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EFFECTS OF HOECHST 33258 ON CONDENSATION PATTERNS OF HETERO- AND EUCHROMATIN IN MITOTIC AND INTERPHASE NUCLEI OF *DROSOPHILA NASUTA*

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SUMMARY

Embryonic and third instar larval brain cells of *D. nasuta* were cultured in vitro in the presence of Hoechst 33258 (H) and H+5-bromodeoxyuridine (BUdR) for periods varying from 2 to 24 h at 24°C. Air-dried chromosome preparations were made with and without hypotonic pretreatment and stained with Giemsa. Metaphase chromosomes from H-treated (2 h) embryonic preparations show typical inhibition of condensation of the A-T-rich heterochromatin as in mouse. Presence of BUdR with H causes inhibition of condensation in fewer embryonic metaphase cells, but in the affected metaphases the degree of inhibition is more severe. In larval brains, however, even a 24 h H or H+BUdR treatment does not cause any significant inhibition of heterochromatin condensation. It is suggested that the differences in H effect on metaphase chromosomes of embryos and larval brains is related to differences in chromosome organization in the two cell types. Exposure of H-treated embryonic as well as larval brain cells to a hypotonic salt solution prior to fixation causes a 'supercondensation' of the heterochromatic chromocentre in most interphase nuclei. Presence of BUdR along with H reduces the frequency of cells showing such 'supercondensed' chromocentre. The euchromatin region in H-treated interphase nuclei is, on the other hand, slightly more diffuse than in control nuclei. Apparently, H-binding to DNA affects the nucleoprotein organization in hetero- and euchromatic regions of interphase nuclei in specific ways.

Since the initial study by Hilwig & Gropp [1], Hoechst-33258 (H) has been known to inhibit or delay the chromatin condensation preparatory to mitosis, so that at metaphase stage the chromosome or chromosome regions appear highly extended [2, 3]. Furthermore, it is also known that A-T-rich centric heterochromatin or 5-bromodeoxyuridine (BUdR)-substituted chromatin is much more susceptible to H-induced inhibition of condensation [1, 4, 5]. Compared with studies on mammalian cells, there have been fewer studies on the effect of H on live cells of *Drosophila*. In one study, Gatti et al. [4] have reported H-induced inhibition of condensation of certain A-T-rich heterochromatin regions in metaphase chromosomes in a few species of *Drosophila*. In the present report, we present our observations on the effects of H treatment on heterochromatin in interphase and metaphase nuclei in embryos and larval brain ganglia of *Drosophila nasuta*. In *D. nasuta* all the major chromosomes carry large blocks of heterochromatin and all these heterochromatic segments share asymmetric A-T-rich sequences [6, 7] and in this later respect, the heterochromatin in *D. nasuta* appears similar to that in mouse. However, our present observations show that while the heterochromatic regions in metaphase chromosomes from embryonic cells of *D. nasuta* show drastic inhibition of condensation after H treatment as in

mouse, the metaphase chromosomes from larval brains of *D. nasuta* do not show such inhibition of condensation even after prolonged H treatment. Our observations further show that in H-treated interphase nuclei of *D. nasuta* (from embryos as well as from larval brains) the heterochromatic chromocentre region becomes 'supercondensed' if these H-treated cells are exposed to a hypotonic salt solution prior to fixation. A similar effect on interphase heterochromatin has not been reported earlier.

MATERIALS AND METHODS

A wild-type strain (Varanasi) of *D. nasuta* has been used for these studies. Eggs were collected and larvae were reared on the standard agar–cornmeal–brown sugar food at 24±1°C. The following experiments were carried out.

