessary for in vitro work.

Of the standard methods for detecting DNA denaturation, most (e.g. viscosity) are obviously unusable for samples not in solution. The most

obvious usable criterion is optical density at 260 $m\mu$; however, the very short sample path length of a microscopic section, and interference by other cellular components, reduce the likelihood of success with this approach; several attempts we have made in this direction have not succeeded.

Other reported cytological methods for detecting DNA denaturation are: (a) the ability to incorporate tritiated thymidine, after acid denaturation, when the cell is incubated with the appropriate DNA polymerase (6); (b) the color of acridine orange-stained chromosomes, following heat and formalin denaturation (7). Both of these methods are nonquantitative, in that the actual process of denaturation cannot be followed and T_m values cannot be determined for a single specimen; in addition, the reliability of the acridine orange color criterion has recently been found questionable (8).

We have previously described a method for the detection of DNA orientation in microscopic

preparations, utilizing polarization of the fluorescence from acridine orange (AO) molecules bound to oriented DNA (9). This polarization of fluorescence is present because the mode of binding of AO to DNA, at the low AO concentrations used, is such that the flat rings of the AO molecules are rigidly held perpendicular to the DNA helix axis. Orientation of the DNA, therefore, produces orientation of the bound AO molecules. Much evidence has been presented which supports an intercalation model for this binding mode, in which the flat dye molecules are actually inside the DNA helix, sandwiched between adjacent base pairs (10-13). With such an arrangement, both absorption and fluorescence by the dye molecules take place with high preference for light quanta having electric vectors parallel to the planes of the fused rings of the dye, i.e., perpendicular to the DNA helix axis.

It seemed possible that the orientation of AO molecules bound in this fashion could be correlated with the intactness of the DNA double helix, and monitoring the fluorescence polarization as a function of temperature (for suitably oriented DNA) might provide a measure of thermal denaturation. We have made such measurements microscopically on the giant polytene chromosomes of *Chironomus thummi* salivary glands. Correlative studies have also been made on the temperature dependence of DNA-AO binding, using purified AO and DNA in solution.

We find that the effect observed for stretched polytene chromosomes appears not to be a direct measure of the DNA denaturation process usually measured by hyperchromic shift, but apparently derives from a closely related phenomenon; it also seems to depend, in a way similar to T_m , upon histone association. Studies on the thermal dependence of the rotational diffusion rates of AO in AO-DNA solutions suggest that the depolarization phenomenon observed in the microscope would also depend on the base composition of the chromosomal DNA.

MATERIALS AND METHODS

Polarized Fluorescence Microscope

The instrument used to measure fluorescence polarization in microscopic preparations has been briefly described elsewhere (9); the optical path and a block diagram are shown in Fig. 1. The exciting light from the mercury arc source passes through the primary filter (which cuts off wavelengths longer

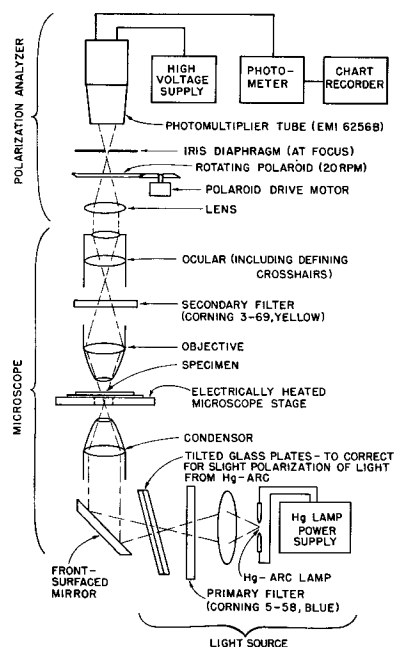


FIGURE 1 Optical path and block diagram for the polarized fluorescence microscope, including an electrically heated microscope stage. Not shown are swing-out mirrors located above the ocular for photography and viewing through side tubes.

