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Differential Fluorescent Staining of *Drosophila* Chromosomes with Quinacrine Mustard* **

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Abstract. The polytene and mitotic chromosomes of *D. melanogaster*, *D. simulans*, *D. ananassae*, and *D. virilis* were stained with the fluorescent dye, quinacrine mustard (QM). In all these species except *D. ananassae*, we have detected species-specific chromosomal loci which exhibit an extremely brilliant fluorescence. Most, but not all, of the brilliantly fluorescing areas are located in heterochromatic chromosome regions. Cytochemical and chemical methods have been employed to demonstrate that the brilliant fluorescence represents regions of acid labile non-covalent binding between DNA and QM whereas the moderate overall fluorescence is primarily due to covalent bonding (by alkylation) of the QM to DNA. The exact mode of binding of QM in the brilliant areas and the nature of the DNA in these areas are not known. The possible biological significance of the DNA in the brilliant regions is discussed.

Introduction

Although heterochromatin in *Drosophila* has been recognized and studied for over forty years, its functional role is largely unknown. In *Drosophila melanogaster*, heterochromatin was initially regarded as genetically inert because nearly all of the hundreds of known genes were located in euchromatin. In spite of this dearth of functional genes, pronounced genetic effects can be attributed to heterochromatin in the phenomenon of heterochromatin-induced position effect (see reviews by Lewis, 1950; and Hannah, 1951).

At the present time, Heitz's (1928) original cytological definition of heterochromatin as darkly staining, highly condensed chromatin, which remains in the condensed state throughout the cell cycle, still serves to distinguish it from euchromatin. More recent work has shown that heterochromatin can also be distinguished from euchromatin by

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** This paper is dedicated with respect and affection to Professor Berwind P. Kaufmann on the occasion of his 74th birthday, April 23, 1971.

its time of DNA replication, since the DNA of heterochromatin replicates later in the synthetic period than euchromatin (Lima-de-Faria and Jaworska, 1968). Except for these distinguishing characteristics, very little that is helpful for an understanding of its genetic function is known.

However, staining of *Drosophila* chromosomes with the fluorescent dye, quinacrine mustard (QM), may shed some light on this problem because QM appears to be capable of distinguishing qualitative differences in the DNA of heterochromatin and euchromatin.

The binding of this fluorochrome to plant and mammalian chromosomes has been studied by Caspersson *et al.* (1968, 1969a, 1970), and they have noted marked differences in the intensity of QM fluorescence along the length of some chromosomes. These authors have hypothesized that regions of intense fluorescence may represent areas of the chromosome which contain a relatively guanine-rich DNA. This interpretation is supported by the known affinity of alkylating agents (such as QM) for the N-7 atom of guanine in DNA (Lawley and Wallick, 1957; Lawley, 1958; Lawley and Brooks, 1960), and by the observation that binding of QM to chromosomes is probably mediated largely by the alkylating group of the mustard moiety (Caspersson *et al.*, 1969a).

Whatever the precise binding mechanism may be, it is of great interest to note that the most intensely fluorescent regions coincide with sections of chromosomes that have been identified cytologically or cytochemically as heterochromatin (Caspersson *et al.*, 1969a, b). It is possible then, that QM may have a special affinity for heterochromatin or a certain class of heterochromatin.

In the experiments described in this paper we have investigated the possibility that QM may be a useful dye for the identification and characterization of heterochromatic regions in *Drosophila* polytene and mitotic chromosomes. Cytochemical tests have been used to determine whether components of the chromosomes other than DNA are involved in the binding of QM, especially in the highly fluorescent areas. In addition, we have studied the binding of QM to purified DNA and its components in solution, under conditions that approximate the cytological staining conditions.

Materials and Methods

1. Cytological Preparations

The *Drosophila* species used in this study were *D. melanogaster* [Swb-9 and T(X:4)$w^{m258-21}$ stocks], *D. simulans*, *D. ananassae*, and *D. virilis*.

Mitotic chromosomes of neural ganglia and polytene chromosomes of salivary glands were prepared from late third-instar larvae. The ganglia and salivary glands were fixed in 45% acetic acid for 3 min and squashed on slides in 45% acetic acid. The preparations were then frozen on "dry ice". After 20–30 min the cover slips

were removed and the slides bearing the tissue were placed in absolute ethanol at 4° C for storage.

After rehydration from absolute ethanol, the chromosome preparations were either stained with QM or subjected to one or another of the treatments described below and then stained with QM.

Staining Procedure. The QM (Sterling Winthrop Research Institute) was used at a concentration of 50 μg/ml in MacIlvaine's disodiumphosphate/citric acid buffer at pH 4 for 20 min at 24° C. Before placing a slide in the staining solution it was rinsed in the buffer, and after staining the cells, the slides were rinsed in 3 changes of buffer and mounted for observation in the buffer.

DNA Removal. This was effected by placing the slide in a solution of DNase I (Worthington), 0.1 mg/ml in 0.003 M $MgSO_4$ at pH 6 for three hours at 24° C. Control preparations were stained by the DNA-specific Feulgen procedure to determine the effectiveness of the enzyme treatment.

