Friedman, S. (1973), *Nature (London), New Biol. 244,* 18.
Funamizu, M., Terahara, A., Feinberg, A., and Nakanishi, K. (1971), *J. Amer. Chem. Soc. 93,* 6706.
Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38,* 470.
Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7,* 3459.
Gray, M. W., and Lane, B. G. (1968), *Biochemistry 7,* 3441.
Hall, R. H. (1971), The Modified Nucleosides in Nucleic Acids, New York, N. Y., Columbia University Press, p 160.
Harada, F., Kimura, F., and Nishimura, S. (1971), *Biochemistry 10,* 3269.
Hayashi, H., Nakanishi, K., Brandon, C., and Marmur, J. (1973), *J. Amer. Chem. Soc. 95,* 8749.
Hayes, N. V., and Branch, C. E. K. (1943), *J. Amer. Chem. Soc. 65,* 1555.
Krebs, H. A., and Hems, R. (1952), *Biochim. Biophys. Acta 12,* 172.
Levitt, M. (1969), *Nature (London) 224,* 759.
McCloskey, J. A., Lawson, A. M., Tsuboyana, K., Krueger, P. M., and Stillwell, R. N. (1968), *J. Amer. Chem. Soc. 90,* 4183.
Murao, K., Tanabe, T., Ishii, F., Namiki, M., and Nishimura, S. (1972), *Biochem. Biophys. Res. Commun. 47,* 1332.
Nakanishi, K., Furutachi, N., Funamizu, M., Grunberger, D., and Weinstein, I. B. (1970), *J. Amer. Chem. Soc. 92,* 7617.
Ohashi, Z., Maeda, M., McCloskey, J. A., and Nishimura, S. (1974), Submitted for Publication.
Sharkey, A. G., Jr., Friedel, R. A., and Langer, S. H. (1957), *Anal. Chem. 29,* 770.
Uchida, T., and Egami, F. (1967), *Methods Enzymol. 12,* Part A, 239.
Yaniv, M., and Barrell, B. G. (1971), *Nature (London), New Biol. 233,* 113.
Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun. 43,* 729.

Biophysical Studies on the Mechanism of Quinacrine Staining of Chromosomes†

J. M. Gottesfeld,* J. Bonner, G. K. Radda, and I. O. Walker

ABSTRACT: The fluorescence of quinacrine was measured in solution in the presence of interphase chromosomal material (chromatin) and in the presence of chromatin which had been fractionated into extended and condensed regions (euchromatin and heterochromatin). Quinacrine fluorescence is quenched most effectively by the euchromatin fraction, intermediately by unfractionated chromatin, and least effectively by the heterochromatin fractions. These differences are abolished when the fluorescence of quinacrine is measured in the presence of DNA isolated from chromatin and the chromatin fractions. Spectrophotometric titrations indicate that the association constants for quinacrine binding to the various chromatin fractions differ by only a factor of two, and that the number of sites per nucleotide available for quinacrine binding at saturation are nearly identical for all fractions. Circular dichroism spectroscopy suggested that the conformation of the DNA in the euchromatin fraction is most like that of protein-free DNA in aqueous solution ("B"-form DNA) while the DNA in the heterochromatin fractions is partially in the "C" conformation. These results suggest that protein–DNA interactions in chromatin are responsible for the fluorescence patterns observed and that chromosome banding with quinacrine might arise from differences in protein–DNA interactions (and DNA conformation) along the chromatids of metaphase chromosomes.

Caspersson *et al.* (1968) and others have shown that the fluorescent dye quinacrine mustard stains specific regions of chromosomes with a very brilliant intensity, leaving other areas of chromosomes relatively dark. It was originally thought that the linear differentiation of chromosomes into fluorescent bands and poorly staining interband regions was due to the specific alkylation of guanine residues by the mustard function of the dye. The finding that quinacrine itself produces identical banding patterns suggested that alkylation of guanine residues was not the primary mechanism of fluorescence staining of chromosomes (Caspersson *et al.*, 1969). Ellison and Barr (1972) have suggested that enhancement of quinacrine fluorescence might be a function of base ratio, with (A + T)-rich regions fluorescing brightly. Weisblum and de Haseth (1972) and Pachmann and Rigler (1972) have investigated quinacrine fluorescence *in vitro* with a series of natural and synthetic polynucleotides, and found that A·T base pairs are responsible for fluorescence enhancement. Guanine residues were shown to give rise to quenching of quinacrine fluorescence. These data, and several other lines of evidence (Schreck *et al.*, 1973; Lomholt and Mohr, 1971), suggested that the fluorescent bands observed with quinacrine-stained chromosomes are indeed (A + T) rich.

This investigation was undertaken to determine whether quinacrine flourescence in the presence of isolated chromosomal material is due solely to intrachromosomal differences in DNA base composition, or whether DNA–protein interactions in chromatin play a role in producing banding patterns. Chromatin has been fractionated into extended and condensed regions (euchromatin and heterochromatin) (Billing and Bonner, 1972; Bonner *et al.*, 1974; Gottesfeld *et al.*, 1974). It has been suggested that the extended chromatin fraction corresponds to

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TABLE I: Distribution of DNA in the Chromatin Fractions.

Sample	% of Total DNA: 5-min DNase Exposure	% of Total DNA: 15-min DNase Exposure
Rat chromatin		
Euchromatin (S2)	11.3 ± 3.9[a]	25.0 ± 3.0[b]
Heterochromatin (P1)	84.6 ± 4.8	23.9 ± 2.9
Heterochromatin (P2)	4.1 ± 2.5	50.6 ± 3.3
Drosophila chromatin		
Euchromatin (S2)		25.6 ± 3.2[c]
Heterochromatin (P1)		28.2 ± 1.9
Heterochromatin (P2)		45.4 ± 5.8

[a] Mean of 11 determinations ± SD. [b] Mean of 9 determinations ± SD. [c] Mean of 3 determinations ± SD.

the template active portion of chromatin *in vivo* and that the heterochromatin fractions correspond to the repressed or template inactive portion of chromatin *in vivo* (Bonner *et al.*, 1973, 1974; Gottesfeld *et al.*, 1974). Fluorescence and binding data have been obtained for the interaction of quinacrine with chromatin and the chromatin fractions. Significant differences in the quantum yield of quinacrine bound to these fractions were found; these differences were found to be due to protein–DNA interactions and not differences in DNA base composition. In addition, circular dichroism studies of the chromatin fractions have yielded information on the protein-induced conformational alteration of DNA in these fractions.

Materials and Methods

Preparation of Chromatin. Chromatin from rat liver was prepared by the method of Marushige and Bonner (1966). *Drosophila* embryo chromatin was a gift of Dr. S. C. R. Elgin (Elgin and Hood, 1973). The crude chromatin from both rat and *Drosophila* was purified by centrifugation through 1.7 M sucrose as described by Marushige and Bonner (1966). The sucrose pellet was homogenized in 0.01 M Tris-Cl (pH 8) and centrifuged at 27,500*g* for 20 min.

Fractionation of Chromatin. Purified chromatin was homogenized with a motor-driven glass-Teflon homogenizer (20 strokes at 10 strokes per minute) in approximately 20–30 ml of 0.01 M Tris-Cl (pH 8). The homogenate was dialyzed overnight *vs.* 6 l. of 0.025 M sodium acetate (pH 6.6). After dialysis, the dialysate was homogenized as before with the motor-driven glass-Teflon homogenizer. The volume of the chromatin solution was adjusted to give 10 A_{260} units/ml when the absorbance of an aliquot (100 μl) was measured in 0.9 N NaOH. DNase II (Worthington, HDAC) was added to 10 units of enzyme/A_{260} unit of chromatin (100 units of enzyme/ml). The digestion was allowed to proceed for 5–15 min at ambient temperature (24°). At the end of the incubation period, the pH of the chromatin suspension was brought to 7.5 with 0.02 M Tris-Cl (pH 11). The chromatin suspension was cooled on ice and then centrifuged at 27,500*g* for 15 min at 0–4°. To the supernatant, 0.2 M $MgCl_2$ was added dropwise to give a final concentration of 0.002 M. After 30-min stirring at 0–4°, the solution was centrifuged again at 27,500*g* for 15 min.