Hoechst treatment to embryonic cells

Some 200–300 eggs (4–5 h after laying) were collected and transferred to a modified Schneider's medium [8]. In the modified medium, yeastolate was omitted and the various amino acids were replaced by 1.75% lactalbumin hydrolysate (Sigma). Eggs were washed 2–3 times with the medium to remove the adhering food particles, dechorionized with 2–3% sodium hypochlorite (5 min) and washed again in sterilized medium. Each egg was fragmented into pieces with fine needles. The egg fragments were distributed in three cavity blocks, each with 0.5 ml of the medium. In the first set, Hoechst 33258 (H) was added (40 μg/ml); in the second set, in addition to H (40 μg/ml), 5-bromodeoxyuridine (BUdR) was also added (40 μg/ml). The third set to which neither H nor BUdR was added, served as a control. All cultures were maintained at 24±1°C. After 2 h, colchicine (1 μg/ml) was added to each culture and, 1 h later, egg pieces were transferred to the hypotonic solution (0.67% tri-sodium citrate) for 25 min at 24±1°C. They were subsequently fixed in 1:3 acetomethanol and air-dry preparations were made [6] taking several egg fragments on each slide. The preparations were stained with 5% Giemsa (pH 7.0) for 2–5 min, rinsed, dried and mounted.

Hoechst treatment to late third instar larval brain ganglia

Brain ganglia from late third instar larvae (post black spiracle stage [9]) were dissected out under aseptic conditions and cultured in modified Schneider's medium. The ganglia were exposed to H or H+BUdR treatments as above for 2, 4, 16 or 24 h. Parallel control cultures were also maintained. Air-dried chromosome preparations from control and H-treated ganglia were made either without any hypotonic pretreatment or after a brief (2–10 min) hypotonic (0.67% tri-sodium citrate) pretreatment or after the optimal (35 min) hypotonic pretreatment. In case of H-+BUdR-treated ganglia, only the optimum hypotonic pretreatment was given. Giemsa-stained preparations were examined for the organization and morphology of hetero- and euchromatin in interphase and metaphase nuclei. In each case, at least ten preparations were examined.

OBSERVATIONS

Effect of H on metaphase chromosomes

The metaphase karyotype of *D. nasuta* has been described earlier [6]. It may be noted here that in earlier studies we had reported the X-chromosomes in *D. nasuta* to be acrocentric [6, 7]; however, recently we also observed a new form of submetacentric X in the same wild-type strain in addition to standard acrocentric X-chromosome (fig. 1*a*). The submetacentric X appears to be of spontaneous origin in the stock. This aspect would be reported in detail separately. Suffice it to mention that in the present study, both forms of X-chromosome in homozygous or heterozygous conditions were encountered.

Data on the effects of H or H+BUdR treatment on the condensation patterns of heterochromatic and euchromatic regions in metaphase chromosomes from embryonic or larval brain cells are presented in table 1. A few representative examples are shown in fig. 1*b–i*. It is seen that the metaphase chromosomes from these two cell types respond very differently to H treatment (40 μg/ml). Thus, whereas the condensation of hetero- and euchromatin regions in all H-treated embryonic metaphase cells is inhibited to varying degrees (fig. 1*b–f*), in H-treated brains very few (4 h) or none (24 h) of the metaphases show condensation inhibition in hetero- and euchromatic regions (fig. 1*g–i*). Even in the

