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Characterization of *Drosophila* Heterochromatin

I. Staining and Decondensation with Hoechst 33258 and Quinacrine

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Abstract. A number of preliminary experiments have shown that the fluorescence pattern of Hoechst 33258, as opposed to that of quinacrine, varies with the concentration of dye. The metaphase chromosomes of *D. melanogaster*, *D. simulans*, *D. virilis*, *D. texana*, *D. hydei* and *D. ezoana* have therefore been stained with two concentrations of H 33258 (0.05 and 0.5 μg/ml in phosphate buffer at pH 7) and with a single concentration of quinacrine (0.5% in absolute alcohol). The three fluorescence patterns so obtained were shown to be somewhat different in some of the species and the coincide in others. All three stainings gave an excellent longitudinal differentiation of heterochromatin while euchromatin fluoresced homogeneously. – Living ganglion cells of the six species mentioned above were treated with quinacrine and H 33258. Quinacrine induced a generalized lengthening and swelling of the chromosomes and H 33258 the decondensation of specific heterochromatic regions. – A correlation of the base composition of the satellite DNAs contained in the heterochromatin of the species studied with the relative fluorescence and decondensation patterns showed that: 1) the extremely fluorochrome bright areas and those decondensed are present only in species containing AT rich satellite DNA; 2) the opposite is not true since some AT-rich satellite DNAs are neither fluorochrome bright nor decondensed; 3) there is no good correspondence between Hoechst bright areas and the decondensed ones. – AT richness therefore appears to be a necessary but not sufficient condition both for bright fluorescence and decondensation. Some cytological evidence suggests that similarly AT rich satellite DNA's respond differently in fluorescence and decondensation because they are bound to different chromosomal proteins. – A combination of the results of fluorescence and decondensation revealed at least 14 types of heterochromatin; 4–7 of which are simultaneously present in the same species. Since closely related species (i.e. *D. melanogaster* and *D. simulans*; *D. virilis* and *D. texana*) show marked differences in the heterochromatic types they contain, it can be suggested that within the genus *Drosophila* qualitative variations of heterochromatin have played an important role in speciation.

Introduction

In various species of *Drosophila* the mitotic chromosomes exhibit large heterochromatic regions (Heitz, 1934a, b; Kaufmann, 1934). A series of studies have shown that the heterochromatin of *Drosophila* has the following characteristics: (1) it contains few mappable genes (Hannah-Alava, 1951), (2) it contains highly repetitive DNA including satellite DNA (Hennig et al., 1970; Jones and Robertson, 1970; Rae, 1970; Gall et al., 1971; Peacock et al., 1973), (3) it is late replicating (Barigozzi et al., 1966), and (4) it is underreplicated in polytene chromosomes (Heitz, 1934a, b; Rudkin, 1969; Gall et al., 1971). Neither these studies nor those concerning the genetic effects of heterochromatin (Hannah-Alava, 1951; Baker, 1968) however, have made it possible to understand the functional role of this kind of genetic material.

Recently it has been shown that the heterochromatin of *Drosophila* fluoresces differently after staining both with quinacrine (Vosa, 1970b; Adkisson et al., 1971; Ellison and Barr, 1971; Faccio Dolfini, 1974) and with Hoechst 33258 (Holmquist, 1975a) and decondenses differently when living cells are Hoechst treated (Pimpinelli et al., 1975). It is reasonable to assume that this cytological heterogeneity corresponds to a heterogeneity at the structural and/or molecular level.

We therefore feel that a thorough characterization of heterochromatin constitutes an important prerequisite a) for any study on the functions of this genetic material b) for an understanding of the role played by heterochromatin in speciation.

In this context, making use of an improved technique for the preparation of mitotic chromosomes (Gatti et al., 1974a, b), we have undertaken a systematic study of the cytological organization of *Drosophila* heterochromatin using the modern techniques for the longitudinal differentiation of chromosomes (Hsu, 1973; Schnedl, 1974). Here we describe the comparative results of the response of *Drosophila* heterochromatin to H 33258 and quinacrine staining and decondensation. In order to investigate the chemical nature of the various heterochromatic portions revealed by these techniques, we have used six species of *Drosophila* containing satellite DNAs of different base compositions in their heterochromatin. In fact, *D. melanogaster* (Peacock et al., 1973), *D. simulans* (Travaglini et al., 1972), *D. virilis* (Gall et al., 1971), *D. texana* (Gall et al., 1973) are known to contain various AT rich satellite DNAs. *D. hydei* contains a GC rich satellite DNA (Hennig et al., 1970) and *D. ezoana* is devoid of satellite DNAs (Gall et al., 1973).

Materials and Methods

a) Stocks. Drosophila melanogaster Oregon-R and T (2,3) bw^{V4} stocks were used. Stocks of *D. simulans* and *D. hydei* from natural populations collected in Padua (Italy) were supplied by Dr. A. Danieli. The *D. virilis* and *D. texana* stocks were obtained from Dr. Figueroa (Chicago, U.S.A.). The *D. ezoana* stock was provided by Dr. E. Momma (Sapporo, Japan). All these stocks were grown in standard medium at $25 \pm 1°$ C.

b) Hoechst 33258 and Quinacrine Staining. Neural ganglia of third instar larvae were incubated for 1 h in an aqueous solution of NaCl (0.7%) containing 10^{-5} M colchicine. After hypotonic treatment they were fixed with a 1:1 mixture of acetic acid and methyl alcohol (see Gatti et al., 1974a, b) and then squashed in 45% acetic acid under a siliconized coverslip. The coverslip was then removed by freezing on dry ice and the slides were air-dried. The method of Latt (1973) was used for H 33258 staining. The slides were rehydrated for 5 min in 0.15 M NaCl–0.03 M KCl–0.01 M phosphate (pH 7), stained for 10 min with various concentrations of H 33258 dissolved in the same buffer and, after rapid washing, mounted in 0.16 M sodium phosphate 0.04 M sodium citrate (pH 7). For quinacrine dihydrochloride staining (Gurr) the slides were first soaked for 5 min in ethyl alcohol, then stained for 10 min in a 5% solution of quinacrine in absolute alcohol, rapidly washed in alcohol, and air dried. They were then mounted in distilled H_2O. Some of the slides were sequentially stained with H 33258 and quinacrine according to the method of Holmquist (1975a). Both slides, stained with H 33258 and quinacrine, were stored before observation for at least 1–2 d in the dark at room temperature.

c) Photography and Densitometry. Chromosome fluorescence was observed under a Zeiss fluorescence microscope equipped with incident illumination using a 200 W mercury light source. The combinations of filters 2×BG12, FT510, LP515 and UG5+BG3, FT460, LP475 were used for H 33258 and quinacrine respectively. For both dyes and in all species microphotographs were taken on Ilford Pan-F film which was then developed with Hifin (Ilford) for 18 min at 20° C.

In order to evaluate the fluorescence intensity of the various fluorochromatic blocks shown by H 33258 and quinacrine staining the negatives were then analyzed with a Joyce Loebl Mark III microdensitometer. For each species an for each staining the densitometric tracings of at least five different chromosomes were examined.

d) Experiments on Living Neuroblasts. By the method of Pimpinelli et al. (1975) the neural ganglia were incubated for various lengths of time in an aqueous solution of NaCl (0.7%) complemented with foetal calf serum (20%), containing various concentrations of H 33258 or quinacrine. After an hour's pretreatment with colchicine the ganglia were fixed and squashed in acetic orcein according to our usual procedure (Gatti et al., 1974a, b).

Results

H 33258 and Quinacrine Staining

In the H 33258 staining of the metaphase chromosomes of *Drosophila* various concentrations of this fluorochrome between 0.01 and 2 μg/ml were preliminary used. We have observed that the fluorescence pattern of the chromosomes varies with the concentration of H 33258. At concentrations between 0.01 and 0.1 μg/ml Hoechst-negative chromosomal regions can in fact be identified in the various species. But at concentrations above 0.2 μg/ml some of these regions are more or less brightly fluorescent (see Figs. 3, 8 and 10). We have carried out similar experiments with quinacrine. Only the average chromosome fluorescence increases, however, with quinacrine concentration, the relationships of intensity of fluorescence between the various chromosome regions remaining unchanged.

On the basis of these preliminary experiments the chromosomes of all the species were stained with two concentrations of H 33258 (0.05 and 0.5 μg/ml) in order to study possible variations in the fluorescence pattern. A 0.5% solution in absolute alcohol was used in the quinacrine staining.