The final supernatant from above is termed S2 (extended euchromatin). A 5-min nuclease treatment of rat liver chromatin yields 10–15% of the total chromatin nucleic acid in S2. The DNA prepared from the S2 chromatin fraction (5-min DNase treatment) is double stranded and 700 base pairs in length (number-average length). Longer exposure to DNase II yields up to 25% of the chromatin DNA in S2. When prepared from *Drosophila* embryo chromatin, the euchromatin fraction also contains 25% of the total chromatin DNA (15-min digestion). Table I lists the amounts of nucleic acid found in the fractions obtained from both rat liver and *Drosophila* chromatin.

The pellet heterochromatin fractions are termed P1 (first pellet) and P2 (second pellet). Gottesfeld and Bonner (1974) have shown that the P1 fraction from rat chromatin is relatively inaccessible to enzyme attack. Thus the DNA in this fraction is likely to be more highly compacted and may correspond to the constitutive or centric heterochromatin in metaphase chromosomes. Measurement of template activity and DNA–RNA hybridization studies (Gottesfeld *et al.*, 1974) show that the pellet or heterochromatin fractions correspond to the template inactive fraction of interphase chromatin. Table I indicates that very little nucleic acid is found in the second pellet fraction (P2) after a 5-min enzyme treatment. Bonner *et al.* (1973, 1974) have given the chemical compositions of the fractions obtained from rat chromatin. Evidence for the validity of this fractionation procedure is also presented in Bonner *et al.* (1973, 1974) and in Gottesfeld *et al.* (1974).

Preparation of DNA. DNA was prepared from chromatin and the chromatin fractions in the following manner. The chromatin preparations were first extracted with phenol (saturated with 0.01 M Tris-Cl, pH 8) until no visible material remained at the interface. The aqueous phase was extracted repeatedly with an equal volume of chloroform–isoamyl alcohol (24:1, v/v). The aqueous phase was then dialyzed overnight *vs.* 0.01 M Tris-Cl (pH 8), containing 0.5 M NaCl. The dialysate was treated with ribonuclease (Worthington, RAF; preincubated at 80° for 10 min) at 50 μg/ml for 1 hr at 37°. After ribonuclease treatment, EDTA (pH 8) was added to 0.01 M and sodium dodecyl sulfate to 1% (w/v). Pronase (Calbiochem, CB; preincubated at 37° for 1 hr) was added to 50 μg/ml, and the mixture was incubated at 60° for 1–2 hr. After enzyme treatments, the DNA was phenol and chloroform–isoamyl alcohol extracted as before. The final DNA solution was dialyzed extensively *vs.* distilled water and then lyophilized.

Estimation of Nucleic Acid Concentrations. DNA concentrations were determined spectrophotometrically using an $\epsilon_{260\,nm}$ value of 6600. DNA concentrations of chromatin samples were estimated spectrophotometrically on solutions diluted with 1 N NaOH (final NaOH concentrations were 0.9–0.98 N). An $A_{260\,nm}$ reading of 1.0 corresponds to 37 μg/ml of chromatin DNA.

Fluorescence Measurements. Fluorescence spectroscopy was performed with a Hitachi Perkin-Elmer spectrofluorimeter Model MPF-2A. All measurements were made at 24–25°. Fluorescence of quinacrine in the presence of nucleic acid and nucleoprotein preparations was measured at a quinacrine concentration of 2.0×10^{-6} M. The buffers used in this study were 0.1 M sodium phosphate (pH 6.8–7.0) and 0.01 M Tris-Cl (pH 8). Fluorescence values reported herein are given relative to the fluorescence of quinacrine measured in the same solvent. Quinacrine was the dihydrate hydrochloride (mol wt 506.9) obtained from the Sigma Chemical Co., St. Louis, Mo. The excitation wavelength was 424 nm, and an emission spectrum was obtained for each sample. The excitation and emission slits were maintained at 6 nm.

To test the effect of chromosomal proteins on the fluorescence of a quinacrine–chromatin complex, Pronase was added to the mixture and the fluorescence output of the solution was monitored with time. To 0.5 ml of a solution containing rat

liver P1 fraction heterochromatin (at 83 μg/ml of chromatin DNA) and quinacrine (at 2.0×10^{-6} M) in 0.01 M Tris-Cl (pH 8), 50 μl of a 200-μg/ml solution of Pronase (preincubated at 37° for 1 hr) was added at time zero. The excitation wavelength was 424 nm, and emission was monitored for 30 min at 495 nm. No significant instrument drift was observed with quinacrine alone measured at intervals over a 30-min period. Pronase (in the absence of nucleoprotein) had no effect on the fluorescence of quinacrine.

Measurement of Quinacrine Binding to DNA, Chromatin, and the Chromatin Fractions. Owing to the fact that quinacrine fluorescence is affected differently by A·T and G·C base pairs (Weisblum and de Haseth, 1972; Pachmann and Rigler, 1972), fluorescence titrations with natural DNAs cannot be used to estimate association constants or the number of sites per nucleotide available for dye binding. To study the interaction of quinacrine with DNA and chromatin quantitatively, we made use of the fact that the optical extinction coefficient of quinacrine is reduced on binding to DNA. Pachmann and Rigler (1972) have shown that both A·T- and G·C-containing polynucleotides cause a hypochromic shift in the absorption spectrum of quinacrine. Blake and Peacocke (1968) have presented a detailed treatment of the use of spectrophotometric titrations and the Scatchard plot in studying the binding of aminoacridines to nucleic acids. Briefly, if one plots r/C_F *vs.* r, where r is defined as the ratio of moles of bound dye (quinacrine) to moles of DNA nucleotides and C_F is the molar concentration of free dye, then a straight line is obtained for each class of binding sites present if the sites within each class are equivalent and independent of one another. The slope of the straight line is $-K_a$, the association constant, and the intercept on the abscissa is $\bar{n}$, the maximal number of moles of dye bound per mole of DNA nucleotide.

The molar extinction coefficient of bound quinacrine, $\epsilon_{420\,nm}{}^b$, was obtained experimentally for each DNA and nucleoprotein sample. At a constant concentration of quinacrine (4.4×10^{-5} M) in 0.01 M Tris-Cl (pH 8), the nucleic acid concentration was varied over the range of 1.0×10^{-5} to 3.0×10^{-4} M. The absorbancies of the quinacrine–DNA or quinacrine–nucleoprotein complexes were measured at 420 nm with a blank of DNA or nucleoprotein in the absence of quinacrine. This serves to correct for light scattering at high nucleoprotein concentrations. The absorbance at 420 nm was obtained from an absorption spectrum performed with a Cary Model 11 recording spectrophotometer. All absorption measurements were made at ambient temperature (24°). The value of $\epsilon_{420\,nm}{}^b$ was obtained from the absorption value at infinite nucleotide concentration. This latter value was obtained from a computer fit of a double-reciprocal plot of the experimental data by the method of least squares. Table II lists the extinction coefficients for free quinacrine and for quinacrine bound to various DNA and nucleoprotein preparations.