than about $480 \text{ m}\mu$), through a series of tilted glass plates which correct for a slight polarization of the mercury arc output, and through the condenser (an inverted $10\times$ objective, NA 0.25). The light striking the sample is unpolarized. Fluorescence from the sample passes through the objective ($43\times$, NA 0.85) and the secondary filter (which has a high frequency cutoff at about $510 \text{ m}\mu$), then through the ocular ($8\times$) and an additional lens which brings the image to focus about 10 cm above the ocular. An iris diaphragm is at this position and can be closed down so that any desired portion of the field of view may be analyzed. Just below the iris diaphragm in the optical path is a polaroid, which is motor driven at a rate of 20 rpm. The light passes from the iris diaphragm to a photomultiplier tube (EMI 6256B). The signal from the photomultiplier tube is amplified by a photometer (Pacific Photometer, model 10), and is recorded on a chart recorder (Varian, model G-11A). For oriented DNA, the signal has an oscillatory component (as in Fig. 3), and from the signal the factor R is calculated. R is defined as follows:

$$R = \frac{\text{max} - \text{min}}{\text{max}} (100\%);$$

all signal heights are measured from a background determined by shifting the sample slightly and measuring the "open space" beside the chromosome.

In order to provide for sample heating and continuous measurement of R , we built a special microscope stage. In the center of a piece of Transite (pressed asbestos) (Johns-Manville), $\frac{1}{4}$ inch thick by about 10 cm square, a 4-mm hole was drilled, through which the exciting light beam passes. On the upper surface three grooves, concentric with the hole, were milled, the largest with a radius of about $1\frac{1}{2}$ cm. Coiled heating elements of nichrome wire were placed in the grooves and sealed in with Sauereisen (a liquid porcelain, which also provides excellent heat conduction, manufacturer, Sauereisen Cements Co., Pittsburgh). The microscope slides are held tightly against this heating area with spring clamps. A transformer and Variac (made by Varian Associates) provide power for the heating elements, and a thermistor is embedded next to the central hole for temperature monitoring. This reference thermistor was calibrated with organic crystals of known melting points mounted at the specimen position.

Chromosomal Preparations

The salivary glands of fourth instar *Chironomus thummi* larvae were dissected out into a drop of the physiological salt solution of Ito and Lowenstein (14). They were then either squashed immediately in 45% acetic acid, or were treated with trypsin (2.5% in isotonic saline at room temperature) for varying lengths of time, and then squashed. The glands were squashed between two slides, which were then frozen on dry ice, pried apart, and washed in tap water. The slides were then washed in pH 4.5 acetate buffer (0.1 M), placed for 30 sec in an acridine orange solution (Harleco, with no further purification, 0.25 mg/ml made up in the acetate buffer), washed again in the acetate buffer, and briefly dipped in 70% alcohol before air drying. The use of alcohol in this fashion appears to remove the loosely or "side" bound AO, leaving only that which is presumably intercalated. After being dried, the preparations were mounted in a drop of SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and analyzed.

In general, the DNA of normal polytene chromosomes is not oriented, although some orientation is found in the interband regions (9). However, fairly frequently in the squashing process, chromosomes become stretched, usually between two "islands" of cytoplasm, and such stretched chromosomes can have significantly high R values. Therefore, for this work chromosomes were sought which had become sufficiently stretched in the squashing process as to have an appreciable initial R (usually 10% or more), and which were also free of cytoplasm in the areas

to be analyzed. Results obtained seemed to be independent of initial R , i.e. degree of stretching. Generally, a long portion of an appropriate chromosome, including many bands, interbands, and puffs, was included in the region analyzed (see Fig. 3). In the analysis of a Balbiani ring region, however, the iris diaphragm at the focus of the instrument was closed down to include only that region.

Hyperchromic Shift Measurements

In vitro measurements of DNA thermal denaturation, taken by monitoring the OD at 260 μ , were performed in a Beckman model DU spectrophotometer equipped with a heated sample chamber. For these measurements, purified salmon sperm DNA (Calbiochem, Los Angeles) and AO purified by the procedure of Freifelder and Uretz (15) were used in SSC buffer.