Staining with the two concentrations of H 33258 and quinacrine was repeated several times. For a true comparison of the three fluorescence patterns, three

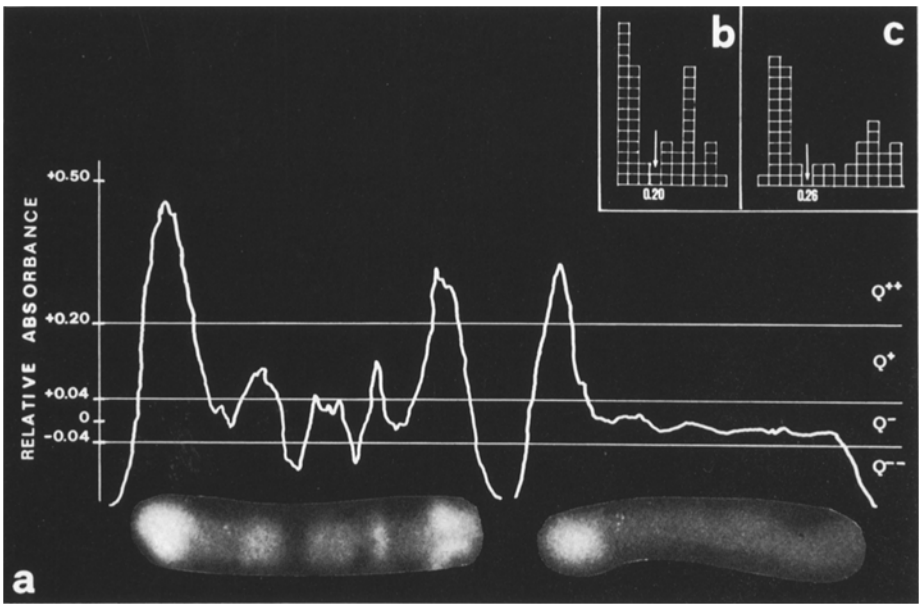


Fig. 1a–c. a Scheme of procedure used in the classification of fluorochromatic blocks of quinacrine stained chromosomes Y (on left) and 2 (on right) of *D. virilis*. o: average fluorescence of euchromatin; ±0.043: euchromatin fluorescence intensity variation range. It was found that the fluorescence intensity of the Q-bright blocks of the various species have a bimodal distribution (**b**). It was therefore selected as the limit between Q^+ and Q^{++} areas the value +0.2 as the best discriminant one between the two distributions. – Also the H. 0.05[1] bright blocks showed a bimodal distribution (**c**). The discriminant value which separates the categories H^+ 0.05 and H^{++} 0.05 was 0.26. This value was then used to classify the H. 0.5 bright blocks

groups of slides were stained each time. Each group contained equally aged preparations of every species. The preparations were then all stained with Giemsa and the metaphases previously photographed in fluorescence were photographed again. It was thus possible to study the relationships between fluorescence pattern and distribution of heterochromatin along the chromosomes. Those areas in which sister chromatids appear closely apposed were regarded as heterochromatic areas. They generally have positive heteropycnosis during interphase and are positive in C-band preparations (Pimpinelli et al., 1976b).

An examination of the densitometric tracings showed that after each staining the euchromatic arms of the chromosomes of the various species fluoresce homogeneously and with similar intensity. With respect to this basic fluorescence, extremely fluorochrome-bright areas, fluorochrome-bright areas, fluorochrome-dull areas and fluorochrome-negative regions were identified in the heterochromatin. These areas will be respectively termed Q^{++} H^{++}, Q^+ H^+, Q^- H^- and Q^{--} H^{--}. Areas having a fluorescence intensity lying within the range of euchromatin fluorescence variation were considered to be Q^- and H^-, those below this range Q^{--} and H^{--}. To decide whether a heterochromatic region brighter than euchromatin falls into the category Q^{++}, H^{++} or into

[1] Abbreviations: H 0.05 – Hoechst at 0.05 μg/ml, H 0.5 – Hoechst at 0.5 μg/ml

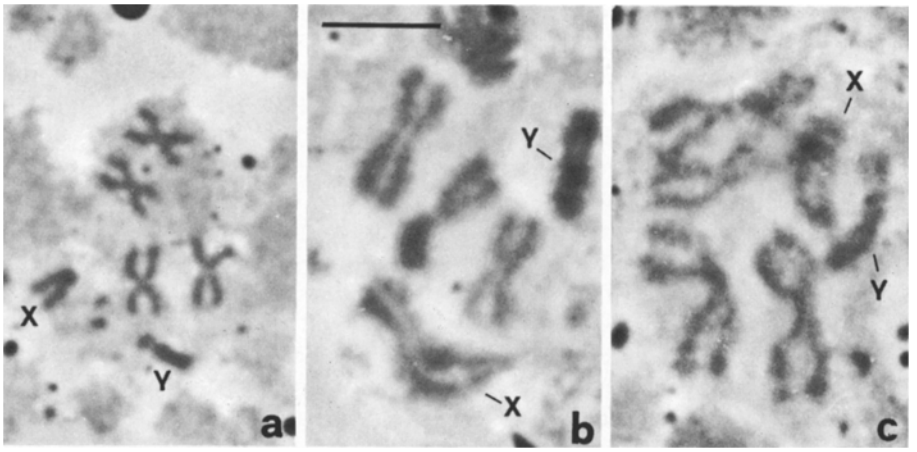


Fig. 2a–c. Effects of quinacrine dihydrochloride on living neuroblasts of *D. melanogaster*. **a** Control metaphase; **b** and **c** metaphases treated for 4 h with 20 and 40 μg/ml of quinacrine respectively. The bar represents 5 μm

the category Q^+, H^+ a value of fluorescence intensity separating these categories (Fig. 1) was set for each staining.

Unlike Holmquist (1975a), we have found that in all species, whether with H 33258 or with quinacrine, the fluorescence pattern does not appreciably vary with chromosome condensation. We have, in fact, observed the same banding pattern in highly condensed metaphase chromosomes as in the long prometaphase chromosomes. Only in the latter, however, can some fluorochromatic blocks be resolved into different bands.

Effect of H 33258 and Quinacrine on Living Neuroblasts

Living neuroblasts of *D. melanogaster* and *D. virilis* were treated with various concentrations of H 33258 (10, 40, 80 and 200 μg/ml) and then fixed after 6 h of treatment. At the lowest concentration the metaphase chromosomes were affected only very slightly and at all the other concentrations they always gave an identical species-specific heterochromatin decondensation pattern (see Figs. 5, 7, 9 and 11). Nor did the frequency of affected metaphases vary with variation of the concentration of Hoechst in the medium.

Previous experiments carried out in *D. melanogaster* (Pimpinelli et al., 1975) had, however, shown that the frequency of the metaphases affected as well as the decondensation pattern varied with length of H 33258 treatment. On the basis of these data, the neural ganglia of all six species were treated with 40 μg/ml of H 33258 and then fixed after 4 and 8 h. Additional 12-h treatments were given to *D. hydei* and *D. ezoana*.

We wish to point out that in none of the species treated did we observe chromosomal aberrations. Often, however, the decondensed areas seemed to cling together (see Figs. 5, 9). A similar phenomenon was observed in other

materials in which undercontraction of the chromatin was induced (Hilwig and Gropp, 1973; McGill et al., 1974; Pathak et al., 1975). The adhesion of the undercondensed areas could be due to the entanglement of submicroscopic chromosome fibrils which failed to condense properly in early stages of mitosis (McGill et al., 1974; Pathak et al., 1975).

D. melanogaster and *D. virilis* were used to study the effect of quinacrine on living neuroblasts. The neural ganglia of these two species were treated with 10, 20 and 40 μg/ml of quinacrine dihydrochloride and then fixed after 1, 3 and 5 hours. After a 1 h treatment, in accordance with the observations of Hsu et al. (1973) in Chinese hamster cells, there were no effects found. In more than 50% of the metaphases treated for 3 and 5 h a general lengthening and swelling of chromosomes (Fig. 2) was, however, clearly observed. The extent of this phenomenon also seemed to increase with the concentration of quinacrine. Since the quinacrine was unable to alter the process of mitotic condensation of specific chromosomal areas, it did not seem to be worthwhile to extend these experiments to the other species.