RNA Removal. Slides were immersed in RNase A (Worthington, code RAF), 0.3 mg/ml in distilled water adjusted to a pH of 6.5 for 3 hours at 24° C. Previous autoradiographic experiments had shown that this treatment was sufficient to remove nearly all detectable RNA from polytene chromosomes.

Depurination. Purines were removed by hydrolysis in 1 N HCl at 60° C for 12 min.

Protein Removal. Proteins were removed by digestion in trypsin or pepsin. Trypsin (Worthington) was used at a concentration of 0.1 mg/ml in disodiumphosphate/citric acid buffer at pH 6.2 for 30 min at 24° C. Pepsin (4× crystallized, salt free) was used at a concentration of 0.1 mg/ml in 0.02 N HCl for 1–1½ hours at 24° C. Control preparations were stained with acid fast green after enzyme treatment to determine the effectiveness of the digestion.

Observations were made with a Zeiss fluorescence microscope with an HBO-200 mercury lamp, exciter filters BG-38 and BG-12 and barrier filters 53 and 44.

2. *Chromatography*

Samples of several commercially available DNAs (calf thymus, salmon sperm and chicken blood) were purchased from California Biochemicals, Inc. *D. melanogaster* DNA was extracted in our laboratory by the Laird and McCarthy (1968) procedure. *Micrococcus lysodiekticus* DNA was isolated by the Marmur (1961) method and *Tetrahymena pyriformis* DNA was a gift of Dr. Sally L. Allen. All DNA samples were dissolved in standard saline-citrate (0.15 M NaCl and 0.015 M Na-citrate) and adjusted to 1 mg/ml. Nucleobases, nucleosides and other DNA components were obtained from California Biochemicals, Inc. These substances were dissolved in disodiumphosphate/citric acid buffer at pH 7, and brought to a concentration of 4 mg/ml, with the exception of guanine which saturated at a considerably lower concentration.

Depurination of extracted DNA was effected by the addition of an equal volume of 2 N HCl to the DNA samples followed by a 30 min incubation at 60° C. The solution was chilled to 4° C and centrifuged for 10 min at 7000 rpm in the Sorvall SS-34 rotor. The supernate was decanted and the precipitate was washed twice with ethanol-ether (3:1), air dried and redissolved in standard saline-citrate.

Samples (0.5 ml) of each of the DNA's were reacted with 0.1 ml of QM (500 μg/ml in pH 7 buffer) for 20 min at 37° C. The DNA was precipitated by the addition of two volumes of ethanol, and collected by centrifugation. The precipitate was resuspended in 0.5 ml of distilled water for chromatography.

Chromatography was carried out on Whatman No. 1 papers using either 65% isopropanol in 2 N HCl or 30% butanol in water.

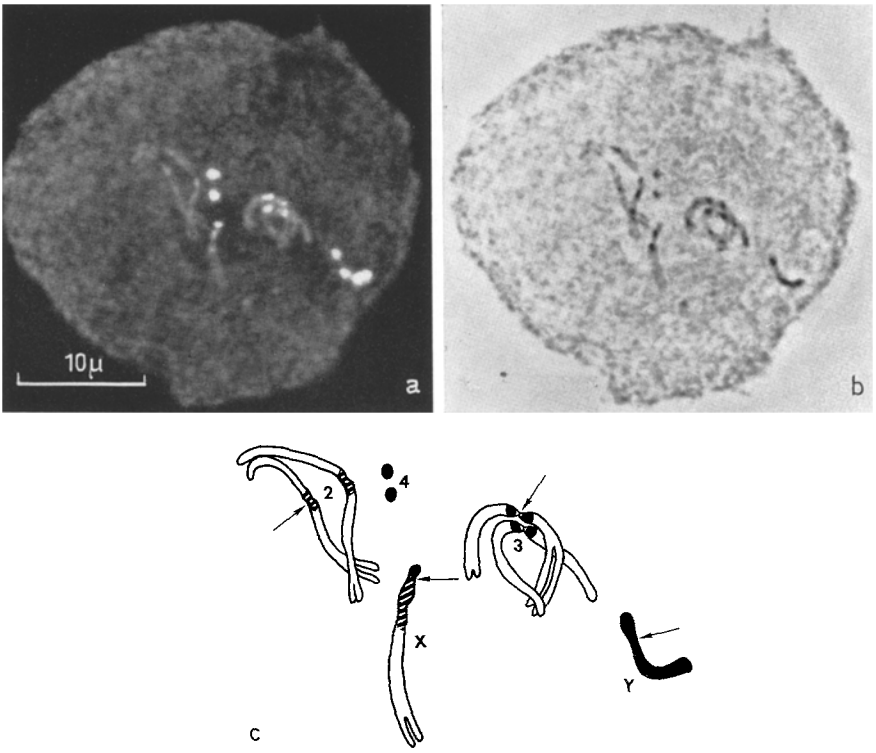

Fig. 1a–c. Late prophase chromosomes from a neural ganglion cell of *D. melanogaster*. a Stained with QM. b Phase contrast. c Diagram of chromosomes in a and b. Areas which stain brilliantly with QM are solid black. These are all heterochromatic with the exception of the fourth chromosomes. Areas of heterochromatin which do not stain brilliantly are striped and euchromatin is white. Arrows point to the location of the centromeres

Results

1. Fluorescence in Mitotic Chromosomes after QM Staining

In all the species of *Drosophila* studied, except *D. ananassae*, the condensed chromosomes exhibit small areas of extremely brilliant fluorescence which contrast strongly with an overall moderate chromosomal fluorescence (see Figs. 1 and 2).