Data for the Scatchard plot were obtained from experiments where the concentration of quinacrine was varied over a 15-fold range at a fixed concentration of nucleic acid (DNA or chromatin). These experiments were performed as follows. To 3.0 ml of a solution containing DNA or nucleoprotein (ranging in concentration from 1.4 to 2.5×10^{-4} M in DNA nucleotides) in 0.01 M Tris-Cl (pH 8.0), 5- to 20-μl aliquots of a 5.0×10^{-3} M solution of quinacrine were added. After each addition of quinacrine an absorption spectrum was taken. A blank containing DNA or nucleoprotein in the absence of quinacrine was used to compensate for light scattering. The absorption spectrum of free quinacrine at each concentration ($1.0–15 \times 10^{-5}$ M) was also obtained. Beer's law was found to be obeyed over this range of quinacrine concentrations. The molar fraction of bound quinacrine was obtained from the absorbance readings as described by Blake and Peacocke (1968). To obtain K_a, the association constant, and $\bar{n}$, the maximal number of moles of quinacrine bound per mole of DNA nucleotides, a computer fit of the Scatchard plot by the least-squares method was performed.

TABLE II: Extinction Coefficients of Quinacrine (Free) and Quinacrine Bound to Rat Liver DNA, Chromatin, and Chromatin Fractions.

Sample[a]	$\epsilon_{420\,nm}$
Quinacrine, free	7.6×10^3
Quinacrine, bound to	
DNA	3.9×10^3
Chromatin	3.8×10^3
Euchromatin	3.9×10^3
Heterochromatin	4.1×10^3

[a] All measurements were made in 0.01 M Tris-Cl (pH 8).

Circular Dichroism Spectroscopy. Circular dichroism spectra were obtained with a Roussel-Jouan Dichrograph II and with a Durrum-Jasco ORD/UV-5. Spectra were recorded at room temperature with a sample cell of 1-cm path length. Spectra were scanned from 350 nm downward to lower wavelengths until the noise level became too high to record meaningful data (usually around 210 nm). CD spectra were obtained with samples at $A_{260\,nm}{}^{1\,cm}$ values of 0.8–1.5. Spectra are reported in terms of the difference in extinction coefficients for left and right circularly polarized light, respectively. $\epsilon_l - \epsilon_r$ is defined as $(A_l - A_r)/lc$, where A_l and A_r are the absorbancies for left and right circularly polarized light, respectively, l is the path length of the sample in centimeters, and c is the molar concentration of the sample. All spectra presented herein are reported in terms of molar concentrations of DNA nucleotides.

Thermal Denaturation. The mean temperature of thermal denaturation (T_m) of the DNA samples was determined with a Gilford spectrophotometer–multiple sample absorbance recorder equipped with a Haake circulating bath. The temperature of the bath was increased at a linear rate of about 0.5°/min. DNA samples were dialyzed against standard saline citrate (0.15 M NaCl–0.015 M trisodium citrate) prior to denaturation.

Determination of DNA Size. The size of the DNA prepared from chromatin and the chromatin fractions was determined by electron microscopy. The DNA samples were spread from aqueous ammonium acetate by the method of Davis *et al.* (1971).

Results

Quinacrine Fluorescence. FLUORESCENCE OF QUINACRINE ON BINDING TO DNA AND SYNTHETIC POLYNUCLEOTIDES. When excited at 424 nm, the fluorescence maximum of quinacrine in aqueous solution is 490–500 nm. The addition of naturally occurring DNAs of moderate A·T content (less than 60% A·T) or G·C-containing polynucleotides to a solution of quinacrine causes the quenching of fluorescence. On the other hand, (A + T)-rich DNAs and polynucleotides cause an enhancement of quinacrine fluorescence (Weisblum and de Haseth, 1972; Pachmann and Rigler, 1972). Neither quenching nor enhancement of fluorescence affects the shape or the position of the maximum of the quinacrine emission spectrum.

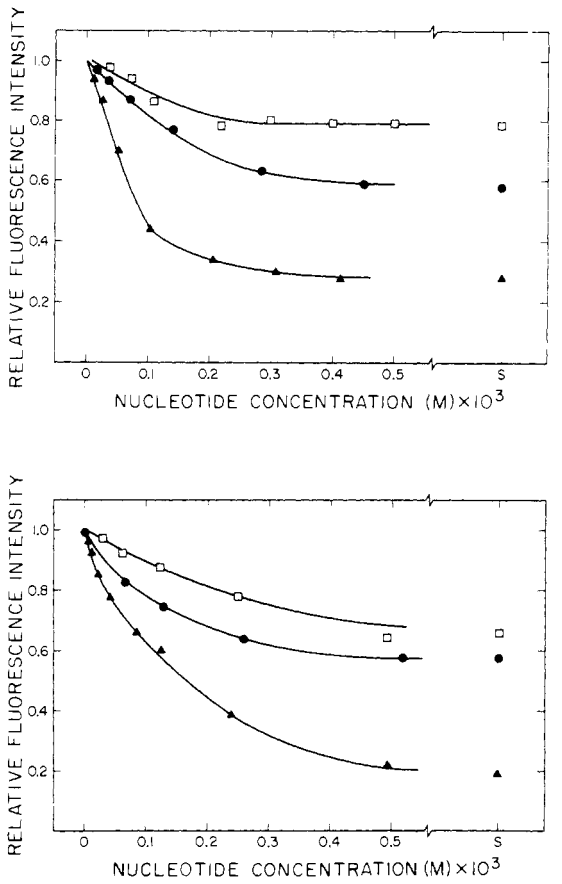

FIGURE 1: Relative fluorescence intensity of quinacrine measured in the presence of increasing concentrations of unfractionated chromatin (●), S2 euchromatin (▲), and P1 heterochromatin (□). The data for P2 heterochromatin (not shown) were essentially identical with that for unfractionated chromatin. Chromatin fractions were isolated after 15-min exposure to DNase. Quinacrine fluorescence was measured in 0.1 M phosphate buffer (pH 6.8) as described in Methods. Data are included for (a) rat liver (top panel) and (b) *Drosophila melanogaster* chromatin (bottom panel). "S" indicates computer fit value of fluorescence at infinite nucleotide concentration.

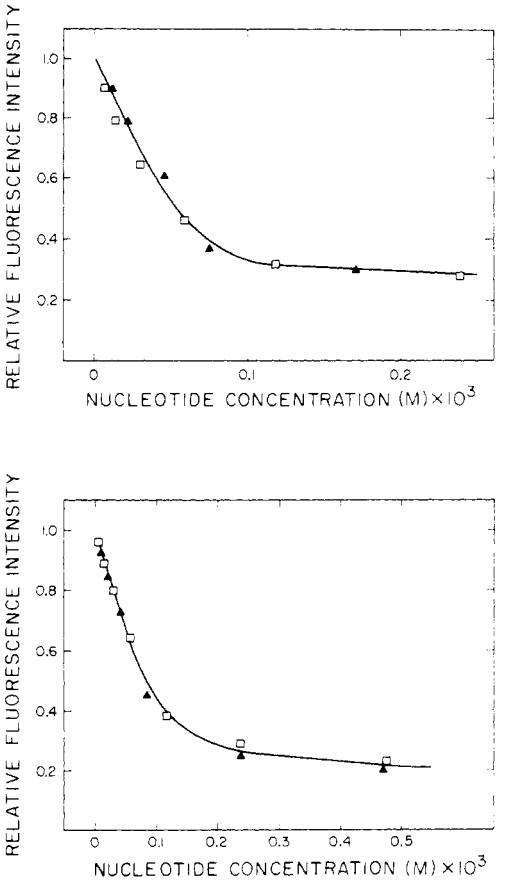

FIGURE 2: Relative fluorescence intensity of quinacrine measured in the presence of increasing concentrations of DNA prepared from S2 euchromatin (▲) and P1 heterochromatin (□). DNA was prepared from chromatin fractions obtained after 15-min exposure to DNase as described in Methods. Fluorescence was measured at a quinacrine concentration of 2×10^{-6} M in 0.1 M phosphate buffer (pH 6.8) as described in Methods. Data are included for (a) rat liver (top panel) and (b) *D. melanogaster* chromatin (bottom panel). "S" indicates computer fit value of fluorescence at infinite nucleotide concentration.