Rotational Diffusion Measurements

A Turner model 110 fluorometer, which measures fluorescence at right angles to the exciting beam, was modified by the addition of a heating jacket around the sample tube and of polaroids in front of the primary and secondary filters. The orientation of the polaroids and the optical path of this instrument are illustrated in Fig. 2. Square pyrex tubes of 5-ml capacity were used for sample tubes, and a cork pierced by a thermistor temperature probe was used to cap them.

Purified AO and purified DNA's of salmon sperm (Calbiochem), *Clostridium perfringens* (Worthington Corp., Harrison, N. J.), and *M. lysodeikticus* (a gift of E. P. Geiduschek), were used for these measurements.

Equilibrium Dialysis Measurements

$\frac{3}{4}$ -inch dialysis tubing was prepared as follows: three washes in distilled water; boiling in EDTA solution; washing in distilled water; boiling in sodium bicarbonate solution; three more washes in distilled water. Into each tubing was pipetted 20 ml of purified salmon sperm DNA in SSC (0.1 mg/ml) plus 2 ml

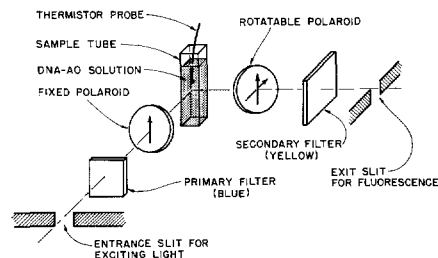


FIGURE 2 Optical path and polaroid orientations for the rotational diffusion experiments.

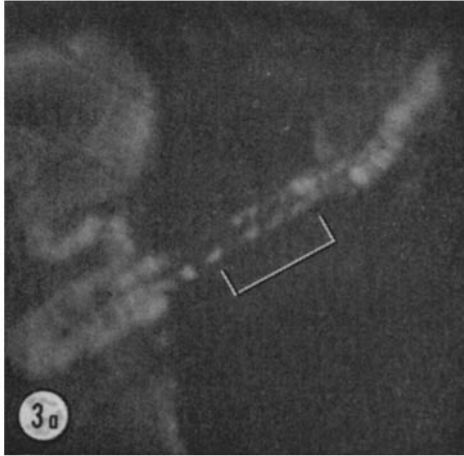


FIGURE 3 a Fluorescence photomicrograph of a pair of stretched *C. thummi* polytene chromosomes stained with acridine orange. Bracket indicates region measured. $\times 600$.

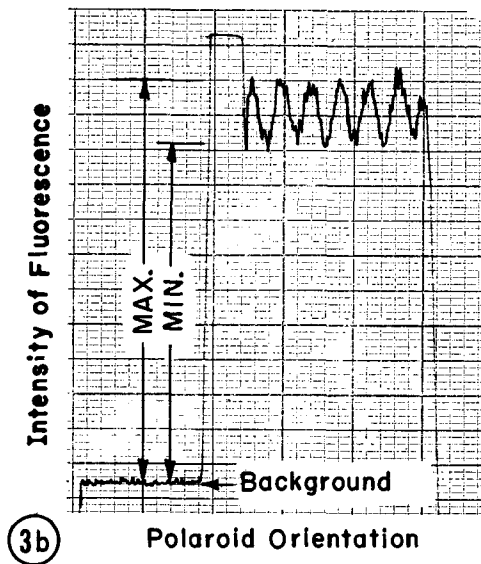


FIGURE 3 b Trace of intensity of fluorescence as the polaroid is rotated, for the specimen above (at 25°C).

of purified AO in SSC (0.1 mg/ml). After sealing, the tubings were placed in flasks containing 78 ml of SSC, the flasks capped, and either shaken or stirred at their particular temperatures for 3 days. Control flasks containing no DNA were also equilibrated at various temperatures so that we could check for uptake of dye by the dialysis tubing and glassware. Aliquots of the external solutions were then taken,

cooled to 25°C, and the AO concentrations measured in a Turner model 110 Fluorometer.