Generally, the brilliant fluorescence is localized in recognizable heterochromatic areas of the *D. melanogaster* late prophase chromosomes. These areas are the short arm of the X chromosome, the whole Y chromosome and the centromeric heterochromatin of the third chromosome. However, the tiny fourth chromosome which contains no cytologically detectable heterochromatin (Kaufmann, 1934) appears to be brilliant

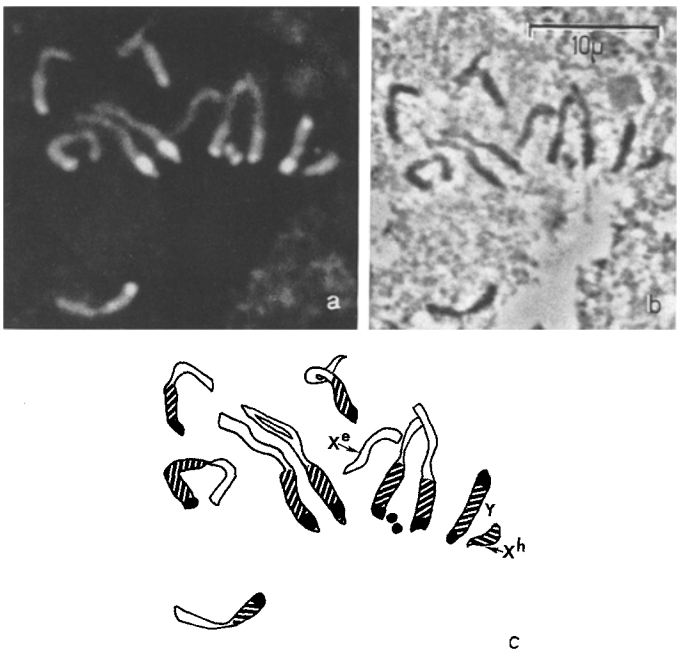

Fig. 2a–c. Late prophase chromosomes from a neural ganglion cell of *D. virilis*. a Stained with QM. b Phase contrast. c Diagram of chromosomes in a and b. Heterochromatic areas which stain brilliantly with QM are solid black. Heterochromatin which is not brilliantly stained is striped and euchromatin is white. During squashing the heterochromatic half of the X chromosome (X^h) was separated from the euchromatic half (X^e)

in its entirety; and in contrast, two sites that are heterochromatic, the centromeric heterochromatin of the second chromosome and the proximal heterochromatin of the X chromosome, have only a moderate degree of fluorescence (Fig. 1). The mitotic chromosomes of *D. simulans* show a fluorescence pattern that is virtually identical to that of *D. melanogaster*.

In *D. virilis* mitotic chromosomes the brilliant areas are also localized in recognizable heterochromatic regions. In this species the proximal half of all the chromosomes and the whole Y chromosome are seen to be heterochromatic at late prophase (Makino, 1940). A small portion of this heterochromatin, which is located near the centromere, in all the rod-shaped chromosomes except the X, is brilliantly fluorescent and the Y chromosome has bright spots on the centromeric end as well as on the distal tip. The tiny dot chromosomes have a moderate to

bright fluorescence, often equivalent to that found in the highly fluorescent areas (Fig. 2).

The interphase nuclei of the three species which exhibit highly fluorescent regions in their mitotic chromosomes invariably display one or more brilliant areas which correspond to the chromocenters as seen in acetic-orcein stained preparations. Since the interphase chromocenters represent the fused heterochromatic regions of the mitotic chromosomes, the brilliance of these regions after QM staining is probably due to the same areas that are highly fluorescent in the mitotic chromosomes. We conclude then, that the characteristic brilliant fluorescence is present in all stages of the mitotic cycle.

2. Fluorescence in Polytene Chromosomes after QM Staining

The polytene chromosomes of all *Drosophila* species studied are characterized by a moderate fluorescence in the bands and very little or no fluorescence in the interbands. In addition, however, the chromosomes of three of these species (*D. melanogaster*, *D. simulans*, and *D. virilis*) possess one or more bands which are readily distinguished by their extremely brilliant fluorescence, as well as small areas which contain brilliant material in the form of droplets (see Fig. 3). The exceptional species is *D. ananassae*, whose polytene chromosomes, like the mitotic chromosomes, are devoid of brilliantly fluorescing areas.