Pachmann and Rigler (1972) have presented absorption and emission spectra of quinacrine alone and quinacrine in the presence of both (A + T)- and (G + C)-rich polynucleotides.

QUINACRINE FLUORESCENCE IN THE PRESENCE OF CHROMATIN AND CHROMATIN FRACTIONS. Figure 1 presents the data obtained from fluorescence titrations of a standard amount of quinacrine (2.0×10^{-6} M) with varying amounts of chromatin and fractionated euchromatin and heterochromatin. Data are presented for two experiments, one performed with rat liver chromatin and another with chromatin prepared from 6- to 16-hr embryos of *Drosophila melanogaster*. The two chromatin preparations gave the same qualitative results. Euchromatin fractions from both rat liver and *Drosophila* embryo chromatin quenched quinacrine fluorescence to a greater extent than did either unfractionated chromatin or heterochromatin. The curves appear to be simple first-order saturation curves. We have performed a fit of the data by the least-squares method (straight-line fit of an inverse plot) to obtain fluorescence values at saturating concentrations of chromatin. The saturation value is the relative fluorescence of completely bound quinacrine. These results are also shown in Figure 1. The relative fluorescence of quinacrine bound to euchromatin is 0.19 for *Drosophila* and 0.28 for rat liver. Thus, the fluorescence of quinacrine is quenched by 70–80% on binding to euchromatin; on the other hand, quinacrine fluorescence is quenched by only 22–35% on binding to heterochromatin. Quenching by the nucleoprotein samples did not shift the emission maximum of quinacrine by more than 5 nm.

PROTEIN–DNA INTERACTIONS AND QUINACRINE FLUORESCENCE. We now attempt to answer the following question: Are the fluorescence patterns observed with quinacrine–nucleoprotein complexes due to differences in the base composition of the DNA of the chromatin fractions, or are they due in part to differences in protein–DNA interactions? The first half of this question can be answered directly by determining the base composition of the DNA isolated from the chromatin fractions. Furthermore, the fluorescence of quinacrine can be measured in the presence of these DNA samples.

The base composition of a DNA preparation (of 400 base pairs or greater in length) can be determined by measuring its T_m (mean temperature of thermal denaturation) in standard saline-citrate (Mandel and Marmur, 1968). Rat DNA (42% G·C) theoretically should exhibit a T_m of 86°. We observed a T_m of 85° for rat liver DNA. The DNA prepared from rat liver euchromatin (5-min DNase treatment) exhibited a T_m of 84°. Similarly, the DNA of rat liver P1 heterochromatin had a T_m of 85°. To a first approximation, therefore, the base composition of the DNAs from the chromatin fractions are identical (with 42 ± 2% G·C).

When the fluorescence output of quinacrine was measured in the presence of varying amounts of euchromatin and heterochromatin DNA, the saturation curves of Figure 2 were obtained. Data for euchromatin and heterochromatin DNA of rat

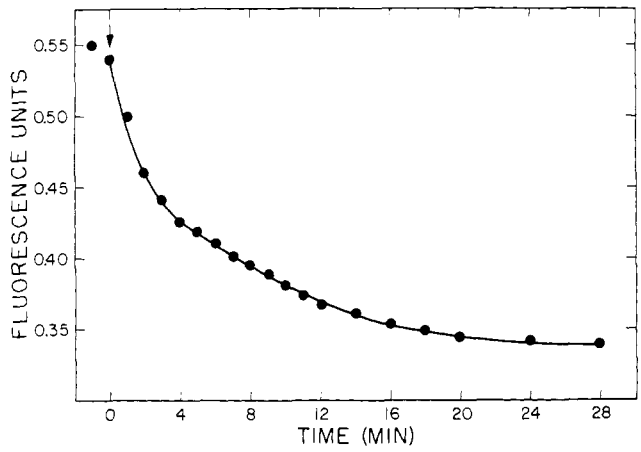

FIGURE 3: Time course of Pronase treatment of a quinacrine–heterochromatin mixture. At time zero (arrow), 50 μl of a 200-μg/ml solution of Pronase was added to 500 μl of a solution containing quinacrine (2×10^{-6} M) and rat liver P1 heterochromatin (83 μg/ml of nucleic acid). The heterochromatin sample was that obtained after 5-min exposure of chromatin to DNase. Other experimental details are given in Methods. Fluorescence is expressed in arbitrary units.

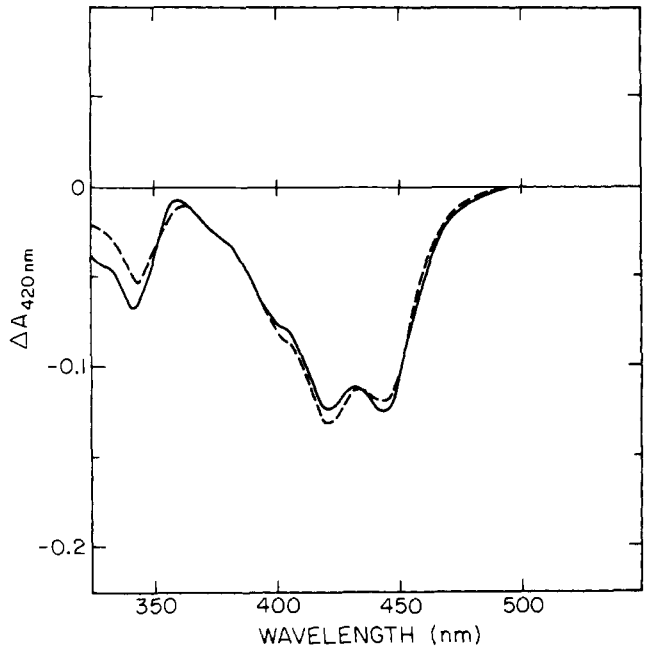

FIGURE 4: Visible difference spectra of quinacrine in the presence of near saturating concentrations of rat DNA (0.31×10^{-3} M in nucleotides) and chromatin (0.23×10^{-3} M in nucleotides) as compared to free quinacrine. Absorption spectra were measured at a quinacrine concentration of 4.4×10^{-5} M in 0.01 M Tris-Cl (pH 8). DNA = solid line; chromatin = dashed line.

(Figure 2a) and *Drosophila* (Figure 2b) are shown. As expected from base composition determinations, the data for euchromatin and heterochromatin DNA (from the same organism) are identical. The fluorescence titration curves for unfractionated DNA were also found to be the same as those for euchromatin and heterochromatin DNA from the same organism. The fluorescence titrations for euchromatin (Figure 1) most closely resemble those for deproteinized DNA (Figure 2), and the relative fluorescence of quinacrine at saturating concentrations of rat euchromatin (Figure 1a) was the same as that value at saturating concentration of rat DNA (Figure 2a).