RESULTS

Thermal Depolarization of Fluorescence in Polytene Chromosomes

Fig. 4 shows representative curves obtained by monitoring R as the temperature is increased, for *C. thummi* salivary gland chromosomes. The results are summarized in Table I (errors indicated are mean deviations). $T_{1/2R}$ is defined as that temperature at which R is one-half of the R at 25°C. $T_{1/2R}$ decreases with increasing removal of histones and other proteins by trypsin, and the difference between untreated and extensively trypsin-treated chromosomes is about 17°C. This should be compared with the in vitro finding that the presence of histone raises T_m , as measured by hyperchromic shift, by up to 20°C (5, 16, 17).

Of particular interest is the fact that the Balbiani ring regions (Fig. 5) show a $T_{1/2R}$ considerably lower than the rest of the chromosomes. There are three Balbiani regions located on the fourth chromosome, one of which is responsible for the nucleolus in this particular chironomid. Although the functions of the nucleolar organizer region are different from those of the other Balbiani regions, no distinction has been made between them in the present study. Because the fourth chromosome is so short, discovering one appropriately stretched is difficult. However, a search of 30 preparations turned up four of these regions with sufficiently large initial R values.

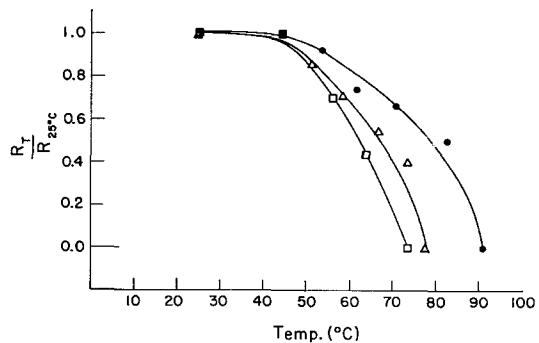


FIGURE 4 Representative curves of the decrease in R with increasing temperature, for *C. thummi* polytene chromosomes. (—●—●—●—), untreated stretched chromosomes. (—□—□—□—), stretched chromosomes extensively treated with trypsin. (—△—△—△—), untreated Balbiani ring regions.

TABLE I
Temperature of Half Depolarization for Stretched Chromosomes

Object	$T_{1/2R}$
Chromosomes, no trypsin	$78.0^\circ \pm 2.8^\circ\text{C}$ (average of 6 experiments)
"Moderately" trypsin-treated chromosomes	62.5° to 71°C (5 different experiments)
Extensively trypsin-treated chromosomes	$61.1^\circ \pm 1.5^\circ\text{C}$ (average of 3 experiments)
Balbani regions, no trypsin	$65.6^\circ \pm 1.1^\circ\text{C}$ (average of 4 experiments)

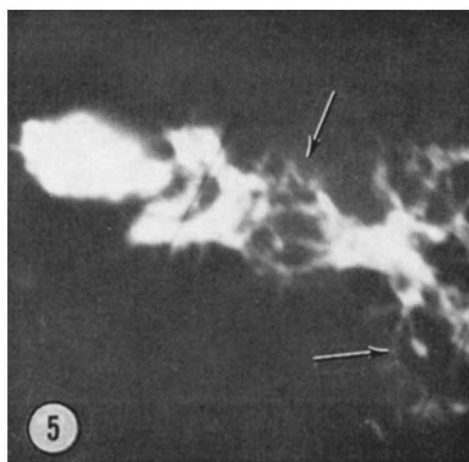


FIGURE 5 Fourth chromosome of *C. thummi* salivary glands, showing two of the expanded Balbiani regions (arrows). The larger is the "nucleolar organizer" ring. $\times 2000$.

Since the $T_{1/2R}$'s obtained for the chromosomal preparations are about 20°C lower than the expected T_m 's in the SSC buffer, it appears that denaturation is not the process being observed. Therefore, several in vitro experiments with purified DNA were performed.