The location of the brilliant areas in *D. melanogaster* polytene chromosomes (Fig. 3) corresponds fairly well to those found in the mitotic chromosomes. There is a brilliant doublet band near the centromere of the right arm of the third chromosome in section 81F1,2 and the fourth chromosome has a bright band in section 101F near the centromere, another bright band in 102D, and the tip (102EF) is covered with bright droplets. It should be pointed out that these droplets were not present in prepupae or in larvae which were very close to the prepupal stage. It is also interesting to note that when the fourth chromosome is translocated onto the X chromosome [in the T(X:4) stock], there is a noticeable increase in the amount of brightly fluorescing material in region 101F (Fig. 4). In addition, there are bright droplets of varying size and number in the centromeric heterochromatin. It is possible that some of these droplets represent the Y chromosome and the short arm of the X chromosome since these chromosomes which do not participate in the process of polytenization are known to be located in the chromosomal heterochromatic mass.

The correspondence between the location of brightly fluorescent areas of the polytene chromosomes and the location of heterochromatin is less obvious than in the mitotic cells, except for the bright droplets which are localized in the centromeric heterochromatin. However, the

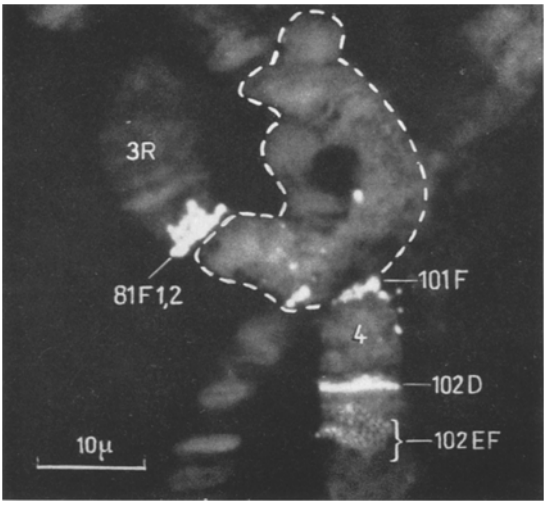

Fig. 3. *D. melanogaster* polytene chromosomes stained with QM. Arrows point to the brilliant bands. Bright droplets are present in the centromeric heterochromatin outlined with a dashed line. Bright droplets fill the tip (102EF) of chromosome 4

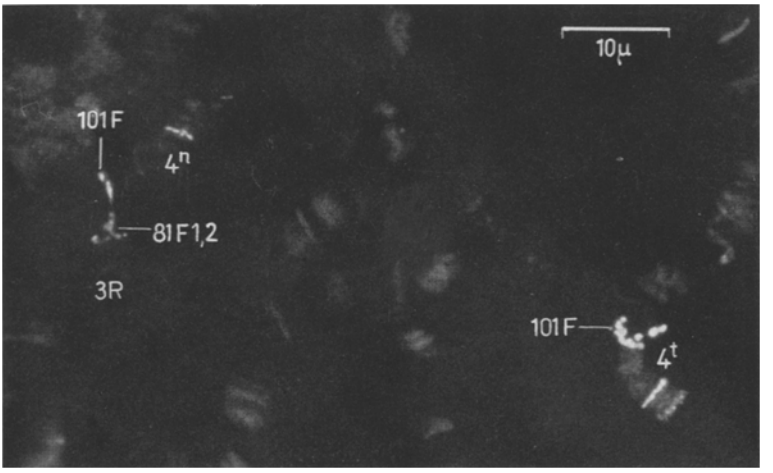

Fig. 4. Polytene chromosomes from *D. melanogaster* stock T(X:4)$w^{m258-21}$ stained with QM. Here one fourth chromosome (4^t) has been translocated on to the X chromosome. In this position 4^t has more brightly stained material in 101F than its homolog (4^n) which is located in its normal position

brilliant bands of the fourth and 3R chromosomes may be intercalary heterochromatin since they often pair ectopically with each other and with the bright droplets in the centromeric heterochromatin.

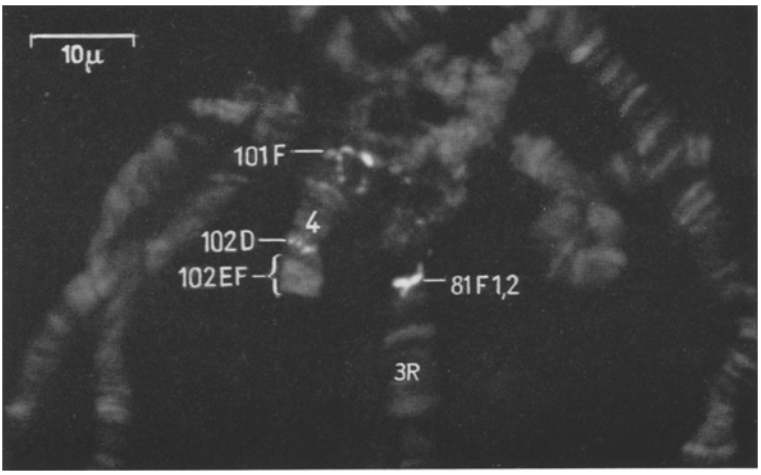

Fig. 5. *D. simulans* polytene chromosomes stained with QM

D. simulans exhibits brilliant areas in the polytene chromosomes that are essentially identical to those described for *D. melanogaster* except that the tip of the fourth chromosome has little bright material and it is not localized into discrete droplets (Fig. 5).