Since base composition differences cannot reasonably account for the data of Figure 1, some other component of chromatin must be responsible. In order to examine the role of chromosomal proteins, the fluorescence of a quinacrine–heterochromatin mixture was monitored with time after addition of Pronase to the solution (Figure 3). Over a 20-min period a marked decrease in fluorescence output was noted. This is the change one would expect as heterochromatin is deproteinized and transformed into DNA. Pronase by itself did not alter the fluorescence of quinacrine. Furthermore, chromosomal proteins by themselves (histone and non-histone proteins) did not affect quinacrine fluorescence under our experimental conditions (0.01 M Tris-Cl (pH 8) or 0.1 M sodium phosphate (pH 6.8)). Thus it appears that protein–DNA interactions in chromatin are responsible for the observed results (Figure 1).

We have indicated that the fluorescence of quinacrine bound to heterochromatin is substantially greater than the fluorescence of quinacrine bound to euchromatin or DNA. We attribute this difference to the different quantum yields of the bound species. This conclusion rests on the value of relative fluorescence at saturating nucleotide concentrations. However, if the fractions are vastly different with respect to either binding affinities for quinacrine or the number of sites per nucleotide available for binding, the conclusions reached above must be reassessed. Therefore, a quantitative estimation of these binding parameters has been obtained.

Binding of Quinacrine to Chromatin and DNA. SPECTRAL CHANGES ON BINDING. Quinacrine in aqueous solution has absorption maxima at 345, 424, and 446 nm. On binding to DNA, the absorption maxima are shifted to longer wavelengths; moreover, the entire spectrum undergoes a hypochromic shift. This is characteristic of the interaction of planar dye molecules with nucleic acids (Blake and Peacocke, 1968). Pachmann and Rigler (1972) have presented data which indicate that both (G + C)- and (A + T)-rich polynucleotides have the same effect on the absorption spectrum of quinacrine as naturally occurring DNA (<60% A·T).

On binding to either chromatin or the chromatin fractions, the absorption spectrum of quinacrine undergoes the same hypochromic shift as on binding to rat DNA. Absorption difference spectra are presented in Figure 4. The absorption spectra of quinacrine–DNA and quinacrine–chromatin complexes were measured at near saturating nucleotide concentrations. Under these conditions nearly all the dye present in solution is bound. Spectra were taken with two cuvets in the reference position of the spectrophotometer; one cuvet contained quinacrine alone while the second cuvet contained either DNA or chromatin. An instrument baseline was obtained by measuring the absorption of quinacrine with an identical cuvet containing quinacrine in the reference position. The absorption difference spectra of quinacrine–DNA and quinacrine–chromatin complexes are nearly identical (Figure 4). Thus it appears that quinacrine forms a similar complex with chromatin as with DNA. Lerman (1963) has shown that the mode of interaction of quinacrine with DNA is intercalation. It is tempting, therefore, to speculate that the mode of interaction of quinacrine with chromatin is also *via* intercalation of the dye between the stacked bases of DNA. Since the absorption spectrum of quinacrine undergoes the same transition upon interaction with DNA or chromatin, spectrophotometric titrations can be used to investigate the binding of quinacrine to DNA and nucleoproteins quantitatively.

A spectrophotometric titration of quinacrine with increasing concentrations of rat chromatin, euchromatin, and heterochromatin was performed in order to obtain the value of the extinction coefficient of bound quinacrine. Figure 5 and Table II present the results of this experiment. The values of $\epsilon_{420\,nm}{}^{b}$ are nearly identical (±3.5%) for DNA, chromatin and the chromatin fractions. Even though the absorption values at sat-

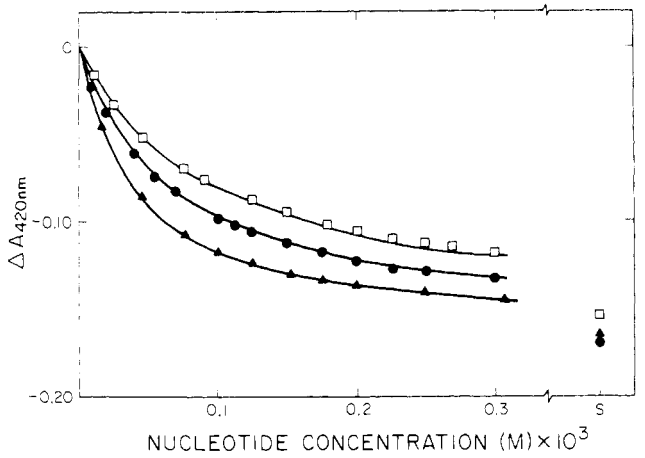

FIGURE 5: Spectrophotometric titration of quinacrine (4.4×10^{-5} M) with increasing concentrations of rat chromatin (●), P1 heterochromatin (□), and S2 euchromatin (▲). The absorption of quinacrine in 0.01 M Tris-Cl (pH 8) in the absence of nucleoproteins was 0.335 at 420 nm. "S" indicates computer fit value of absorption at infinite nucleotide concentration. Chromatin fractions were obtained after 15-min exposure to DNase.

uration are nearly identical for the nucleoprotein preparations, the spectrophotometric titrations are somewhat different. An equivalent hypochromic shift in the absorption of quinacrine was found at lower concentrations of euchromatin than heterochromatin; that is, more heterochromatin than euchromatin was required to produce the same decrease in absorption. This holds true for nonsaturating concentrations (less than 3×10^{-4} M in nucleotides). Again, quinacrine fully bound to chromatin, euchromatin or heterochromatin has essentially the same extinction at 420 nm.

SCATCHARD PLOTS. Spectrophotometric titrations were also performed under conditions where the concentration of nucleic acid was fixed and the concentration of quinacrine was varied over a 15-fold range (see Methods). The results of such titrations were plotted according to the method of Scatchard (1949). Figure 6 illustrates Scatchard plots for rat DNA, chromatin, euchromatin and heterochromatin. The intercept on the r axis (the abscissa) is $\bar{n}$, the maximal number of moles of quinacrine bound per mole of nucleotides, and the slope is $-K_a$, the association constant. The values of $\bar{n}$ obtained for DNA, chromatin and the chromatin fractions are nearly identical (Table III); the average value of $\bar{n}$ was 0.048 ± 0.002. This corresponds to about one site available for quinacrine binding per turn of double-helical "B"-form DNA.

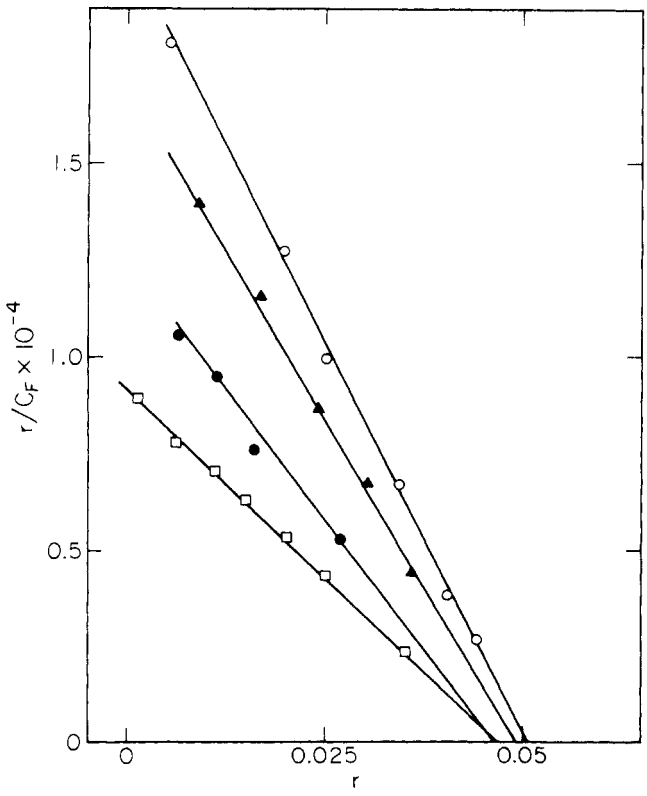

FIGURE 6: Scatchard plots of data obtained from spectrophotometric titrations of a standard amount of DNA or chromatin with increasing concentrations of quinacrine (see Methods): DNA (○); unfractionated chromatin (●); S2 euchromatin (▲); and P1 heterochromatin (□). Chromatin fractions were obtained after 15-min exposure to DNase.