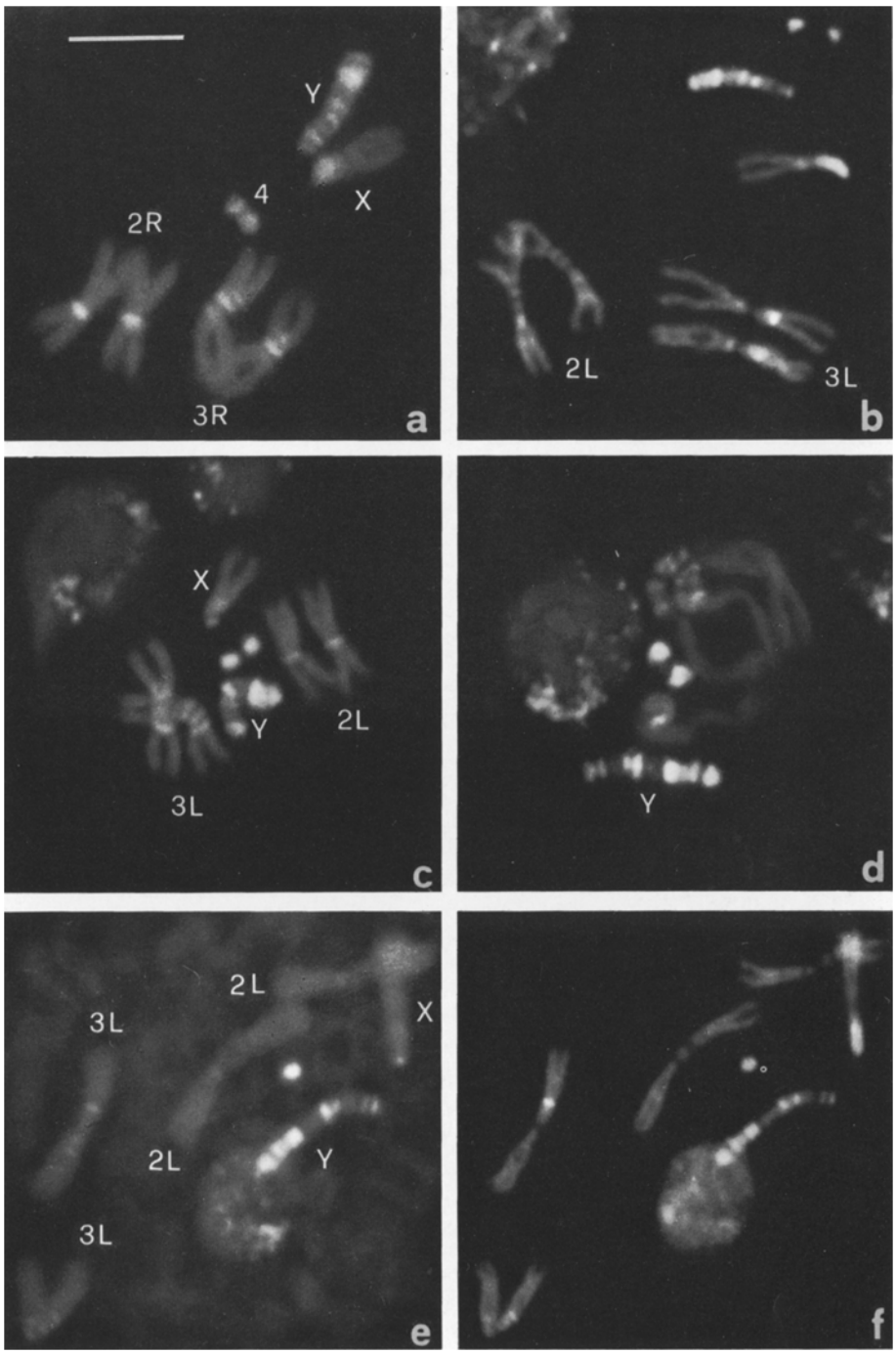
D. melanogaster and D. simulans

The karyotype of *D. melanogaster* is well known – two pairs of metacentric autosomes, a pair of dot chromosomes and, in males, an acrocentric X chromosome and a sub-metacentric Y chromosome. The entire Y chromosome, the proximal half of the X chromosome and the centromeric areas of the autosomes are heterochromatic and C-band positive (Hsu, 1971; Pimpinelli et al., 1976b). The metaphase chromosomes of *D. melanogaster* stained with H 33258 show a fluorescence pattern which differs with the concentration (0.05 or of 0.5 μg/ml) (see Fig. 3). X, Y and the fourth chromosomes fluoresce similarly at both concentrations. At the lowest concentration the chromosomes 2 show only a few H^{--} regions on the centric heterochromatin, but when stained with 0.5 μg/ml of H 33258 they show a H^{++} band on arm 2L. At this concentration (0.5 μg/ml) the chromosomes 3 have two further H^{++} heterochromatic blocks besides the one which can be identified on the 3L arm by staining with 0.05 μg/ml.

Using a concentration of 0.5 μg/ml of H 33258, Holmquist (1975a) demonstrated a cytological differentiation of heterochromatin rather similar to that obtained by us. In his preparations, however, the heterochromatic H^{++} blocks shown in Figure 3 were well-defined but moderately fluorescent.

The quinacrine fluorescence pattern corresponds roughly to that of H 33258 at 0.5 μg/ml. In the X chromosome, however, only a proximal portion of the large H^{++} block is quinacrine bright. As can be seen in Figure 3, in the

Fig. 3a–f. Male metaphase chromosomes of *D. melanogaster* stained with **a** H 33258 at 0.5 μg/ml; **b** H 33258 at 0.05 μg/ml; **c** and **d** quinacrine; the prometaphase **d** lacks the chromosomes 2. **e** and **f** The same metaphase sequentially stained with quinacrine (**e**) and with H 33258 (**f**) at 0.05 μg/ml. To localize the fluorescent heterochromatic areas with respect to the centromere, the fluorescence pattern of the metaphase chromosomes of larvae heterozygous for the translocation (2,3) bw^{V4} was studied. This translocation was previously used for the localization of the areas decondensed by H 33258 (Pimpinelli et al., 1975). The bar represents 5 μm


Y
4
X
2R
3R
a
2L
3L
b
X
Y
2L
3L
c
Y
d
2L
X
3L
2L
Y
3L
e
f

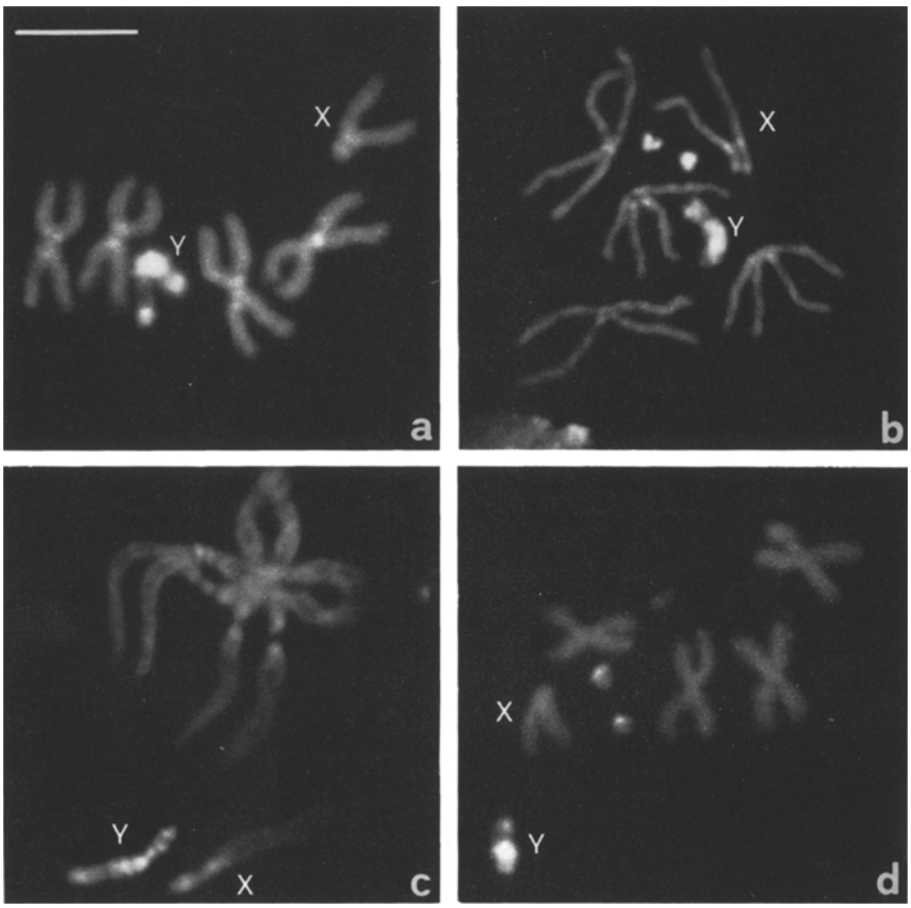
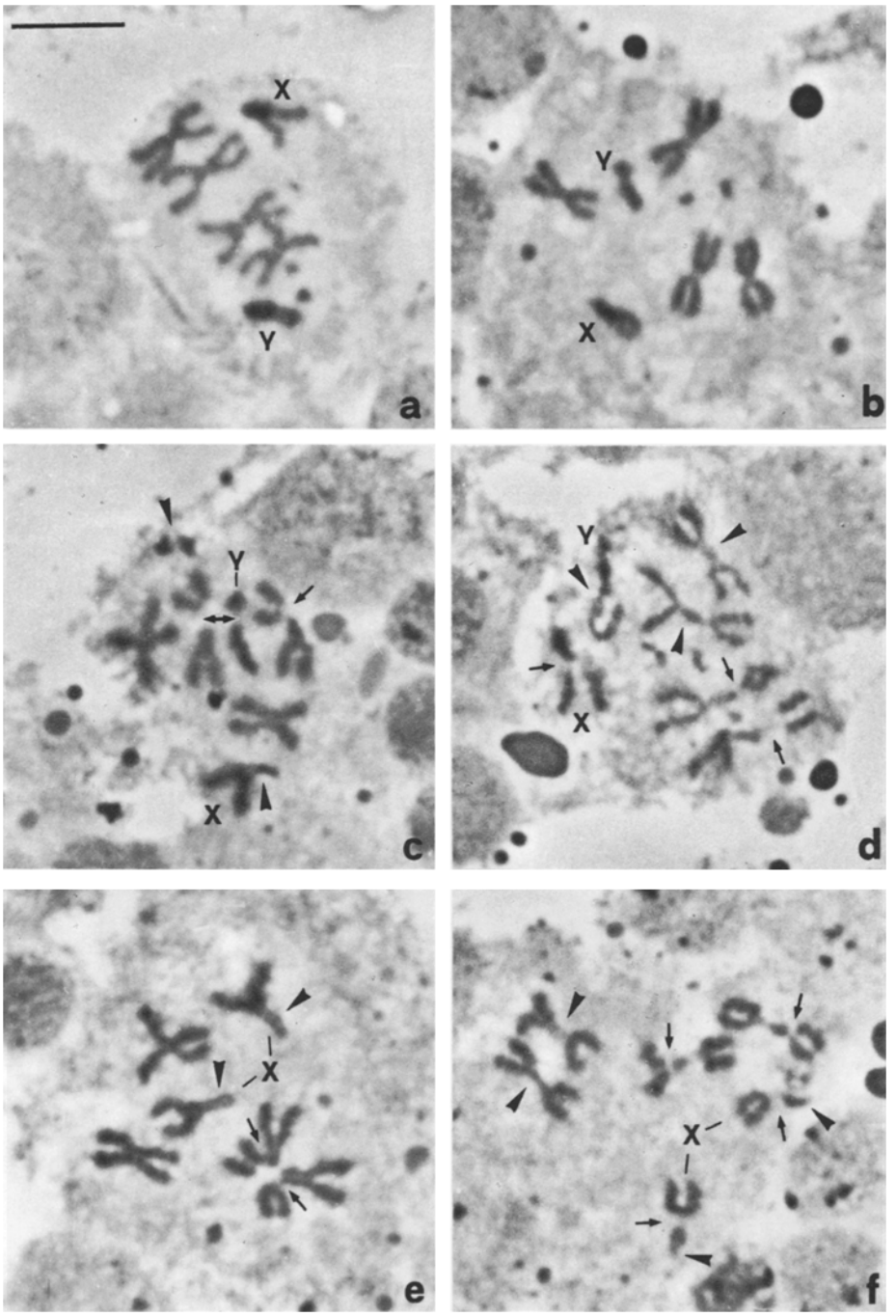