Hyperchromic Shift Experiments

Thermally induced change of optical density at $260\text{ m}\mu$ was measured for DNA samples with and without AO. The results are shown in Fig. 6. A low temperature hump occurs in the sample containing AO, and the sample then goes on to show a normal hyperchromic shift, with a T_m identical to the sample without AO. About half of the absorption at $260\text{ m}\mu$ is by AO, and the normal (high temperature) shift by the sample containing AO is nearly half of the total shift by the pure DNA sample. Therefore, the low temperature hump of the sample with AO is probably due to a change in the AO molecular configuration, perhaps ejection

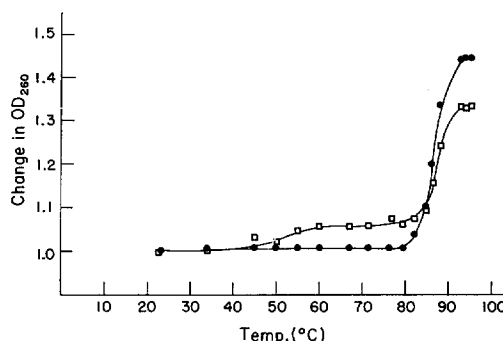


FIGURE 6 Change of optical density at $260\text{ m}\mu$ with increasing temperature. (—●—●—●—), purified salmon sperm DNA alone, conc. = $20\text{ }\mu\text{g/ml}$. (—□—□—□—), purified salmon sperm DNA, conc. = $10\text{ }\mu\text{g/ml}$, plus purified AO, 1 AO/8 DNA-phosphates.

of the intercalated AO molecules from the DNA helix, rather than a change in the DNA base-pairs. This finding is in contradiction to that of Kleinwachter and Koudelka, who found that the presence of AO raised T_m (18); however, their experiments were performed with a buffer of much lower ionic strength. Walker found that proflavine (an acridine dye closely related to AO) increases the T_m of DNA in a buffer of low ionic strength but not in one of high ionic strength (19).

Rotational Diffusion Experiments

The time constant for rotational diffusion for an acridine orange molecule rigidly held to a DNA helix is the same as that of the DNA molecule itself, which may have a molecular weight of 10^6 or more, and thus a large time constant (on the order of milliseconds). For acridine orange molecules free in solution, the time constant is on the order of nanoseconds, since the AO molecule has a molecular weight less than 300. The mean fluorescent lifetime of monomeric AO molecules, bound or unbound, is 10^{-6} – 10^{-8} sec, and thus lies between these two rotational time constants. Therefore,

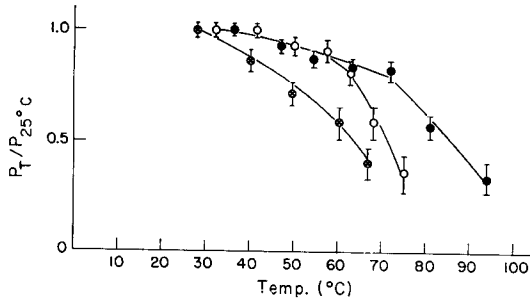


FIGURE 7 The change with temperature of the rotational diffusion rates of AO-DNA mixtures. DNA conc. = 100 $\mu\text{g}/\text{ml}$, 1 AO/8 DNA-phosphates. (●-●-●) *M. lysodeikticus* DNA, GC = 72%. (○-○-○) salmon sperm DNA, GC = 43%. (⊗-⊗-⊗) *Clostridium perfringens* DNA, GC = 26.5%.