TABLE III: Data from the Scatchard Plots.

Sample	K_a (10^5 M^{-1})	K_d (10^{-6} M)	$\bar{n}$ (Sites/ Nucleotide)	Sites/10 Base Pairs
DNA	4.0	2.5	0.051	1.02
Chromatin	2.6	3.9	0.047	0.94
Euchromatin	3.5	2.9	0.049	0.98
Heterochromatin	2.0	5.0	0.047	0.94

The association constants obtained from the Scatchard plots are listed in Table III. The values of K_a for the interaction of quinacrine with DNA and for quinacrine with heterochromatin differ by only a factor of two. The values of K_a for euchromatin and unfractionated chromatin lie between the values for DNA and heterochromatin. The data of the Scatchard plots appear to lie on one straight line; this indicates that only one class of binding sites for quinacrine exists in either DNA or chromatin (Blake and Peacocke, 1968). This finding was quite surprising since A·T and G·C base pairs affect the fluorescence of quinacrine so differently. From the Scatchard plots we must conclude that quinacrine binds to A·T and G·C base pairs by the same thermodynamic process. Furthermore, since the maximal number of sites available for quinacrine binding ($\bar{n}$) is the same for DNA, chromatin and the chromatin fractions, chromosomal proteins do not occupy potential binding sites. Chromosomal proteins do, however, decrease the association constant for the interaction of quinacrine with the nucleic acid in chromatin. These data are quite similar to those obtained by Simpson (1970) for the interaction of a reporter molecule with DNA and chromatin. The number of sites available for binding the reporter ($\bar{n}$) was found to be the same for DNA and chromatin; however, the association constant for the interaction of the reporter with chromatin was one-half that for the interaction of the reporter with DNA.

Pachmann and Rigler (1972) and Modest and Sengupta (1973) have also presented results of binding experiments with quinacrine and DNA. Using fluorescence polarization, Pachmann and Rigler (1972) report an association constant of 6–10 $\times 10^5$ M^{-1} and $\bar{n}$ = 0.08–0.16. These values are in close agreement with those of Figure 6 and Table III. On the other hand, Modest and Sengupta (1973) have reported values of $K = 1.6 \times 10^7$ M^{-1} and $n = 0.72$. These data were obtained by ultrafiltration and probably reflect the ionic interaction of quinacrine with DNA phosphates. The spectroscopic binding data reported herein and by Pachmann and Rigler (1972) reflect binding *via* intercalation. The second (ionic) class of binding sites was not detected in our experiments (Figure 6); higher dye to nucleotide ratios, however, have revealed this class of binding.

ESTIMATION OF BINDING PARAMETERS FROM FLUORESCENCE DATA. We have mentioned before that the binding parameters K_a and $\bar{n}$ cannot be determined independently from fluorescence titrations with natural DNAs due to the fact that A·T and G·C base pairs have different effects on quinacrine fluorescence. Hélène *et al.* (1971), however, have shown that the product of $K_a\bar{n}$ can be obtained directlyly from a titration of a fluorescent molecule with DNA. Equation 6 of Hélène *et al.* (1971) can be modified to give a general equation for the binding of fluorescent molecules to DNA

$$\phi_F/(\phi_F - \phi) = [\sum_i K_a{}^i \bar{n}_i \phi_F / \sum_i K_a{}^i \bar{n}_i(\phi_F - \phi_B{}^i)] + (1/C_{DNA})[\phi_F/\sum_i K_a{}^i \bar{n}_i(\phi_F - \phi_B{}^i)] \quad (1)$$

This equation assumes that i classes of binding sites exist. ϕ_F is the quantum yield of free quinacrine; $\phi_B{}^i$ is the quantum yield of quinacrine bound to sites of class i; and ϕ is the observed quantum yield. $\phi_F/(\phi_F - \phi)$ is equal to $1/\Delta f$, where Δf is 1 minus the quantum yield ϕ/ϕ_F. Equation 1 describes a straight-line plot of $1/\Delta f$ *vs.* $1/C_{DNA}$. Figure 7 illustrates such a plot for rat DNA and for poly(rG)·poly(rC). The ratio of y intercept to slope is $\Sigma_i K_a{}^i \bar{n}_i$. From Figure 7, $\Sigma_i K_a{}^i \bar{n}_i$ is 2.07×10^4 M^{-1} for rat DNA, and 2.21×10^4 M^{-1} for poly(rG)·poly(rC). From the Scatchard plot for rat DNA, K_a is 4.0×10^5 M^{-1} and $\bar{n}$ is 0.051. The product $(K_a\bar{n})$ is 2.04×10^4 M^{-1}. Thus by two independent measurements (fluorescence and spectrophotometric titrations), we arrive at the same value of $K_a\bar{n}$. This indicates that the values of K_a and $\bar{n}$ obtained from the Scatchard plots are reliable.

We must reemphasize that fluorescence titrations cannot be used to estimate K_a and $\bar{n}$ independently for *natural* DNAs. Binding parameters can be estimated, however, from fluorescence titrations of quinacrine with synthetic homopolymer duplexes. From a Scatchard plot of the fluorescence titration data for poly(dA)·poly(dT) (Weisblum and de Haseth, 1972), a K_a of 3.3×10^5 M^{-1} was calculated. This value is quite similar to the value of K_a calculated from the spectrophotometric titration for rat DNA (4.0×10^5 M^{-1}). Furthermore, the value of K_a for poly(rG)·poly(rC) is approximately 4.4×10^5 M^{-1}. Thus quinacrine appears to have about the same affinity for A·T and G·C base pairs. Moreover, the number of sites per nucleotide available for binding ($\bar{n}$) appear to be the same for all polynucleotides and nucleoproteins investigated ($\bar{n} = 0.048 \pm 0.002$).

DNA Conformation. CIRCULAR DICHROISM SPECTROSCOPY. In recent years circular dichroism spectroscopy has come into wide use as a tool for investigating nucleic acid conformation and protein-induced alterations in DNA conformation. We have measured the CD spectra of chromatin, euchromatin, heterochromatin, and DNA under a variety of model conditions. Figure 8 illustrates the CD spectra for these nucleoproteins and DNA isolated from rat liver. The CD spectrum of DNA in aqueous solution is characterized by a major positive band at 275 nm and a major negative band at 245 nm with a crossover at the wavelength of maximal absorption, 258 nm. A second positive band is centered at about 220 nm. The CD spectrum of chromatin differs from that of DNA in several respects. The absolute magnitude of the positive band at 275 nm is reduced from that of DNA in chromatin. By treating chromatin with deproteinizing agents (sodium dodecyl sulfate, NaCl, or proteolytic enzymes), one can transform the CD spectrum from that of chromatin to one resembling the CD spectrum of DNA (Shih and Fasman, 1970; Simpson, 1972; Henson and Walker, 1970). Below 250 nm, the major features of the CD spectrum of chromatin are the protein CD band at 220 nm and the nucleic acid shoulder at 245–250 nm.