Fig. 4a–d. Male metaphase chromosomes of *D. simulans* stained with **a** H 33258 at 0.5 μg/ml; **b** and **c** H 33258 at 0.05 μg/ml; **d** quinacrine. In the prometaphase shown in **c** the autosomes and the sex chromosomes, excessively far apart on the slide, have been artificially brought closer together. Note in this prometaphase the fine banding of the Y chromosome. If **a** is compared with **b** it can be seen that there are no substantial differences in the fluorescence pattern at the two concentrations of H 33258. The bar represents 5 μm

Fig. 5a–f. Effects of H 33258 on metaphase chromosomes of *D. melanogaster* and *D. simulans* neuroblasts **a** untreated male cell of *D. melanogaster*; **b** untreated male cell of *D. simulans*; **c** male cell of *D. melanogaster* treated for 4 h with H 33258; **d** male cell of *D. simulans* treated for 8 h with H 33258; **e** female cell of *D. melanogaster* treated for 4 h with H 33258; **f** female cell of *D. simulans* treated for 4 h with H 33258. (↑) Drastically decondensed areas; (△) Intermediately decondensed areas. For *D. melanogaster*, differences in the decondensation pattern after treatments of 4 and 8 h have been previously described (Pimpinelli et al., 1975). The gangliar cells of *D. simulans* do not show substantial differences in the decondensation pattern with the variation of time of treatment. The Y chromosome appears already lengthened after a 4 h treatment; after 8 h its lengthening is slightly accentuated. Note in **f** the aspect of the X of *D. simulans*: they have a drastically decondensed distal area and a moderately decondensed proximal one, separated by a rather compact heterochromatic block. This pattern result from the decondensation of the two H^+ blocks which can be seen in Figure 3 after H 33258 staining. The bar represents 5 μm


X
Y
a
Y
X
b
Y
X
c
Y
X
d
X
e
X
f

quinacrine-stained metaphases there is a fine banding of the centric heterochromatin of the chromosomes 3 and of the Y chromosome. Previous studies (Vosa, 1970b; Adkisson et al., 1971; Ellison and Barr, 1971; Faccio Dolfini, 1974) have shown a greater fluorescence of these areas without, however, succeeding to resolve them into well-defined fluorescent bands. The present data therefore show that quinacrine staining, like that of H 33258, is a useful and efficient cytochemical method for the longitudinal differentiation of *D. melanogaster* chromosomes.

The *D. simulans* karyotype differs from that of *D. melanogaster* essentially in the shortness of the Y chromosome (see Figs. 3 and 4). The metaphase chromosomes of *D. simulans*, however, are markedly different from those of *D. melanogaster* when they are stained with H 33258 and with quinacrine (see Fig. 4). At both concentrations of H 33258 the two autosome pairs show moderately fluorescent heterochromatic blocks on both sides of the centromere. Also the heterochromatin of the X chromosome is composed of two H^+ blocks separated by an H^- band. The only H^{++} areas present in *D. simulans* are limited to the Y chromosome and to the dot chromosomes. These chromosomes exhibit very bright areas also when stained with quinacrine. The autosomes and the X chromosome, in agreement with the observations of Ellison and Barr (1971), fluoresce homogeneously with quinacrine without any longitudinal differentiation.

The effects of H 33258 on the living ganglion cells of *D. melanogaster* have already been described in detail (Pimpinelli et al., 1975). In Figure 5, however, some metaphases of *D. melanogaster* are compared with those of *D. simulans*. As can be seen, these two sibling species show a markedly different heterochromatin decondensation pattern. In *D. melanogaster* a distal portion of the heterochromatin situated on arm 3L and a block near the centromere of the Y chromosome are drastically decondensed. A proximal portion of the heterochromatin of the X chromosome, the chromosomes 4 and, after a 6–8 h treatment, the entire Y chromosome are also intermediately decondensed.

In *D. simulans*, all the heterochromatin except for a few blocks, is decondensed. Areas of drastic decondensation are present in a pair of autosomes and on the chromosome X while the other heterochromatic areas are moderately decondensed.

If the decondensation patterns of *D. simulans* and *D. melanogaster* are compared with the respective fluorescence patterns after H 33258 staining, it is evident that practically all the H^+ and H^{++} material of *D. simulans* is decondensed. In *D. melanogaster* the distal H^{++} block on arm 3L, the H^{++} block on the X and, probably, all the H^{++} bands of the Y are decondensed.

D. virilis and D. texana

D. virilis has 5 pairs of acrocentric chromosomes and a pair of dot chromosomes. The proximal half of the acrocentric chromosomes and the entire Y chromosome are heterochromatic and C-band positive (Pimpinelli et al., 1976b). When stained with 0.05 μg/ml H 33258, all the chromosomes except the Y show a large distal

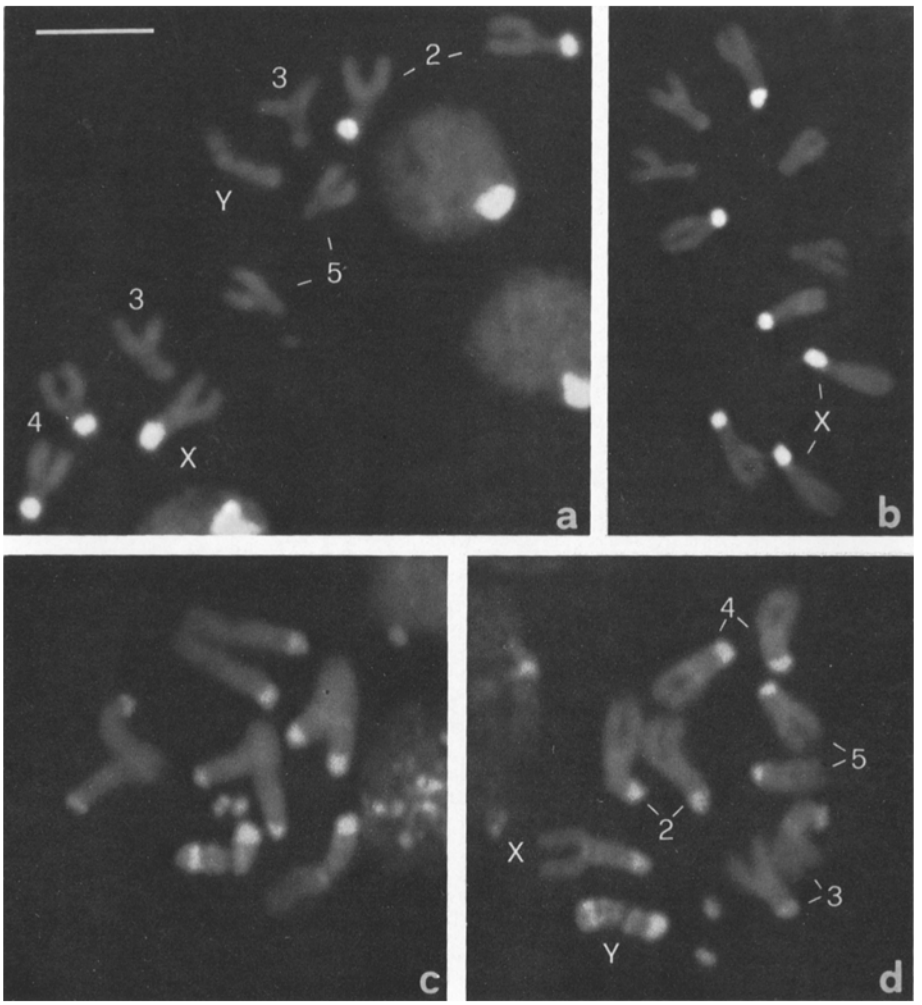


Fig. 6a–d. Metaphase chromosomes of neuroblasts of *D. virilis*. **a, b** Stained with H 33258 at 0.05 μg/ml, **a** ♂ and **b** ♀. **c** and **d** stained with quinacrine, **c**♂ and **d** ♀. For the identification of the chromosomes we applied the data of Holmquist (1975a) that the chromosomes 2 and 4 possess a proximal H^+ block but chromosomes 3 and 5 do not. As can be seen in both these groups of chromosomes, one pair appears larger than the other. Thus for a definitive identification it is sufficient to establish the largest pair for each group. This was done by studying the fluorescence pattern of metaphases of hybrids between *D. virilis* and *D. texana*; in these metaphases the chromosomes 2 and 3 of *D. virilis* can be easily recognized in that they are somatically paired with the metacentric chromosome of *D. texana* originating in the centric 2–3 fusion. This procedure obviously also served to identify the chromosomes of *D. texana* without ambiguity. For the identification of the chromosomes of the metaphase **d** stained with quinacrine, the slide was destained and subsequently restained with H 33258. Note that the dot chromosomes are quinacrine bright and Hoechst negative. The bar represents 5 μm