In *D. virilis* there appears to be little relationship between the location of bright areas in the polytene and mitotic chromosomes, for there is only one brilliant area, a band located in the centromeric end of chromosome 4 (Fig. 6). In this species the centromeric heterochromatin can be easily differentiated into two types α- and β-heterochromatin (Fujii, 1942). In some preparations the α-heterochromatin is obviously brighter than the β-heterochromatin and the banded euchromatin (Fig. 7). It is possible that the α-heterochromatin of polytene cells is homologous to the heterochromatic segments of the mitotic chromosomes (Fujii, 1942). If so, the brightness of the α-heterochromatin may be caused by chromatin homologous to the brilliant ends of the mitotic chromosomes.

3. Results of Enzymatic Treatments

Chromosomes are complex structures composed of DNA, RNA and proteins. Since all these components are potentially capable of binding to QM we have enzymatically removed one or another of these substances from the chromosomes to determine which one(s) are responsible for the characteristic fluorescent patterns described above. Treatment of both mitotic and polytene chromosomes with DNase results in extreme diminution or total loss of fluorescence in both brilliantly

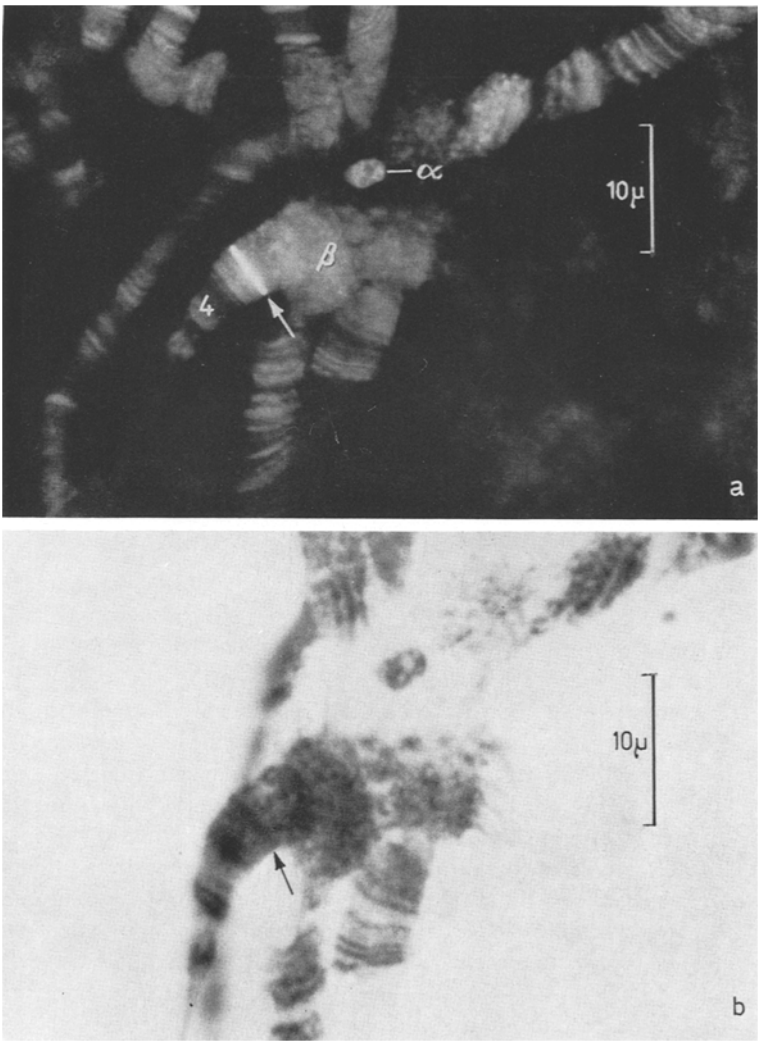

Fig. 6a and b. *D. virilis* polytene chromosomes. a Stained with QM. The centromeric heterochromatin is divisible into α- and β-heterochromatin. b Stained with Feulgen. The band in chromosome 4 which stains brilliantly with QM is not more heavily stained than other bands in these chromosomes

and moderately fluorescing regions. The digestion of RNA from both polytene and mitotic chromosomes or proteins from polytene chromosomes had essentially no effect on the intensity of either the brilliant or moderate fluorescence. We conclude, therefore, that it is the DNA