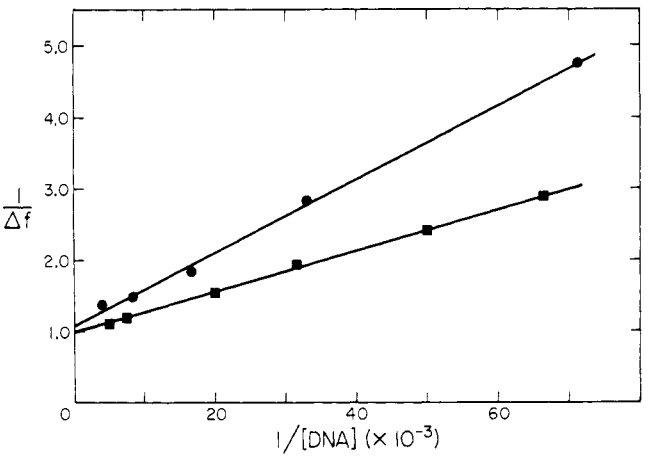

FIGURE 7: Plot of $1/\Delta f$ *vs.* 1/DNA concentration for rat DNA (●) and poly(rG)·poly(rC) (■). The data for poly(rG)·poly(rC) were obtained from Weisblum and de Haseth (1972). $1/\Delta f$ is defined as the inverse of (1 − relative fluorescence intensity). The value of $\Sigma_i K_a{}^i \bar{n}_i$ was 2.21×10^4 M^{-1} for poly(rG)·poly(rC) and 2.07×10^4 M^{-1} for rat DNA.

The reduced value of the CD band at 275 nm has been interpreted by many authors as evidence for the altered conformation of DNA in chromatin. It has been suggested that the DNA in chromatin is partially in the "C" conformation (Johnson *et al.*, 1972). DNA is thought to exist in the "C" conformation under a variety of conditions; for example, Li salts of DNA at 95% relative humidity (Marvin *et al.*, 1961; Tunis-Schneider and Maestre, 1970) and DNA in ethylene glycol

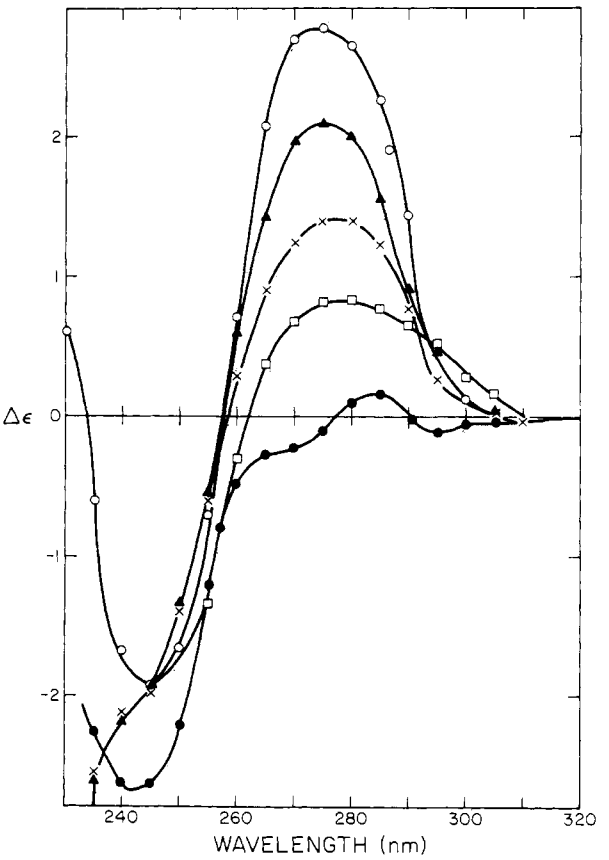

FIGURE 8: Circular dichroism spectra of rat DNA (○), chromatin (X), S2 euchromatin (▲), P1 heterochromatin (□) in 0.01 M Tris-Cl (pH 8) and rat DNA in 90% ethylene glycol (●). The chromatin fractions were obtained after 15-min exposure to DNase.

TABLE IV: CD and Nucleic Acid Conformation.

Sample	$\Delta\epsilon_{275\ nm}$	% B conformation
DNA, aqueous solution	2.64	100
DNA, 90% ethylene glycol	−0.01	0 ("C" form)
Chromatin	1.40	53
Euchromatin	2.00	76
Heterochromatin	0.83	32

(Nelson and Johnson, 1970) show characteristics of the "C" form. The CD spectrum of DNA in ethylene glycol (Figure 8 and Nelson and Johnson, 1970) is nearly identical the spectrum of films of Li salt DNA known to be in the "C" form (Tunis-Schneider and Maestre, 1970). We will therefore refer to the CD spectrum of DNA in ethylene glycol as a "C"-form spectrum. The B → C transition is characterized by an almost complete loss of the 275-nm positive band. Minor CD bands are seen at 284 and 295 nm, respectively. A negative shoulder is seen at 265–270 nm. The major negative band of DNA is increased in the "C"-form spectrum; the value of $\Delta\epsilon_{245\ nm}$ for "B"-form DNA is −1.96, while $\Delta\epsilon_{245\ nm}$ in the "C"-form spectrum is −2.67.

The values of $\Delta\epsilon_{275\ nm}$ for DNA ("B"- and "C"-form spectra) and the nucleoprotein samples are listed in Table IV. The spectrum of euchromatin is most like that of "B"-form DNA while heterochromatin exhibits a spectrum more like that of the "C"-form of DNA. The spectrum of unfractionated chromatin is intermediate between those of euchromatin and heterochromatin. Johnson *et al.* (1972) have assigned a per cent "B" contribution to chromatin DNA from the CD spectrum. If we assume that all the base pairs of rat DNA in aqueous solution are in the "B" form, and that all the base pairs of rat DNA in 90% ethylene glycol are in the "C" form, then we can ascribe a per cent "B"-form conformation to the nucleic acid of the nucleoprotein samples. Since protein does not contribute to the CD spectrum of DNA above 250nm, we make use of $\Delta\epsilon_{275\ nm}$ in making the assignment of conformation. Our only reservation in making these assignments is that we do not know what effects, if any, light scattering may have on the circular dichroism spectrum of chromatin (Dorman and Maestre, 1973). The heterochromatin fraction (P1) showed considerably more light scattering than either euchromatin or unfractionated chromatin ($A_{320}/A_{260} \geq 0.1$ for heterochromatin; $A_{320}/A_{260} < 0.02$ for euchromatin and unfractionated chromatin). It is clear that the CD spectrum of heterochromatin does not fit either the "B"- or "C"-form spectra above 285 nm; this tailing of the positive CD band to longer wavelengths is most likely due to light scattering (Dorman and Maestre, 1973). Thus, we do not know to what degree our assignment of per cent "B" conformation to heterochromatin DNA is influenced by scattering artefacts; nevertheless, the conformations of DNA in the chromatin fractions are clearly different (Polacow and Simpson, 1973).

Conclusions

In this study, we have concerned ourselves with the biophysical basis of differential staining of chromosomes with quinacrine. We have chosen to work with fractionated interphase heterochromatin and euchromatin. We have shown that the quantum yield of quinacrine bound to either unfractionated chromatin or heterochromatin is significantly greater than the quantum yield of quinacrine bound to either euchromatin or DNA in aqueous solution. Deproteinization abolishes this differential fluorescence; furthermore, no differences in DNA base composition were found between the euchromatin and heterochromatin fractions. These results cast doubt on whether the banding patterns seen with quinacrine-stained chromosomes are solely a reflection of intrachromosomal differences in DNA base composition. It must be stressed, however, that this work *does not* dispute the findings of Weisblum and de Haseth (1972) and Pachmann and Rigler (1972) pertaining to the *in vitro* base specificity of quinacrine fluorescence with purified nucleic acids. In fact, the very sharp (and very narrow) fluorescent bands seen on the Y chromosome of *Drosophila* (Vosa, 1970) could indicate the localization of (A + T)-rich satellite DNA.