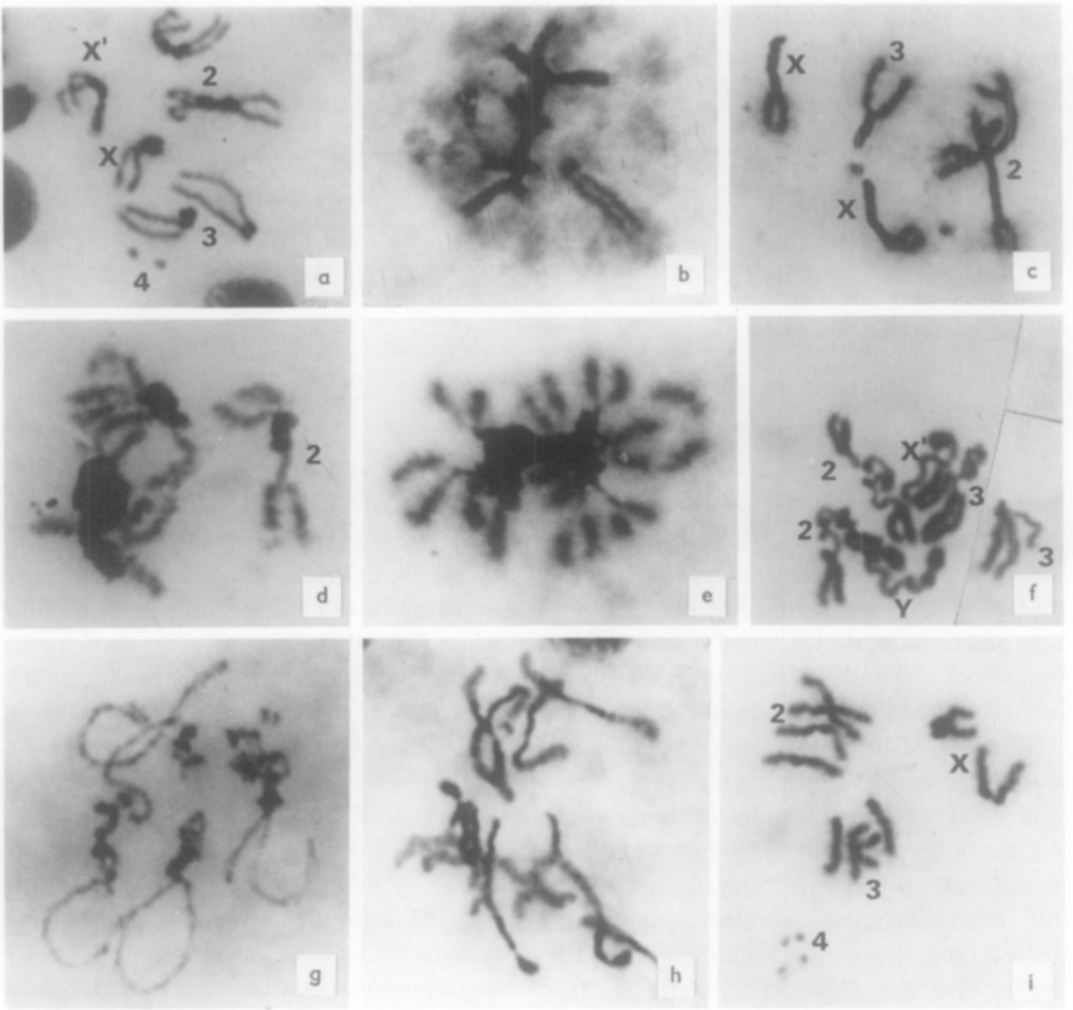

Fig. 1. Giemsa-stained (*a*) normal or (*b–i*) treated metaphases from (*b–f*) embryos or (*g–i*) larval brains of *D. nasuta*. (*a*) Metaphase chromosomes from control larval brain—note the large centric heterochromatic regions on X, 2nd and 3rd chromosome pairs, the acrocentric and submetacentric forms of X are designated X and X′, respectively; (*b–e*) 2 h H-treated metaphases from embryos showing (*b*) extended/diffused hetero- and euchromatic regions; (*c*) normally consensed euchromatin but moderately extended heterochromatin; or (*d, e*) fusion of parts of heterochromatic segments of different chromosomes into (*d*) several or (*e*) one dense mass; (*f*) 2 h H-+BUdR-treated metaphase from embryos showing a severe inhibition of condensation in heterochromatic regions only—there are traces of differential staining of sister chromatids in the normally condensed euchromatic regions; (*g–i*) metaphases from (*g, h*) 4 h or (*i*) 24 h H-treated larval brains showing abnormal patterns of chromatin condensation in (*g, h*) 4 h, but no effect in (*i*) 24 h treated samples. ×1600.

affected metaphase plates from brain tissue, the degree of condensation inhibition, particularly of heterochromatic regions, is far less than that seen in H-treated embryonic cells. It should also be noted that the H-induced inhibition of condensation occurs independently in hetero- and euchromatin regions, since in different plates either one of these regions may appear normal or extended/diffused. In some of the H-treated

Table 1. *Effect of H (40 μg/ml) and H+BUdR (each 40 μg/ml) treatments on condensation of metaphase chromosomes in embryonic and larval brain cells of D. nasuta*

Cell type	Treatment	Duration (hours)	No. of metaphases observed	Frequency (%) of metaphases with: Heterochromatin normal Euchromatin — Normal	Diffused	Total	Heterochromatin extended Euchromatin — Normal	Diffused	Total
Embryonic	H	2	104	0.0	0.0	0.0	2.88	97.11	100.00
	H+BUdR	2	259	54.44	8.88	63.32	13.89	22.78	36.67
Larval brain	H	4	240	61.66	14.58	76.24	4.16	19.58	23.74
	H	24	44	100.00	0.0	100.00	0.0	0.0	0.0
	H+BUdR	4	151	67.55	26.49	94.03	2.65	3.31	5.96

embryonic preparations, parts of heterochromatic regions of different chromosomes appear as highly condensed irregular masses which may be fused with each other into one or more dark-stained mass(es) (see fig. 1*d, e*). A similar effect was not seen in any H-treated brain preparations.