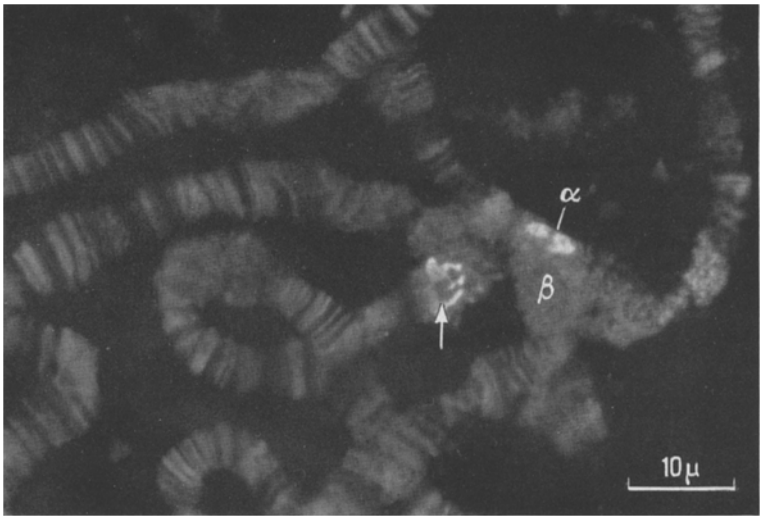

Fig. 7. *D. virilis* polytene chromosomes showing α-heterochromatin highly stained with QM. The arrow points to the brilliant band in chromosome 4. Its appearance is due to smearing during the squash procedure

component of these chromosomes which is the predominant site of binding to QM in both the brilliantly and moderately fluorescing regions.

4. Results of Acid Treatments

Caspersson *et al.* (1969a), have obtained indications of at least two different modes of binding of QM to chromosomal DNA. The first mode, which is characteristic of the bright bands seen in some plant chromosomes, is acid labile (5 min in 1 N HCl), and is presumed to represent non-covalent bonding (intercalation or ionic bonding) of the QM molecule to chromosomal DNA. The second mode of binding is stable to this mild acid treatment and probably represents covalent bonding to one or more of the DNA bases, most likely alkylation of the N-7 of guanine.

To determine whether the brilliant areas in the *Drosophila* chromosomes represent regions of differential QM binding we repeated the mild acid treatment used by Caspersson *et al.* (1969a). Polytene chromosome preparations were stained with QM and examined to determine that brilliant areas were present. When the same preparations were then treated for 5 min in 1 N HCl at 24° C or 11° C, the brilliant areas were no longer distinguishable from the surrounding chromosome regions in intensity of fluorescence. The moderate overall fluorescence of the

chromosomes was apparently unaffected by this treatment. Subsequent restaining of the chromosomes with QM is possible and the brilliant areas reappear with the same intensity as before the acid treatment. The process of staining and removal of stain by HCl can be repeated on the same preparation without noticeable change in the relative intensities of the brilliant and moderate regions. We have noted that even milder acid treatments may be used (1 min in 1 N HCl, or 5 min in 0.1 N HCl) to achieve the same result, namely, the abolition of the brilliant fluorescence without obvious effect on the moderate overall fluorescence.

The interpretation which we place on these experiments is that the brilliantly fluorescing regions of *Drosophila* chromosomes, like the bright bands seen in some plant chromosomes, are produced as the result of an acid labile binding of QM to DNA. The ease with which the QM is removed from these areas and then restored by subsequent restaining suggests some as yet unknown non-covalent binding site on the DNA molecule in these regions.

The general moderate fluorescence is most likely the result of alkylation of the DNA purines since depurination of the chromosomes either before or after staining with QM results in nearly a complete loss of fluorescence in the moderate and brilliant regions. This conclusion is supported by our finding that depurinated DNA in solution does not bind to QM (see chromatography results).

5. Determination of DNA Content or Compaction in Highly Fluorescent Areas

As Caspersson *et al.* (1968, 1969a) have made clear, differential QM fluorescence may reflect varying concentrations of DNA along the length of the chromosomes. Although we did not make quantitative measurements of the DNA/unit chromosome, phase contrast microscopy and Feulgen staining of polytene chromosomes show that the highly fluorescent areas are neither particularly dense (Fig. 8), nor are they regions of exceptionally high Feulgen stainability (see Fig. 6).

Phase contrast microscopy of the mitotic chromosomes of *D. melanogaster* and *D. virilis* shows that in general the denser heterochromatic regions can be divided into two types on the basis of their staining with QM (see Figs. 1 and 2). One type fluoresces very brilliantly and the other type fluoresces slightly more strongly than the euchromatin. This latter property is probably due to the greater compaction of the DNA in heterochromatin, but the brilliant spots are difficult to explain on this basis since their loci are not obviously more condensed than the other heterochromatic areas. Thus, in *Drosophila* as in plant material (Caspersson *et al.*, 1968, 1969a) the presence of brilliant fluorescing areas