Quinacrine bands are generally thought to be localized in the heterochromatic regions of chromosomes (Vosa, 1970; Adkisson *et al.*, 1971; Gagné *et al.*, 1971). Our results suggest that the conformation of the DNA in the heterochromatic portion of chromosomes is altered from that of the "B" to the "C" form. Chromosomal proteins, in particular the histones, are thought to induce and maintain this change in nucleic acid conformation (Simpson, 1972; Shih and Fasman, 1972; Henson and Walker, 1970; Simpson and Sober, 1970). Furthermore, our results suggest that quinacrine bound to heterochromatin is highly fluorescent while quinacrine bound to euchromatin is only weakly fluorescent. The alternation of heterochromatic and euchromatic regions along the chromatids of metaphase chromosomes would, therefore, lead to fluorescence banding patterns. Caspersson *et al.* (1972) have shown that the mitotic chromosomes of several cell types of the same organism have the same banding patterns. Thus, banding does not reflect the genetic activity of cells *per se*. More likely, banding reflects the ordered packaging of DNA into the chromosome.

One of the most remarkable features of chromosome banding techniques is the similarity of bands produced by a variety of methods. Lee *et al.* (1973) have identified four groups of techniques which produce similar patterns; these are (1) quinacrine fluorescence; (2) Giemsa in combination with alkali-heating techniques; (3) Giemsa in combination with any one of several proteolytic enzymes; and (4) Giemsa in combination with protein-denaturing substances such as 5 M urea and anionic and nonionic detergents. Until recently, it was thought that the Giemsa-alkali heating technique (number 2 above) reflected the differential chromosomal localization of classes of repetitious and nonrepetitious DNA. Comings *et al.* (1973) and Stockert and Lisanti (1972) have shown that *in situ* renaturation of chromosomal DNA (repetitive and single copy) is complete within a few minutes. Thus, preferential reassociation of repetitious DNA cannot explain the banding patterns produced with Giesma. Comings *et al.* (1973) have also shown that the G-banding techniques remove very little DNA or protein from the chromosomes. Harsh treatment of chromosomes with alkali or prolonged exposure to proteolytic enzymes abolishes G bands altogether.

The results of G-banding techniques employing mild treatment with proteolytic enzymes and detergents tend to support the idea that chromosome banding patterns do not reflect intrachromosomal differences in DNA base ratio. These agents (enzymes and denaturants) presumably act only on the protein component of chromosomes. The fact that these techniques give rise to banding patterns which are similar to those obtained with quinacrine support the conclusion that variations in protein–DNA interactions along the chromatids of metaphase chromosomes are responsible for banding. Recently, Rodman and Tahiliani (1973) have shown that Feulgen staining of mouse chromosomes reveals a banding pattern similar to that

obtained with Giemsa or quinacrine-mustard. These authors postulate that "the localization of Feulgen dark and light stain, representing relative DNA densities, reflects the regional protein association of the DNA."

In conclusion, we suggest that chromosome banding with quinacrine reflects differences in protein-DNA interactions, and DNA conformation, along the chromatids of metaphase chromosomes.

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References

Adkisson, K. P., Perreault, W. J., and Gay, H. (1971), *Chromosoma 34,* 190-205.

Billing, R. J., and Bonner, J. (1972), *Biochim. Biophys. Acta 281,* 453-562.

Blake, A., and Peacocke, A. R. (1968), *Biopolymers 6,* 1225-1253.

Bonner, J., Garrard, W. T., Gottesfeld, J., Billing, R. J., and Uphouse, L. (1974), *Methods Enzymol.* (in press).

Bonner, J., Garrard, W. T., Gottesfeld, J., Holmes, D. S., Sevall, J. S., and Wilkes, M. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* (in press).

Caspersson, T., De la Chapelle, A., Schröder, J., and Zech, L. (1972), *Exp. Cell Res. 72,* 56-59.

Caspersson, T., Farber, S., Foley, G. E., Kudynowski, J., Modest, E. J., Simonsson, E., Wagh, V., and Zech, L. (1968), *Exp. Cell Res. 49,* 219-222.

Caspersson, T., Zech, L., Modest, E. J., Foley, G. E., Wagh, V., and Simonsson, E. (1969), *Exp. Cell Res. 58,* 128-140.

Comings, D. E., Avelino, E., Okada, T. A., and Wyandt, H. E. (1973), *Exp. Cell Res. 77,* 469-493.

Davis, R. W., Simon, M., and Davidson, N. (1971), *Methods Enzymol. 21D,* 413-428.

Dorman, B., and Maestre, M. F. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 255-259.

Elgin, S. C. R., and Hood, L. (1973), *Biochemistry 12,* 4984-4991.

Ellison, J. R., and Barr, H. J. (1972), *Chromosoma 36,* 375-390.

Gagné, R., Tanguay, R., and Laberge, C. (1971), *Nature (London), New Biol. 232,* 29-30.

Gottesfeld, J. M. and Bonner, J. (1974), in preparation.

Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F., and Bonner, J. (1974), *Proc. Nat. Acad. Sci. U. S.* (in press).

Hélène, C., Dimicoli, J.-L., and Brun, F. (1971), *Biochemistry 10,* 3802-3809.

Henson, P., and Walker, I. O. (1970), *Eur. J. Biochem. 16,* 524-531.

Johnson, R. S., Chan, A., and Hanlon, S. (1972), *Biochemistry 11,* 4347-4358.

Lee, C. L. Y., Welch, J. P., and Lee, S. H. S. (1973), *Nature (London), New Biol. 241,* 142-143.

Lerman, L. S. (1963), *Proc. Nat. Acad. Sci. U. S. 49,* 94-101.

Lomholt, B., and Mohr, J. (1971), *Nature (London), New Biol. 234,* 109-110.

Mandel, M., and Marmur, J. (1968), *Methods Enzymol. 12B,* 195-206.

Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15,* 160-174.

Marvin, D. A., Spencer, M., Wilkens, M. H. F., and Hamilton, L. D. (1961), *J. Mol. Biol. 3,* 547-565.

Modest, E. J., and Sengupta, S. K. (1973), *in* Chromosome Identification, Nobel Symposium 23, Caspersson, T., and Zech, L., Ed., New York, N. Y., Academic Press, pp 327-333.

Nelson, R. G., and Johnson, W. C. (1970), *Biochem. Biophys. Res. Commun. 41,* 211-216.

Pachmann, U., and Rigler, R. (1972), *Exp. Cell Res. 72,* 602-608.

Polacow, I., and Simpson, R. T. (1973), *Biochem. Biophys. Res. Commun. 52,* 202-207.

Rodman, T. C., and Tahiliani, S. (1973), *Chromosoma 42,* 37-56.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51,* 660-672.

Schreck, R. R., Warburton, D., Miller, O. J., Beiser, S. M., and Erlanger, B. F. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 804-807.

Shih, T. Y., and Fasman, G. D. (1970), *J. Mol. Biol. 52,* 125-129.

Simpson, R. T. (1970), *Biochemistry 9,* 4814-4819.

Simpson, R. T. (1972), *Biochemistry 11,* 2003-2008.

Simpson, R. T., and Sober, H. A. (1970), *Biochemistry 9,* 3103-3109.

Stockert, J. C., and Lisanti, J. A. (1972), *Chromosoma 37,* 117-130.

Tunis-Schneider, M. J., and Maestre, M. F. (1970), *J. Mol. Biol. 52,* 521-541.

Vosa, C. G. (1970), *Chromosoma 30,* 366-372.

Weisblum, B., and de Haseth, P. L. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 629-632.