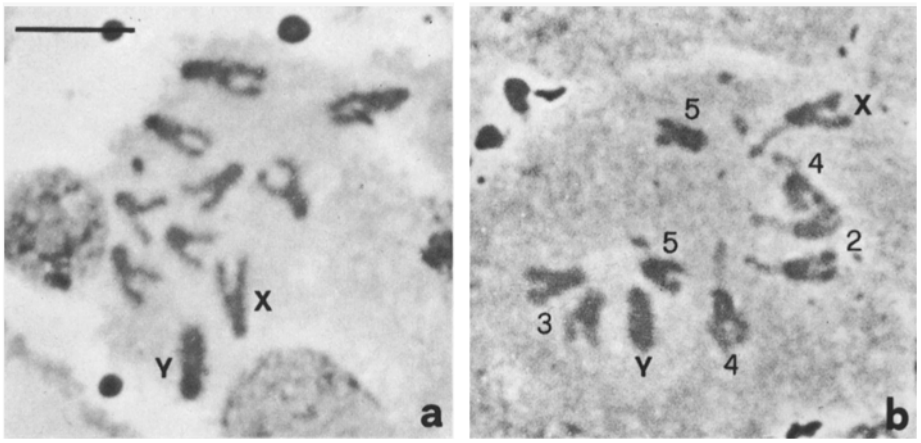


Fig. 7a and b. Effects of H 33258 on living neuroblasts of *D. virilis*. **a** Male control cells; **b** male cells treated for 4 h with H 33258 (40 μg/ml). No differences were observed in the decondensation pattern with the variation in length of treatment. In the cells treated for 8 h all metaphases are decondensed and in most of them the decondensed areas adhere to each other forming a tangle which is difficult to resolve. The decondensed chromosomes have been identified by taking into account their relative dimensions and studying the decondensation pattern in hybrids between *D. virilis* and *D. texana*. The bar represents 5 μm

heterochromatic H^{--} block (Fig. 6). The proximal heterochromatin of these chromosomes is H^{++} in chromosomes 2, 4 and X and H^{-} in chromosomes 3 and 5. The Y chromosome is weakly fluorescent with some H^{--} bands. If 0.5 μg/ml of H 33258 are used in the staining, in accordance with the previous observations of Holmquist (1975a), the H^{--} regions show up as moderately fluorescent.

In chromosome preparations stained with quinacrine, very brightly fluorescent areas can be seen on the proximal heterochromatin of all chromosomes and at both ends of the Y chromosome. The other heterochromatic areas, except for a few narrow Q^{+} and Q^{--} bands, are all Q^{-}. This quinacrine fluorescence pattern is different both from that reported by Adkisson (1971) who found the proximal heterochromatin of the X chromosome to be weakly fluorescent, and from that of Holmquist (1975a) who found the proximal region of the chromosomes 5 to be Q^{-}.

Figure 7 shows the decondensation pattern obtained in *D. virilis*. As can be seen, the proximal heterochromatin of chromosomes 2, 4 and X is moderately decondensed. There is therefore a precise correspondence between H^{++} areas and decondensed areas.

D. texana differs from *D. virilis*, in the presence of two large metacentric chromosomes resulting from centric fusion of chromosomes 2 and 3 (Patterson and Stone, 1952). However, when the chromosomes of *D. texana* are stained with the two fluorochromes (Fig. 8) or decondensed (Fig. 9) they show striking differences from those of *D. virilis*. In the preparations stained with 0.05 μg/ml H 33258 the distal heterochromatic H^{--} blocks already observed in *D. virilis* are present. The proximal H^{++} heterochromatin is, however, absent not only

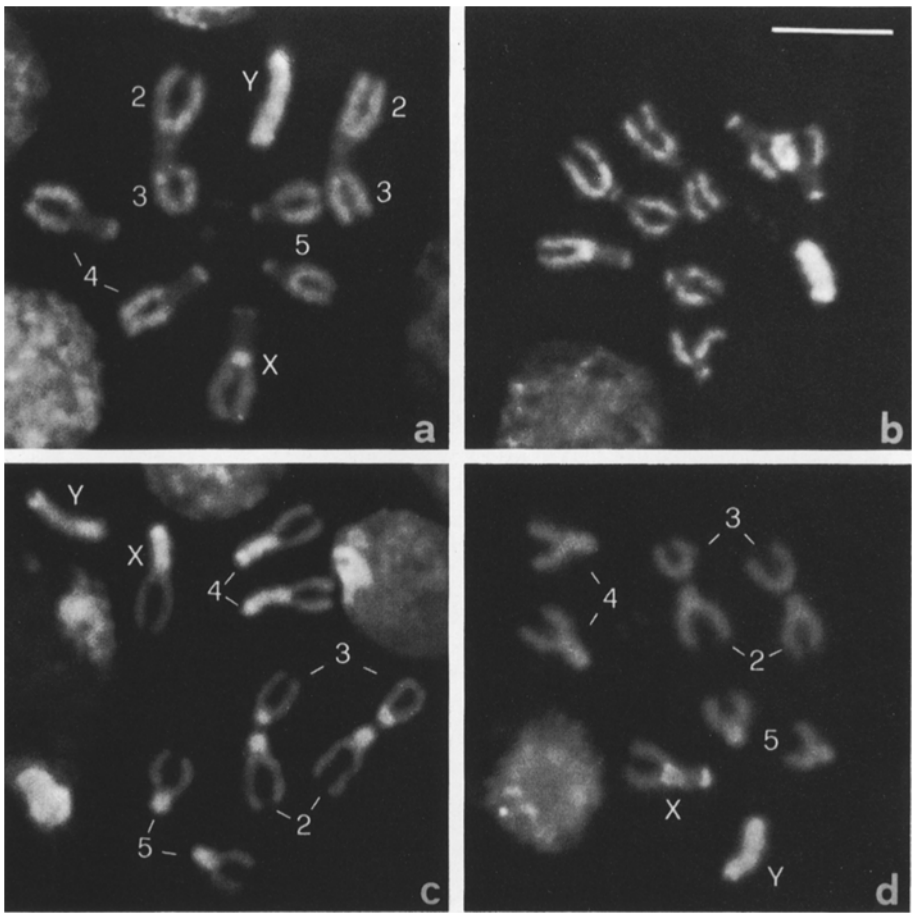


Fig. 8a–d. Metaphase chromosomes of male neuroblasts of *D. texana* stained with **a** and **b** H 33258 at 0.05 μg/ml; **c** H 33258 at 0.5 μg/ml; **d** quinacrine. Note how H^{--} blocks evident in **a** and **b** on staining with 0.5 μg/ml of H 33258 appear in **c** moderately fluorescent. The H^{--} blocks of *D. virilis* behave in the same way. The Y chromosome at both concentrations of H 33258 is a moderately and uniformly fluorescent element. It is moderately fluorescent also in the metaphases stained with quinacrine. The dot chromosomes are always H^{--} and Q^{--}. The bar represents 5 μm

in the metacentric chromosomes resulting from the fusion of chromosomes 2 and 3 but also in chromosomes 4 and in the X chromosome. In the latter chromosomes the proximal H^{++} block evident in *D. virilis* is substituted by a slightly smaller H^{-} block. In *D. texana* there are none of the Q^{++} blocks observed on the *D. virilis* chromosomes.

The decondensation pattern of *D. texana* is completely unexpected and cannot be deduced from the results of fluorescence. Figure 9 shows clearly that the greater decondensed areas include the proximal heterochromatin of chromosomes 4 and 5. Small amounts of material weakly decondensed by H 33258 are also present at the point of junction of chromosomes 2 and 3, in the middle

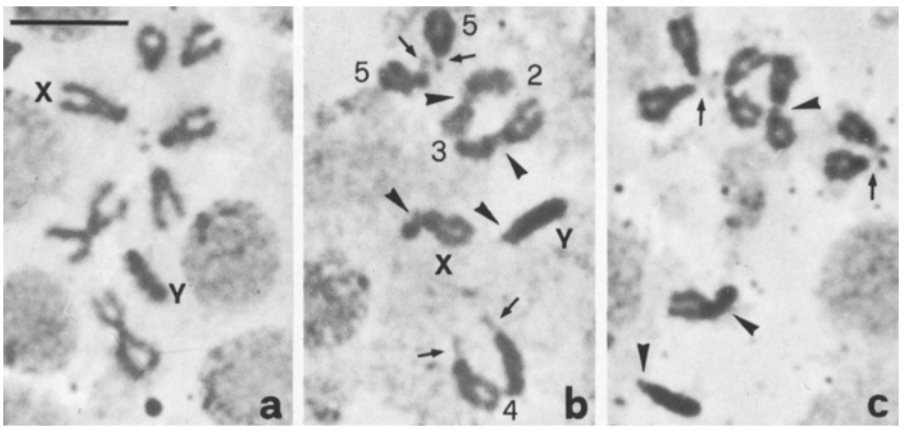


Fig. 9a–c. Effects of H 33258 on living neuroblasts of *D. texana*. **a** Control metaphase; **b** metaphase treated for 4 h with H 33258; **c** metaphase treated for 8 h. There were no differences observed in the decondensation pattern with the variation in treatment time. The decondensation of the proximal heterochromatin of chromosomes 4 and 5 (↑) is always clearly visible. Areas of slight decondensation (△) at the level of the centromere of chromosome 2–3 in the middle of the heterochromatin of the X and at the end of Y are not so evident. From a comparative examination of a good number of decondensed and control cells, we have been able to establish with certainty that these effects are real. The bar scale represents 5 μm

of the heterochromatin of X and at the end of Y. All the decondensed areas seem to correspond to weakly fluorescent areas in the preparations stained with H 33258.