In H+BUdR-treated embryonic preparations, relatively fewer metaphases show inhibition of condensation of hetero- or euchromatin (see table 1). But, it should be noted that in those metaphases in which the heterochromatin condensation is affected, the degree of inhibition is much higher than after H treatment alone (see fig. 1*f*). In most of these plates, euchromatin appears normally condensed. In H+BUdR-treated (4 h) brain preparations too, the frequency of normal metaphases is slightly higher than that seen after only H treatment alone (4 h). But, unlike in embryonic preparations the affected metaphases in H+BUdR-treated brain preparations do not show a greater condensation inhibition than is seen with H treatment only.

Effect of H on interphase nuclei

In *D. nasuta,* the interphase nuclei from embryos as well as larval brains normally have a prominent chromocentre (cc) formed

Table 2. *Effect of H and H+BUdR treatments on the morphology of chromocentre in interphase nuclei in embryonic and larval brain cells of D. nasuta*

Cell type	Treatment	Duration (hours)	No. of interphase nuclei observed	Frequency (%) of interphase nuclei with: Normal chromocentre	'Supercondensed' chromocentre: One chromocentre/nucleus	More than one chromocentre/nucleus	Total
Embryonic	H	2	2 511	19.00	47.51	33.53	81.04
	H+BUdR	2	2 017	68.71	19.48	11.80	31.28
Larval brain	H	4	2 932	55.18	44.78	0.0	44.78
	H	16	5 551	31.26	68.74	0.0	68.74
	H+BUdR	4	1 821	69.13	30.86	0.0	30.86
	H+BUdR	16	2 994	60.75	39.26	0.0	39.26