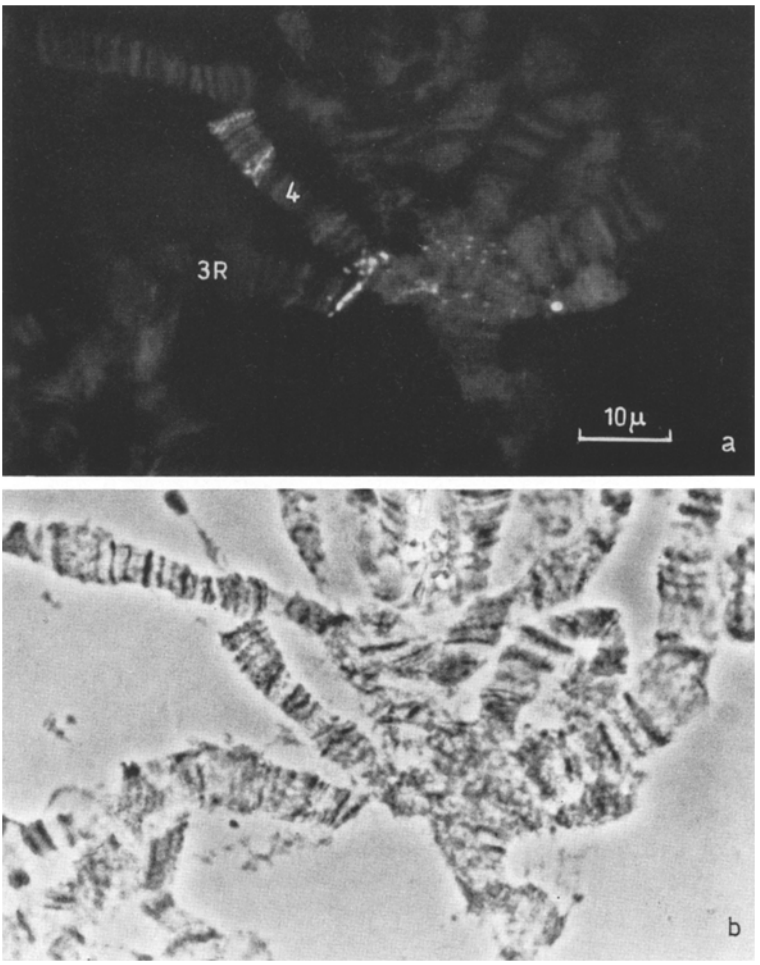

Fig. 8a and b. Polytene chromosomes of *D. melanogaster*. a Stained with QM. The fourth chromosome is unusually long due to stretching during the squash procedure. b Phase contrast of the same preparation in a. The brightly stained areas are not unusually dense

can not be explained as simply the compaction or high concentration of DNA within these regions.

6. Chromatography

Although there is abundant experimental information concerned with the binding of alkylating agents (in general) to DNA, there is

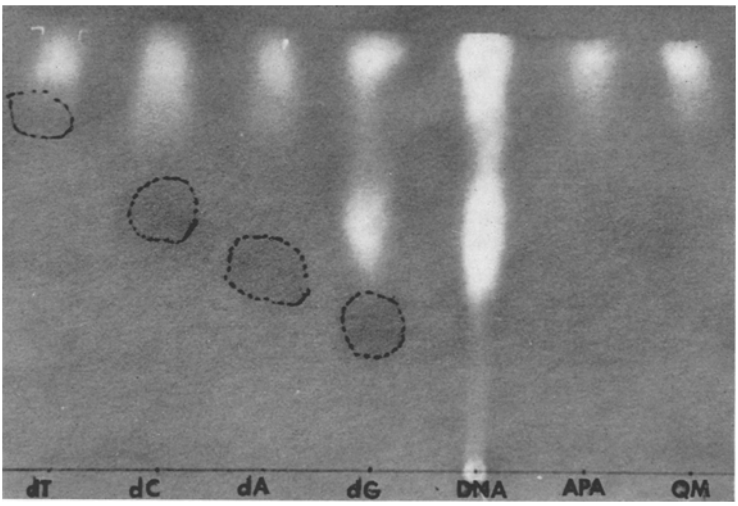

Fig. 9. Paper chromatogram showing the products of the reaction of DNA, depurinated DNA (APA), and four deoxyribonucleosides (*dT*, *dC*, *dA*, *dG*) with QM. The action of the solvent (isopropanol-HCl) releases a highly fluorescent spot from DNA which has an R_f identical to that of *dG* reacted with QM. Depurinated DNA does not react to any significant extent with QM, nor do *dT*, *dC*, and *dA*. QM alone runs at the solvent front. Dotted lines encircle the UV absorbing spots of *dT*, *dC*, *dA*, and *dG*

virtually no information specific for QM. Therefore we have made a chromatographic analysis of the reaction of QM and DNA or its components in solution to determine which parts of the DNA are potentially capable of binding to QM.