D. hydei

The *D. hydei* possesses four pairs of acrocentric and one pair of dot autosomes and, in males, a metacentric X and a sub-metacentric Y. The entire Y chromosome, one arm of the X chromosome and the pericentrometric areas of the autosomes are heterochromatic and C-band positive (Pimpinelli et al., 1976b). After H 33258 staining (0.05 μg/ml), various H^{--} and H^{-} areas appear in the heterochromatin and three brief H^{+} regions on the Y chromosome. Some of the H^{--} areas persist even at higher concentrations of H 33258 (Fig. 10).

Quinacrine fluorescence is very similar to that of H 33258 except, perhaps, for some differences in the banding of the Y chromosome. Neither quinacrine nor H 33258 staining make it possible to identify the four pairs of autosomes which are completely similar to each other both in size and in fluorescence pattern.

After four hours of H 33258 treatment there is no effect observed on the process of condensation of heterochromatin. After 8 or 12 h treatments a small area of intermediate decondensation can be identified near the centromere of the Y chromosome in about 10% of the cells (Fig. 11). Most likely the decondensed area corresponds to a H^{+} region.

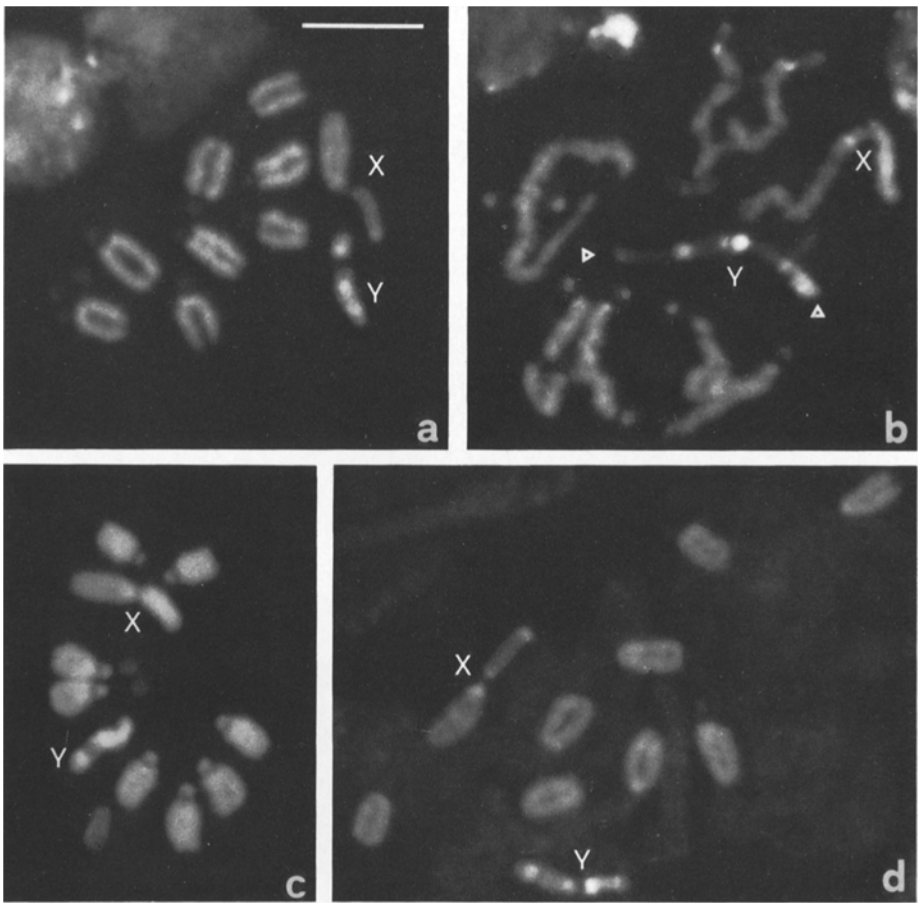


Fig. 10a–d. Chromosomes of male neuroblasts of *D. hydei* stained with **a** 0.05 μg/ml of H 33258; **b** and **c** 0.5 μg/ml of H 33258; **d** quinacrine. The Xh arm at the lowest concentration of H 33258 is slightly less fluorescent than euchromatin. It becomes H^+ when stained with 0.5 μg/ml (**c**). The dot chromosomes are not easily visible in that they are H^{--} and Q^{--}. Note the aspect of the Y in the prophase. The two arrows in **b** indicate the ends of this chromosome. The bar represents 5 μm

D. ezoana

The *D. ezoana* karyotype consists of 5 pairs of autosomes (3 acrocentric, 1 submetacentric, 1 dot) and, in males, an acrocentric X and a large metacentric Y. The entire Y chromosome and the centromeric region of the X chromosome and of the autosomes are heterochromatic and C-band positive (Pimpinelli et al., 1976b).

When the chromosomes of *D. ezoana* are stained with H 33258 (at both concentrations) or with quinacrine they fluoresce rather homogeneously and weakly, with a similar pattern. Slightly more fluorescent areas can be observed

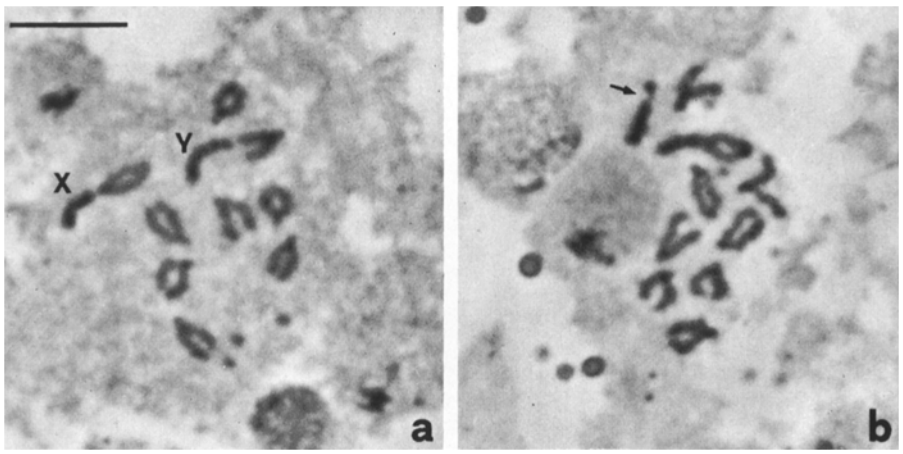


Fig. 11a and b. Effects of H 33258 on living neuroblasts of *D. hydei* **a** Male control cell; **b** male cell treated for 8 h with H 33258. The arrow indicates the area of decondensation on the Y. The bar represents 5 μm

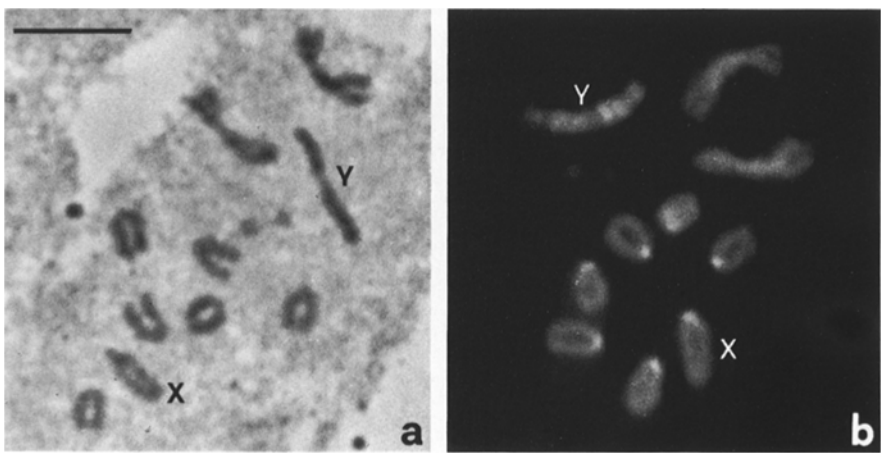


Fig. 12a and b. Male metaphase of *D. ezoana* stained **a** with acetic orcein and **b** with H 33258 (0.05 μg/ml). The bar represents 5 μm

on the centric heterochromatin of the acrocentric chromosomes and on the Y (Fig. 12).

Only if prolonged for 8 and 12 h does in vivo treatment with H 33258 produce 2–3 less stained narrow bands on the Y in a small percentage of cells. It can be assumed that these bands result from the slight undercontraction of specific heterochromatic portions.

Discussion

Chemical and Structural Bases of Quinacrine and H 33258 Fluorescence

Quinacrine binds DNA by intercalation without an appreciable specificity for its base composition. However the fluorescence of the quinacrine bound to DNA is markedly base composition dependent, since AT rich DNA enhances and DNA containing GC bases quenches quinacrine fluorescence (Weisblum and de Haseth, 1972; Pachman and Rigler, 1972; Michelson et al., 1972; Selander and de la Chapelle, 1973; Latt et al., 1974; Comings et al., 1975). Unlike quinacrine, Hoechst 33258 binds specifically with AT rich DNA by an external attachment to the double helix (Comings, 1975; Latt and Wohlleb, 1975). Its fluorescence in vitro is increased with both AT and GC rich DNA, even if the increase in fluorescence with AT rich DNA is greater than that with GC rich DNA (Weisblum and Haenssler, 1974; Simola et al., 1975; Comings, 1975). The fluorometric properties of both these dyes would therefore indicate that they specifically stain AT rich chromosomal regions.