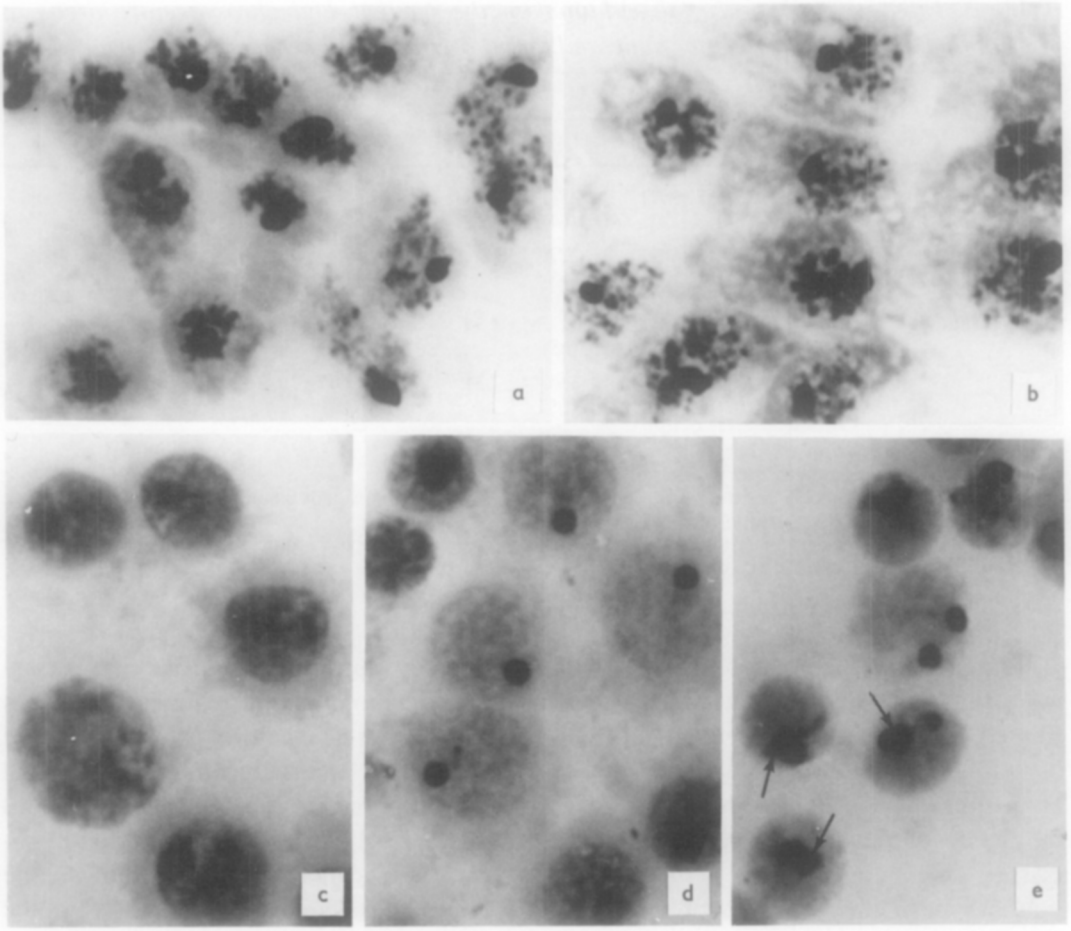

Fig. 2. Giemsa-stained interphase nuclei from (*a–d*) larval brain or (*e*) embryos. (*a*) Control nuclei without exposure to hypotonic salt solution; (*b*) 4 h H-treated nuclei also without exposure to hypotonic salt solution; (*c*) control nuclei after hypotonic treatment—note the partial or complete diffusion of the chromocentre; (*d*) 4 h H-treated nuclei after exposure to hypotonic solution showing the 'supercondensed' chromocentres and uniformly stained euchromatin; (*e*) H-treated nuclei from embryos after a brief exposure to hypotonic solution, a less dense region is seen (→) associated with the chromocentre in many nuclei—note also the presence of more than one 'supercondensed' chromocentre in some embryonic nuclei. ×1600.

by fusion of heterochromatin of all chromosomes [6]. In control hypotonic-pretreated preparations, the heterochromatic cc becomes less prominent and is often not seen at all. However, in hypotonic-pretreated preparations of embryonic or larval brain cells exposed to H, the majority of cells show a very intensely stained and smoothly outlined cc, such as is never seen in control preparations (fig. 2*c*, *d*). As shown in table 2, the frequency with which this type of 'supercondensed' cc is seen after H treatment varies with cell type and duration of treatment, being more common in embryonic cells than in larval brain cells. Furthermore, in H-treated embryonic cells, often (≃33%) there is more than one such supercondensed body (fig. 2*e*), but in

Table 3. *Effect of hypotonic salt solution on the nuclear and chromocentre areas in control and H-treated (4 h) embryonic and larval brain cells of D. nasuta*

Cell type	Treatment	Mean nuclear area (±S.E.)		Mean chromocentre area (±S.E.)	
		Non-hypotonic	Hypotonic	Non-hypotonic	Hypotonic
Embryonic	Control	211.83±9.71 (54)	254.14±11.71 (64)	34.57±1.43 (54)	–
	H	248.55±9.97 (65)	227.71±11.20 (65)	34.37±1.22 (65)	20.52±0.84 (65)
Larval brain	Control	272.48±9.83 (64)	327.65±8.34 (65)	42.58±1.20 (64)	–
	H	351.37±13.85 (62)	366.74±7.31 (65)	47.47±2.05 (62)	27.62±1.66 (65)

The areas are expressed in arbitrary units.
Figures in parentheses indicate the number of nuclei measured.
The cc area in hypotonic pretreated control embryonic and larval brain cells could not be measured, since in most of them the cc is very diffused or not seen at all.
The mean nuclear areas in all H-treated samples, except in embryonic hypotonic series, differ significantly ($P<0.01$) from the corresponding control values.

H-treated brain preparations, nuclei with more than one supercondensed cc are extremely rare or do not occur at all. Embryo fragments or larval brains exposed to H in presence of BUdR showed a lower frequency of nuclei with supercondensed cc (see table 2).