When native DNA and depurinated DNA were reacted with QM and the solutions chromatographed in isopropanol-HCl, the reacted DNA yielded a bright fluorescent spot (under UV irradiation) with an R_f of about 0.6, but the depurinated DNA sample had no corresponding spot (Fig. 9). In this solvent system QM moves very rapidly, having an R_f greater than 0.9. Control DNA (unreacted with QM) remained at the origin, but did display two UV absorbing spots with R_f's corresponding to those of adenine and guanine. These two purines are no doubt hydrolyzed from the DNA molecule by the HCl of the chromatography solvent. When the same experiment was carried out using chromatography solvents which should not release purines (water or water-butanol), most of the fluorescence either moved with the solvent front or remained at the origin with the DNA.

Our interpretation of these results is that the fluorescent spot seen after chromatography of the QM-DNA solution (in isopropanol-HCl)

is due to one or both of the released purines bound to QM. This was verified in other experiments which have shown that of all the nucleic acid components (sugars, bases, nucleosides and nucleotides) only guanosine, deoxyguanosine, guanosine 2′(3′)-monophosphate and to a much lesser extent deoxyadenosine and adenosine 2′(3′)-monophosphate were capable of significant binding to QM within 20 min. Also the R_f of the fluorescent spot seen after guanosine was reacted with QM is identical to that of the fluorescent spot seen when DNA alone is reacted with QM (see Fig. 9).

We feel that the rapid reaction of QM with native DNA in solution and the demonstrated affinity of QM for guanine derivatives supports our interpretation that the overall moderate fluorescence of the cytological preparations is due to alkylation of guanine in chromosomal DNA.

Discussion

These experiments have shown that QM can distinguish a portion of the known heterochromatin of several species of *Drosophila*, for with the exception of the mitotic fourth chromosome in *D. melanogaster* and possibly the few bright bands of the polytene chromosomes, the brilliantly stained material is localized in cytologically defined heterochromatin. It is important to note, however, that not all heterochromatin stains brilliantly.

At this time we believe that QM is bound directly to DNA in the bright areas as well as in the rest of the chromosome. Our results, which indicate that the high affinity of QM for certain regions of the heterochromatin is not entirely due to a higher concentration of DNA, agree with those of Caspersson *et al.* (1968, 1969a), and we concur with their interpretation that there is a differentiation of the DNA in the highly fluorescent areas. The nature of this differentiation is obviously important for an understanding of the biological or genetic function of these areas. The hypothesis of Caspersson *et al.* (1968, 1969a) that the DNA of the bright areas is differentiated from the rest by being G-C rich remains a possibility. Experiments now underway may help to clarify this point in *Drosophila*.

The mode of binding of QM may also reveal some details about the nature of the DNA in the highly fluorescent areas. QM can bind covalently to DNA by alkylation via the mustard moiety or it can bind non-covalently by ionic bonding or intercalation into the DNA double helix. Presently it seems clear that the primary source of the brilliant fluorescence is due to non-covalently bound QM in addition to some covalently bound QM, and the major part of the moderate overall fluorescence is caused by covalently bound QM.

Vosa (1970) using the fluorescent dye, quinacrine, has detected patterns of brilliant fluorescence in *D. melanogaster* polytene and mitotic chromosomes which are nearly identical to the QM patterns described in this paper. In preliminary experiments we have stained *D. melanogaster* polytene chromosomes with quinacrine and have confirmed this specific pattern of brilliant fluorescence with the exception that we find a brilliant doublet band in the base of 3R, rather than in the base of the X chromosome. We have also noted that the fluorescence associated with quinacrine staining is extremely acid labile both in the brilliant and moderately fluorescing areas. Since quinacrine is incapable of alkylating DNA, we conclude that Vosa's (1970) observations and our preliminary experiments both support the interpretation that the mode of QM binding in the brilliantly fluorescing regions does not primarily involve alkylation of DNA guanine.

Two possibilities with a bearing on the biological function of the brilliant areas are suggested by our data. The first is that at least some of the highly fluorescent DNA represents a local proliferation of DNA. This is suggested by our observation that the brilliant material appears in droplets in the tip of the polytene fourth chromosome and that it appears in a much larger quantity in the basal area of the translocated fourth chromosome when compared to the fourth chromosome in its normal position. Also the apparent loss of the bright droplets from the tip of the fourth chromosomes of prepupae points toward a possible relationship between developmental stage and appearance of the bright droplets.

Secondly, reports of Jones and Robertson (1970) and Rae (1970) have shown that reiterated DNA of *Drosophila melanogaster* is located primarily in the centromeric heterochromatin and the fourth chromosome of polytene cells. This localization corresponds fairly well with our localization of the brilliantly stained areas. Perhaps the highly fluorescent areas of *Drosophila* chromosomes represent a special class of heterochromatin composed of reiterated DNA. Studies of QM fluorescence in chromosomes of species such as the mouse where heterochromatin is known to be the site of reiterated DNA (Jones and Robertson, 1970), as well as a cytological localization of the reiterated DNA of *Vicia* and human chromosomes, whose QM fluorescence has already been well characterized by Caspersson *et al.* (1968, 1969a, 1970), may reveal whether the correlation of brilliant fluorescence and reiterated DNA is a more general phenomenon.

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