Cytological studies on fixed chromosomes have shown, however, that the in vitro DNA fluorochrome data do not provide a satisfactory explanation of the mechanisms involved in quinacrine and Hoechst banding (see Jalal et al., 1974 and Holmquist, 1975a for review).

The present study, which extends and completes that of Holmquist (1975a) on *D. melanogaster, D. virilis* and *D. eohydei* represents a further cytological approach to the problem of the chemical and structural bases of chromosome banding. In fact, knowing the base composition of the satellite DNAs contained in the heterochromatin of the six species we studied, we can correlate these data with quinacrine and Hoechst fluorescence. This analysis is reported in Table 1 together with the decondensation results which will be discussed later. The following conclusions can be drawn from Table 1:

The three fluorescence patterns (two with H 33258 and one with quinacrine) are rather different in some species while in others they coincide. It appears therefore that H 33258 and quinacrine have a different cytological specificity.

2. At the highest concentration of H 33258 regions which at 0.05 µg/ml were H^- and H^{--} become H^{++} and H^+. In the various species there is a good correspondence between the percentage of AT rich satellite DNA and that of the brightly fluorescent areas (H^{++} and H^+ areas) with 0.5 µg/ml of H 33258. The percentage of quinacrine bright areas (Q^{++} and Q^+ areas) is often rather less than of AT rich satellite DNA (e.g. *D. simulans, D. virilis, D. texana*). If used at sufficiently high concentrations, H 33258 seems therefore to be a general indicator of AT rich regions in chromosomes, including those which are not demonstrable with quinacrine.

3. Extremely bright fluorescent areas are present only in species which possess AT rich satellite DNAs. The opposite however is not true, since a number of AT rich satellites seem to be moderately fluorochrome bright, fluorochrome dull or even fluorochrome negative. For example from Table 1 it can be inferred that the light satellite of *D. simulans* which has a density of 1.692 g/cm^3 is

Table 1. Relationship between differential fluorescence and decondensation of heterochromatin with the type of satellite DNA contained in it

Species	Heterochromatic areas in male genome[a] (%)	Satellite DNAs contained in heterochromatin[b]				Fluorescence with quinacrine and Hoechst 33258[c]						Decondensation with H 33258[d]		
		Density g/cm^3	% GC	Genome %		Stain	% in genome of areas				Total bright areas	Drastically decondensed	Intermediately decondensed	Total
							++	+	−	−−				
D. melanogaster	33	(L)[e] 1.672	7.0	5.2		H 0.05	9	7	12	5	16	4	5	9
		(L) 1.686	22.0	3.8	13.0	H 0.5	16	2	14	1	18			
		(L) 1.688	–	4.0		Q	8	12	12	1	20			
		(H) 1.705	37.4	4.4										
D. simulans	30	(L) 1.669	–	3	22	H 0.05	4	19	7	–	23	4	17	21
		(L) 1.692	–	19		H 0.5	4	19	7	–	23			
						Q	5	–	25	–	5			
D. virilis	52	(L) 1.671	14.3	8		H 0.05	10	–	17	25	10	–	10	10
		(L) 1.688	14.3	8	41	H 0.5	10	34	8	–	44			
		(L) 1.692	28.6	25		Q	20	3	28	1	23			
D. texana	47	(L) 1.676	–	6	31	H 0.05	–	10	10	27	10	–	7	7
		(L) 1.691	–	25		H 0.5	–	42	5	–	42			
		(H) 1.721	–	4[f]		Q	2	10	35	–	12			
D. hydei	30	a cryptic light satellite DNA				H 0.05	–	4	9	17	4	–	< 1	< 1
		(H) 1.714	55.0	3		H 0.5	–	12	13	5	12			
						Q	3	5	7	15	8			
D. ezoana	33	devoid of satellite DNAs				H 0.05	–	9	24	–	9	–	< 1	< 1
						H 0.5	–	9	24	–	9			
						Q	–	4	29	–	4			

[a] The percentage of heterochromatin was calculated on prometaphases stained with acetic orcein. Similar percentages were obtained on C-banded metaphases (Pimpinelli et al., 1976b). Also dot chromosomes were considered heterochromatic since, as they are often positive in C-band preparations and have a different fluorescence from euchromatin, they have typical heterochromatic properties

[b] The data on satellite DNA were those of Peacock et al. (1973) for *D. melanogaster*, Travaglini et al. (1971) for *D. simulans*, Gall et al. (1971) for *D. virilis*, Gall et al. (1973) and Gall and Atherton (1974) for *D. texana* and *D. ezoana*, Hennig et al. (1970) for *D. hydei*

[c] The proportion of the various fluorescent blocks was calculated on enlarged microphotographs

[d] To estimate the percentage of the decondensed regions, the euchromatin of the affected cells was measured and from this the expected amount of heterochromatin calculated. The amount of non-decondensed heterochromatin was substracted from this expected amount and the percentage per genome of decondensed material obtained

[e] (L) Light and (H) heavy satellite DNAs with respect to the main band

[f] The percentages of satellite DNAs have been estimated from the densitometer tracings obtained by Gall et al. (1973) and Gall and Atherton (1974) after ultracentrifugation in neutral CsCl density gradient

quinacrine dull and moderately Hoechst bright. Satellite I of *D. virilis*, which is localized on all the chromosomes except the Y (Gall et al., 1971) is probably $H^{--}0.05$, $H^{+}0.5$, Q^{-} and of satellites II and III one is $H^{++}0.05$, $H^{++}0.5$, Q^{++} and the other $H^{-}0.05$, $H^{+}0.5$, Q^{++} (see also Holmquist, 1975a; Mayfield and Ellison, 1975). Lastly, the lightest of the *D. texana* satellites seems to be $H^{-}0.05$, $H^{+}0.5$, Q^{-}. It appears therefore that even chromosomal regions containing very AT rich satellite DNAs can be fluorochrome dull. 4. The different cytological specificities of quinacrine and H 33258, as also the other phenomena of differential fluorescence, do not seem to be easily correlated either with the relative AT richness or with the base sequences of the various AT rich satellite DNAs. For example, satellites II and III of *D. virilis* which, as has been mentioned, are cytologically different, both contain 14% of GC (Gall et al., 1971) and are composed of closely related base sequences (Gall and Atherton, 1974).

Taken as a whole, the present data show that chromosome regions containing similarly AT rich satellite DNAs fluoresce differently after H 33258 and quinacrine staining and respond differently to increase in concentration of H 33258. It is therefore reasonable to assume that these regions differ from each other in a number of structural characteristics such as the conformation of the AT rich satellite DNAs (Bram and Tougard, 1972) and/or chromosomal protein bound to them.

We suggest that the non-histone proteins are involved in many of the above phenomena of differential fluorescence. In fact, these proteins could be specifically bound to a number of AT rich satellite DNAs and differently inhibit the binding and/or fluorescence of H 33258 and quinacrine. They could also be involved in the variation of the fluorescence patterns with the concentration of H 33258. Two arguments can be proposed to support this hypothesis. (1) Recent biochemical studies have shown that non-histone proteins inhibit the fluorescence both of quinacrine (Gottesfeld et al., 1974; Comings et al., 1975; Simola et al., 1975) and of H 33258 (Simola et al., 1975). Simola et al. (1975) have also suggested that these proteins inhibit the binding and/or fluorescence of H 33258 to a lesser extent than that of quinacrine in fixed chromosomes. (2) Some chromosomal regions decondensed by Hoechst (which presumably binds specifically to them in interphase competing with the proteins) are only weakly fluorescent (e.g. *D. texana, D. simulans*). Both these regions and those which show an increase in fluorescence with concentration of H 33258 are less or more heavily stained (Pimpinelli et al., 1976b) with the N-banding method, which stains non-histone proteins specifically (Matsui and Sasaki, 1973; Funaki et al., 1975). On the contrary, all the extremely Hoechst bright regions and most extremely quinacrine bright ones are not N-banded (Pimpinelli et al., 1976b).

It is interesting to note that some heterochromatic regions that are completely negative both to quinacrine and to Hoechst, are located on the autosomes of *D. hydei.* Similar areas are present in the centromeric heterochromatin of *D. eohydei* (Holmquist, 1975a) and in that of *Bos taurus* (Schnedl, 1972; Gropp et al., 1973). Since *D. hydei, D. eohydei* (Hennig et al., 1970) and *B. taurus* (Kurnit et al., 1973) contain GC rich satellite DNAs, it might be inferred that this

DNA is located in the fluorochrome negative areas. However, *D. texana*, which also contains GC rich satellite DNA (Table 1), has no Q^{--} and $H^{--}0.5$ areas. Since the heterochromatin of *D. hydei* is much more heavily N-banded than that of *D. texana* (Pimpinelli et al., 1976b), it could be suggested that a high content both of GC bases and of non-histone proteins are required for the complete absence of fluorescence in a given chromosomal region.

In conclusion, the present data clearly show that even chromosomal regions containing very AT rich satellite DNAs can be Hoechst or quinacrine dull. Indirect evidence points to the fact that these regions contain non-histone proteins which inhibit fluorochrome binding and/or fluorescence. AT richness appears therefore to be a necessary but not sufficient condition for both Hoechst and quinacrine brightness.