It has been observed that if hypotonic pretreatment is not given, the control and H-treated interphase nuclei (embryonic as well as larval) show a similar cc organization (fig. 2*a*, *b*). To examine this aspect in some detail, the cc and nuclear areas in control and H-treated brain preparations, made without or with optimal hypotonic pretreatment (0.67% tri-sodium citrate for 35 min at 24°C), have been measured from enlarged negative images (for details of the method see ref. [10]). The data on the cc and nuclear areas under the different conditions are presented in table 3, and they clearly show the following:

(*i*) In preparations made without hypotonic pretreatment, the nuclear area in H-treated cells is significantly greater than in similarly prepared control cells. As expected, hypotonic pretreatment increases the nuclear area in control preparations, but in H-treated embryonic as well as larval brain cells, the mean nuclear areas do not differ ($P>0.1$) in hypotonic and non-hypotonic exposed preparations. Although, the mean nuclear areas in control-hypotonic and H-hypotonic embryonic samples do not differ significantly ($P>0.1$), in larval brain cells the mean nuclear area is significantly ($P<0.01$) larger in H-hypotonic than in control-hypotonic sample.

(*ii*) In preparations of embryos and larval brains made without hypotonic pretreatment, the cc area in control and H-treated cells is similar, but in the H-treated cells exposed to hypotonic solution, the cc area is significantly smaller (see table 3).

A few H-treated larval brain preparations were made after a brief (2, 5 or 10 min) hypotonic pretreatment. Interestingly, in all of these, the cc appeared 'supercondensed' as after the optimal hypotonic treatment. However, in some nuclei in these prepara-

tions a less dense region was seen associated with the supercondensed cc (fig. 2*e*).

DISCUSSION

The present study reveals unexpected but interesting effects of H on live cells of *D. nasuta*. Firstly, while H causes striking inhibition of condensation of heterochromatin in embryonic cells, a similar effect is nearly absent in larval brain cells. Secondly, even a brief exposure of H-treated interphase cells to a hypotonic solution results in a 'supercondensation' of the heterochromatic chromocentre, whereas in non-H-treated tissues the hypotonic treatment causes decondensation of the chromocentre in interphase nuclei. Thirdly, unlike the results of Matsukuma & Utakoji [5] in mouse cells, BUdR substitution in *D. nasuta* nuclei appears to have a 'protective' effect against H treatment, at least in some cells.

The highly extended appearance of hetero- and euchromatin regions of embryonic metaphase chromosomes in *D. nasuta* after H treatment is comparable to the reported effects of H on chromatin condensation in mammalian cells [1–3]. The similar response of all heterochromatic regions in embryonic cells of *D. nasuta* to H treatment further confirms our earlier suggestion that in this species heterochromatin segments of different chromosomes share similar A-T-rich base sequences [6, 7]. In this context, the observed absence of inhibition of heterochromatin condensation in a majority of metaphases in the H-treated larval brains of *D. nasuta* is intriguing.

While considering the insensitivity to the H effect of certain heterochromatic regions in human or other chromosomes, Marcus et al. [3] have suggested that the lack of response may be due to a low permeability of H in certain cell types. In the present case, however, it does not appear that the larval brain cells are less permeable to H than the embryonic cells for two reasons. Firstly, the interphase nuclei in embryonic and larval brain cells respond identically to the H effect ('supercondensation' of heterochromatin and diffusion of euchromatin); secondly, we have directly ascertained, by fluorescence microscopy (data not presented), that in larval brains exposed to H (40 μg/ml) for 4 h or longer, all interphase and metaphase nuclei show the typical fluorescence pattern (intense in all heterochromatic regions and dull in euchromatic regions). Thus, the drug enters the brain cells and also efficiently binds to chromatin. The absence of the H effect in the majority of brain metaphase cells, in spite of the H-binding to chromatin, may alternatively be related to the possibility that the fluorochrome may not reach a high enough concentration in gangliar cell nuclei to prevent the normal pre-mitotic chromatin condensation patterns. To check this possibility, we have made additional experiments (detailed data not presented here) in which the larval brain ganglia were exposed to higher concentrations of H for 4 h (80 or 200 μg/ml) or for 16 h (80 μg/ml). It is significant to note that even at these higher H concentrations, only 20–25% of the observed metaphases (60–100 metaphases observed in different cases) show inhibition of heterochromatin condensation, as has already been noted with 40 μg/ml H treatment (table 1). The only difference seen between 40 μg/ml and the higher concentration treatments is that with increasing concentrations the affected metaphases show higher condensation inhibition. Thus we believe that in larval brain ganglia of *D. nasuta* only a subpopulation of mitotically dividing cells may be responding to the condensation inhibitory effect of H. The insen-

sitivity to H in other mitotic cells is probably not related to factors like low cell permeability or to a sub-optimal concentration of the drug in cell nucleus.