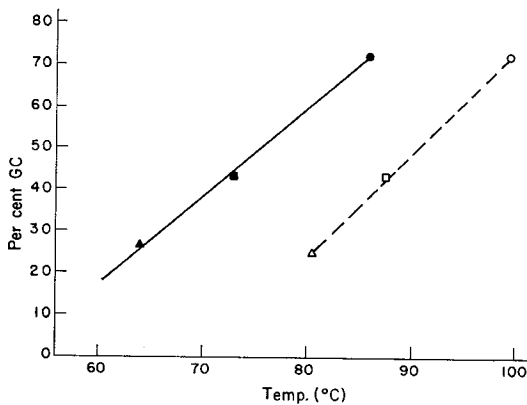


FIGURE 8 The relationship between $T_{1/2P}$ the temperature at which the rotational diffusion rate of the AO increases (solid line), and the DNA GC percentage. This is compared to the relationship between the thermal denaturation temperature (T_m) and GC percentage (dashed line). (●, ○), *M. lysodeikticus* DNA; (■, □), salmon sperm DNA; (▲, △), *C. perfringens* DNA. Closed points, $T_{1/2P}$ (from Fig. 7). Open points, T_m (from Marmur and Doty, 4).

with excitation by polarized light, as in the optical system of Fig. 2, the fluorescence from AO molecules intercalated in DNA helices should be partially polarized, with an electric vector predominantly vertical. For free or very loosely bound AO molecules, the rotational diffusion will be too rapid, and no polarization should be observed. Such is the case: at room temperature, a polarization of about 0.3 is observed for DNA plus AO in SSC, while no polarization is observed for free AO. (Polarization, P , is defined as the difference in fluorescent intensities for the polaroid in the two positions divided by the sum of these intensities.)

As in the microscopic experiments, the polarization of the DNA + AO in solution disappears as the temperature is raised. This is shown in Fig. 7, for DNA's of three different GC/AT ratios. The temperature at which half the initial polarization is gone ($T_{1/2P}$) is about 20°C lower than the T_m 's reported for these DNA's (shown, for example, for salmon sperm DNA in Fig. 6). A plot of per cent GC vs. the temperatures at which half the initial polarizations are gone for the curves of Fig. 7 is shown in Fig. 8, and this is compared with a similar plot for reported T_m 's in the same buffer.

We also find that $T_{1/2P}$ for calf thymus nucleohistone (Worthington) is about 10°C higher than that for purified calf thymus DNA (Sigma Biochemicals, St. Louis).

Equilibrium Dialysis Experiment

For simple binding between two molecular species, a plot of the logarithm of the equilibrium constant vs. $1/T$ gives a straight line, the slope of which is proportional to the heat of binding. A plot of $1/T$ vs. the equilibrium constant is shown in Fig. 9, for salmon sperm DNA and AO. This plot has not been corrected for a slight uptake of dye by the dialysis tubing, and a detailed interpretation has not been attempted. However, it is clear that the binding involves more than a single simple

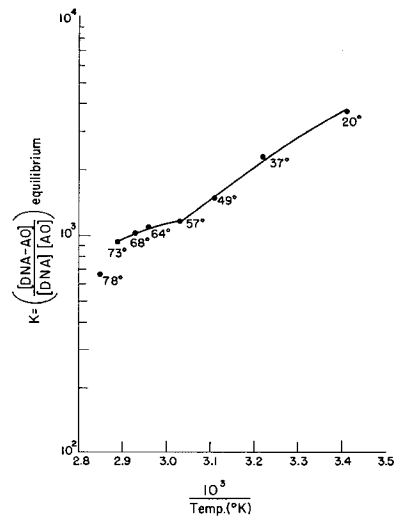


FIGURE 9 Plot of the log of the equilibrium constant for the binding of AO and DNA vs. the inverse of the Kelvin temperature: by the method of equilibrium dialysis. Not corrected for a slight uptake of dye by the dialysis tubing and the glassware.

mode and that there is considerable dissociation at temperatures well below the denaturation temperature of this DNA.