Chemical and Structural Bases of H 33258 Induced Decondensation

The results obtained by treating the living ganglion cells with H 33258 and quinacrine have shown that: (1) H 33258 decondenses specific heterochromatic regions and quinacrine produces a generalized lengthening of the chromosomes; (2) the effects of H 33258 do not depend on its concentration but those of quinacrine do; (3) for both agents the percentage of cells affected increases with time of treatment. Taking into account the length of the cell cycle of the neuroblasts (Pimpinelli et al., 1976a) it is probable, as suggested for Chinese hamster cells (Rocchi et al., 1976), that both H 33258 and quinacrine have an S-dependent effect.

On the basis of these data we suggest that, probably during the S phase, H 33258 binds preferentially with particular chromosome regions interfering with the protein binding which determines the mitotic condensation of the chromatin. The effects of quinacrine could be simply due to the extension of the DNA helix after its binding by intercalation (Comings et al., 1975). However, whatever the mechanism of action of H 33258 and quinacrine may be, their different effects confirm at the cytological level that the molecules of these two stains do not occupy the same sites in DNA (Simola et al., 1975; Comings, 1975; Latt and Wohlleb, 1975).

It has been suggested that the H 33258 decondensed areas contain AT rich DNA (Pimpinelli et al., 1975; Rocchi et al., 1976). The present data seem to confirm this hypothesis. In fact, as is shown in Table 1, only in the species containing AT rich satellite DNAs there are rather extensive decondensed areas. Very small decondensed regions are also presents on the Y of *D. hydei* and *D. ezoana*. It is possible, however, that these chromosomes contain AT rich satellite DNA in amounts so small as not to be revealed by the analysis in CsCl gradients.

From Table 1 it can be clearly seen that not all AT rich satellite DNAs are decondensed by H 33258 (e.g. *D. virilis, D. texana*). This cannot be explained by assuming that the non-decondensed satellites are not sufficiently rich in AT. For instance, in *D. virilis* (see Table 1) most likely only one of the two satellites (II and III) which both contain 14% of GC is decondensed. Also

not decondensed are the two main satellites of *D. virilis* and *D. texana* which contain 27% of GC (Table 1), while mouse satellite DNA which contains 35% GC (Corneo et al., 1968; Jones, 1970) is decondensed by H 33258 (Hilwig and Gropp, 1973).

The specificity of decondensation is also difficult to correlate with the base sequences of the various AT rich satellite DNAs. The heptanucleotide sequences of satellites I, II and III of *D. virilis* are, in fact, strictly related (Gall and Atherton, 1974) and very different from the base sequence of mouse satellite which contains regions of 5 adjacent AT pairs (Southern, 1970). Therefore factors other than the base sequence and the base composition seem to be involved in determining the cytological specificity of H 33258-induced decondensation. Among these factors, as suggested for differential fluorescence, the chromosomal proteins, could be very important. It is to be pointed out that most probably different proteins are involved in the phenomena of decondensation and differential fluorescence, since there is not a good correspondence between decondensation and Hoechst brightness. The non-histone proteins stained with the N-banding method seem to inhibit fluorescence but not always decondensation (e.g. *D. simulans* and *D. texana*). In conclusion, even if the mechanisms and the specificity of H 33258-induced chromosome decondensation are not yet clear, there is no doubt that this constitutes a useful cytogenetic method for revealing very AT rich chromosomal regions, which are often not Hoechst or quinacrine bright. The techniques of selective chromatin decondensation can also supply useful informations on chromosome organization in its dynamics during the cell cycle.

Heterogeneity of Constitutive Heterochromatin of Drosophila

The use of modern banding techniques has revealed a striking cytological heterogeneity of the constitutive heterochromatin (Jalal et al., 1974; Ganner and Evans, 1971; Vosa, 1970a). Comparative results of the response to decondensation and to H 33258 and quinacrine staining make it possible to divide the constitutive heterochromatin of *Drosophila* which is always positive in C-Band preparations (Pimpinelli et al., 1976b)– into various cytological subtypes (Table 2). As shown in Table 2, from 4 to 7 of these subtypes are simultaneously present within a given genome. For the sake of simplicity, we have used only the data obtained with the lowest concentration of H 33258 in constructing Table 2 and we have not distinguished between drastic and intermediate decondensation. Nor have we taken into account the data concerning the N bands which vary in staining intensity between and within species (Pimpinelli et al., 1976b). The number of heterochromatic types described in Table 2 is therefore an underestimate of those which can really be identified with our cytological methods.

The structural bases of this unexpected heterogeneity of the constitutive heterochromatin of *Drosophila* can, with all probability, be explained by the hypothesis already proposed under the two previous headings. It is very difficult to form a hypothesis on the functional significance of the various types of heterochromatin. The cytological heterogeneity of *Drosophila* heterochromatin

Table 2. Types of heterochromatin identifiable in various species of the genus *Drosophila* after staining with two fluorochromes (H 33258 at 0.05 μg/ml and quinacrine) and selective decondensation with H 33258

Species	Type of heterochromatin													
	H^{++} Q^{++} D^{+}	H^{++} Q^{+} D^{+}	H^{++} Q^{-} D^{+}	H^{+} Q^{+} D^{+}	H^{+} Q^{-} D^{+}	H^{+} Q^{++} D^{-}	H^{+} Q^{+} D^{-}	H^{+} Q^{-} D^{-}	H^{-} Q^{-} D^{+}	H^{-} Q^{++} D^{-}	H^{-} Q^{+} D^{-}	H^{-} Q^{-} D^{-}	H^{--} Q^{-} D^{-}	H^{--} Q^{--} D^{-}
D. melanogaster	+	+	−	−	−	−	+	−	−	−	+	+	+	+
D. simulans	+	−	+	−	+	−	−	−	−	−	−	+	−	−
D. virilis	+	−	−	−	−	−	−	−	−	+	+	+	+	+
D. texana	−	−	−	+	−	−	+	−	+	+	−	+	+	−
D. hydei	−	−	−	+	−	+	+	−	−	−	+	+	−	+
D. ezoana	−	−	−	+	−	−	+	+	−	−	−	+	−	−

Q^{++} and H^{++} = Extremely bright areas with quinacrine and H 33258 respectively; Q^{+} and H^{+} = Bright areas; Q^{-} and H^{-} = Fluorochrome dull areas showing the same fluorescence as euchromatin; H^{--} and Q^{--} = areas not fluorescent or less fluorescent than euchromatin; D^{+} = decondensed areas; D^{-} = areas insensitive to in vivo treatment with H 33258

could simply represent a multiplicity of aspects of the same function or reflect a real functional heterogeneity. In this respect we wish to stress that *D. melanogaster* offers unique possibilities for the study of the functions of heterochromatin. It is well known that it is possible to construct genomes of *D. melanogaster* containing different amounts of heterochromatin (Lindsley and Grell, 1968; Lindsley et al., 1972) and then to study the functions of this material.

Evolution of Heterochromatin

The present results fully confirm that in the genus *Drosophila* "heterochromatin is more taxonomically divergent than external morphological characteristics" (Holmquist, 1975a, b). Hoechst decondensation provides a further cytological criterion for sustaining this claim. Indeed the two sibling-species *D. melanogaster* and *D. simulans*, which show few differences in the banding pattern of the polytene chromosomes (Horton, 1939), differ drastically in heterochromatin both after fluorochrome staining and after decondensation with H 33258. The latter cytological procedure also shows that these species both contain some drastically decondensed areas not present in the other species; they differ, however, in the chromosomal localization of these areas.

The two strictly related species, *D. virilis* and *D. texana*, differ in a few euchromatic inversions and in the centric fusion of chromosomes 2 and 3 in *D. texana* (Patterson and Stone, 1952). When studied with our cytological techniques, they present some interesting analogies and differences in the heterochromatin:

1. *D. texana*, to a great extent because of the fusion between the chromosomes 2 and 3, has 10% less heterochromatin than *D. virilis* (Table 1).
2. The Y chromosomes of the two species are clearly different.
3. *D. virilis* and *D. texana* do not differ with regard to the localization and

size of the heterochromatic $H^{--}0.05$, $H^{+}0.5$, Q^{-}, D^{-} blocks, which seem to effectively correspond (Table 1, and Holmquist, 1975a, b) to satellite I which is present in both species (Gall et al., 1973; Gall and Atherton, 1974).

4. The heterochromatic blocks of *D. virilis* $H^{++}0.05$, $H^{++}0.5$, Q^{++}, D^{+} and $H^{-}0.05$, $H^{+}0.5$, Q^{++}, D^{-} which probably correspond one to satellite II and the other to satellite III (Table 1, and Holmquist, 1975a, b) are, like these satellites, absent in *D. texana.*

5. New heterochromatic blocks $H^{-}0.05$, $H^{+}0.5$, Q^{-}, D^{+} appeared in *D. texana.* They include about 7% of the genome. This material decondensed by Hoechst has the same appearance as that of *D. virilis* but not the same chromosomal localization. We suggest that the new type of heterochromatin (as cytologically defined) appearing in *D. texana* contains the lightest satellite of this species (Table 1). It could originate from the heterochromatin $H^{++}0.05$, $H^{++}0.5$, Q^{++}, D^{+} of *D. virilis* which in the course of evolution underwent some change in the base composition of the satellite DNA contained in it, was coated by different chromosomal proteins and finally translocated into new chromosomes. Most of the material $H^{-}0.05$, $H^{+}0.5$, Q^{++}, D^{-} of *D. virilis* has probably been lost with the fusion of chromosomes 2 and 3 which was preceded by a 2–5 translocation.

Taken as a whole, the present data clearly show the importance of the role played by heterochromatin in the evolution of the genus *Drosophila.* Within this genus the qualitative and quantitative variations of the heterochromatin, like perhaps the euchromatic chromosomal rearrangements, seem to be connected with speciation.

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