To some extent, our present observations on the H effect on mitotic chromosomes in larval brain of *D. nasuta* are in conflict with the previous reports [4, 11] on positive effects of H on brain cells of *D. melanogaster* and other species. It may, however, be noted that under our experimental and larval culture conditions, H treatment to *D. melanogaster* cells gives results essentially similar to those reported here for *D. nasuta* cells. Thus in *D. melanogaster* we have also observed (data not presented) that a 100 μg/ml H treatment to embryonic cells for 2–3 h results in condensation inhibition in all metaphases, but in larval brain tissue a 100 μg/ml H treatment for 4–5 h results in condensation inhibition in only about 40% of the metaphases; the remaining 60% metaphases appear normal.

In view of the above, we feel that the larval brain cells differ in some features of nuclear organization from undifferentiated embryonic cells and the former may be responsible for the differential response observed in the two cell types. Two features of the organization of larval brain cell nuclei may be relevant in this context. Different cells in brain ganglia of *Drosophila* larvae undergo varying numbers of endoreduplication cycles and during these the hetero- and euchromatic regions replicate independently and unequally [10, 12]. In addition, there is some evidence to suggest that in late larval brain ganglia, the metaphase chromosomes become polynemic and on the basis of previous [13–15] and other observations [18] we favour the concept, as a working hypothesis, that in *Drosophila* the embryonic cells have unineme chromosomes, whereas in late larval ganglia most of the mitotic cells become polyneme. It is possible that the insensitivity of metaphase chromosomes in the majority of nuclei in larval brain of *Drosophila* to the H effect are related to these two features of nuclear organization in larval brain ganglia. We are examining these aspects further.

In view of the generally known [2, 3] condensation-inhibitory action of H on live cells, the observed 'supercondensation' of interphase heterochromatin is most unexpected. H treatment also affects the euchromatic regions in interphase nuclei of *D. nasuta*, but this effect is different from that on heterochromatin, since the H-treated nuclei (non-hypotonic series) have a significantly larger nuclear area than the control nuclei. Thus H treatment causes a certain degree of decondensation or inhibition of condensation of euchromatic regions in interphase nuclei. The basis for these effects of H on heterochromatin or euchromatin in interphase nuclei is not understood and neither do we know if the apparently opposite effects of H on interphase and metaphase chromatin condensation patterns are independent actions or if they are different manifestations of the same molecular action of H on chromatin. Our observations underscore the need to examine the effects of H on interphase chromatin in other organisms as well. It might be of interest to note here that the observed effects of H on interphase heterochromatin of *D. nasuta* are presumably not basically related to these heterochromatic regions being A-T-rich [6], since in H-treated brain cells of *D. hydei* also, a similar 'supercondensation' of the cc has been observed (Lakhotia, S. C. & Mania, J., unpublished) although *D. hydei* heterochromatin has only a low proportion of A-T-rich sequences [16, 17].

The present results of H+BUdR treatment are also unexpected in view of the

published accounts of the increased sensitivity of BUdR-substituted chromatin to H-induced condensation inhibition [5]. In our study, fewer interphase and metaphase nuclei show the H effect in H-+BUdR-treated than in only H-treated samples. Thus in both cases, BUdR substitution seems to have some 'protective' effect against H action. However, the occurrence of more severe condensation inhibition in those H-+BUdR-treated embryonic metaphase chromosomes which show a positive H effect, indicates that BUdR sensitization [5] does occur at least in some embryonic cells of *D. nasuta*. The absence of any H effect in brain metaphase cells even after BUdR substitution seems to be related to the chromosomal differentiation presumed to occur in this tissue. The variable response of different metaphase cells in embryos of *D. nasuta* to H+BUdR treatment may also presumably be due to a heterogeneity in mitotic cell population of even these early embryonic stages.

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