DISCUSSION

Thermal depolarization of fluorescence, reflecting disorientation of AO molecules bound to oriented DNA in polytene chromosomes, occurs at temperatures substantially lower than those expected for thermal denaturation in the same materials. In a high ionic strength buffer, purified DNA in solution exhibits normal thermal denaturation even in the presence of AO. Therefore, the depolarization of fluorescence measured in polytene chromosomes is probably not directly a measure of thermal denaturation, as usually determined. However, it appears to reflect the presence of bound histones in a manner similar to the way in which T_m reflects this variable. Also, the temperature dependence of the rotational diffusion rate of AO in solution with DNA varies with the GC/AT ratio of the DNA in the same way that T_m does. Although in the microscopic measurements it is the ordering of dye molecules that is being measured, while in the rotational diffusion measurements it is the rigidity of binding of dye molecules that is being observed, it seems likely that the rotational diffusion rate measurements and the microscopic depolarization of fluorescence measurements reflect the same or closely related phenomena, and that a process similar to denaturation, possibly a limited opening of the DNA helix, is involved. Such a model is analogous to the so called DNA "breathing": temporary openings and closings of the helix over short stretches of base pairs, at temperatures well below T_m (20). Since the DNA helix suffers considerable stress by the intercalation of an AO molecule, those locations at which the dye molecules are intercalated may be more prone to such localized helix openings. At temperatures corresponding to $T_{1/2R}$ the helix may be thermodynamically incapable of reclosing with the AO molecules still inside. In such a case, the dye molecules would be expelled, perhaps to form weak ionic bonds with the DNA phosphates.

Swift (21), and Gorovsky and Woodard¹, have found no differences in the DNA-histone ratios for bands and puffs in *Drosophila* polytene chromosomes. To our knowledge, however, no one has made such measurements for the Balbiani rings of dipteran polytene chromosomes. Although both

¹ M. Gorovsky and J. Woodard. Unpublished.

puffs and Balbiani regions are the presumptive locations of metabolic RNA synthesis, the latter differ considerably in morphology from the former. There would seem to be four possible interpretations for our finding that $T_{1/2R}$ is much lower in the Balbiani regions than in the rest of the chromosomal regions: 1) There is a much lower histone/DNA ratio in Balbiani rings; 2) The histone/DNA ratio is the same, but the histone is not so intimately bound to the DNA in Balbiani rings; 3) The GC/AT ratio of the DNA in Balbiani rings is less than that of other chromosomal regions; 4) There is some other, unknown, factor causing the difference in $T_{1/2R}$ —possibly there would be some difference due to the lower DNA concentration in the Balbiani regions, or because of the presence or absence of molecular species other than trypsin-sensitive proteins. The third possibility does not seem likely: Edström and Beermann (22) measured the base ratios of RNA from different portions of *Chironomus tentans* salivary gland chromosomes, including nucleolar and Balbiani regions. They report GC percentages ranging from 43.8% to 48.4%, a difference far too small to account for the difference in $T_{1/2R}$'s that we have found.

Since an increase in the rotational diffusion rate of AO added to DNA is probably also a valid measure of the depolarization of fluorescence, this method might also be used microscopically, with the great advantage of not requiring orientation of the DNA being analyzed. However, there are several difficulties with this method. Molecular species other than DNA which also bind AO—RNA and some proteins, in particular—will contribute to the measurement; also, components other than DNA, even if they do not bind AO themselves, may affect the rotational diffusion rates of AO molecules expelled from DNA helices. When R is monitored, on the other hand, only native DNA is analyzed, in spite of any other AO-bound species present, since native DNA is presumably the only molecular species capable of so orienting the AO molecules as to produce an $R > 0$. If a method exists for orienting the DNA's of a specimen, $T_{1/2R}$, therefore, becomes a good measure of the state of the DNA, since all other molecular species present may be ignored (although it is probably advisable to use a high ionic strength buffer for such measurements in order to suppress localized ion effects).

Nevertheless, since there are, at present, no practical methods of sufficiently orienting the DNA

of many cells, we are undertaking microscopic work employing the rotational diffusion rate criteria.

This research was supported by United States Public Health Service Research Grant No. CA 02739 from the National Cancer Institute. Mr. MacInnes is a recipient of a USPHS traineeship, GM780, and

subsequently a National Science Foundation Graduate Fellowship; Dr. Uretz is a recipient of a USPHS Research Career Development Award. The authors wish to thank Professors E. P. Geiduschek and H. Swift for helpful discussions, and J. Hanacek and G. Gibson for skilled machine work.

Received for publication 21 October 1966.

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