Indian Journal of Experimental Biology
Vol. 20, November 1982, pp. 791-796

Further Observations on Hoechst 33258 Plus Hypotonic Treatment Induced Supercondensation of Interphase Heterochromatin in *Drosophila nasuta* Cells

J K ROY & S C LAKHOTIA*
Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi 221 005
and
M L S MELLO
Department of Cell Biology, I.B. Unicamp, C.P. 6109, 13100 Campinas (SP), Brazil

Received 17 *June* 1982

Extending our earlier observation [*Exp Cell Res*, **132** (1981) 423] on the effects of Hoechst 33258 and hypotonic treatments on condensation of heterochromatin in interphase nuclei of *D. nasuta*, we now show by Feulgen-DNA cytophotometry that the chromocentre heterochromatin in Hoechst 33258 + hypotonic treated interphase cells of *D. nasuta* is supercondensed. Like the Hoechst 33258 effect, exposure of live embryonic (2 hr) or larval brain cells (16 hr) of *D. nasuta* to 20 μg/ml Netropsin or Distamycin A (both A-T specific DNA ligands) when followed by a pre-fixation hypotonic treatment, also causes supercondensation of chromocentre in majority of the treated cells; without the hypotonic treatment chromocentre is not supercondensed in any of the drug treated cells. A G-C specific DNA ligand, Olivomycin, does not cause any such effect. It is suggested that, only A-T specific ligands can cause the supercondensation of interphase heterochromatin when the treated cells are exposed to hypotonic solution prior to fixation. By reverting 16 hr Hoechst 33258 + 15 min hypotonic treated larval brain ganglia to isotonic culture medium for 1 or 4 hr, the supercondensation of chromocentre can be reversed, although a second hypotonic treatment after 4 hr in Hoechst 33258 free isotonic culture medium restores the supercondensation of chromocentre. It appears that the A-T specific drug binding to DNA modifies the nucleoprotein interactions in interphase heterochromatin in such a way that a hypotonic treatment causes a very rapid but reversible reorganization of chromatin constituents to cause the supercondensation.

A prefixation treatment of live cells with Hoechst 33258 (H) leads to inhibition of the mitotic chromatin condensation, specially of the A-T rich segments[1-3] so that at metaphase these chromosome regions appear relatively extended. A comparable inhibition of condensation has also been found with other A-T specific drugs like Netropsin[4,5] or Distamycin A[5-7]. In contrast with the above inhibitory effect of the pre-fixation H-treatment on mitotic chromatin condensation, we had earlier[8] observed an unusual effect of H on the A-T rich heterochromatic chromocentre (cc) exposed to a hypotonic (hy) treatment prior to fixation while in the absence of the hy treatment no change in the size and morphology of the cc was found. We presumed[8] this decrease in the size of the cc in H + hy treated cells to be due to a 'supercondensation' of the interphase heterochromatin.

We have now further examined the effects of three other DNA-specific ligands, viz. Netropsin (NP), Distamycin A (DA) and Olivomycin (OM) on the condensation of interphase heterochromatin in *D. nasuta*. While NP and DA are A-T specific, OM is G-C specific DNA binding agent[9,10]. In addition, we have also extended our studies on the H-effect on interphase heterochromatin by examining the following aspects: (i) Feulgen-DNA cytophotometric measurements on H and H + hy treated larval brain cells to ascertain if the decreased area of cc in H + hy treated cells is due to a 'supercondensation' of heterochromatin or due to other factors and (ii) reversibility of the H + hy effect in larval brain cells by reverting the H + hy treated ganglia to isotonic medium.

Materials and Methods

A wild type strain of *Drosophila nasuta* (Varanasi) brown sugar-yeast food at 24° ± 1°C. The following experiments have been done:

Feulgen-DNA cytophotometry of Hoechst 33258 *treated larval brain cells*— Larval brain ganglia were cultured for 16 hr in the presence (treated) or absence (control) of H (40 μg/ml), as described earlier[8]. Air dried preparations of H-treated ganglia were made with or without any pre-fixation hy treatment in 0.67% tri-Sodium Citrate for 35 min while those of control ganglia were made without any hy treatment. The ganglia were fixed for a very brief period (≃ 1 min) in fresh acetomethanol and the air dried preparations were immediately placed in 70% ethanol for 2 min after which they were again dried in air[11]. The control, H and H + hy treated larval brain preparations were

*Addressee for reprint requests.

subjected to Feulgen reaction, hydrolysis being carried out in 4*N* HCl for 80 min at 22 C. The material was then rinsed in sulphurous and distilled waters, dehydrated, cleaned in Xylene and mounted in a Cargille oil ($n_D = 1.550$).

Microspectrophotometry of the Feulgen stained zones was made with a Zeiss Microscope photometer equipped for automatic scanning in an off line operation. Operating conditions were: Planapo. 100/1.3 objective, optovar 2, measuring diaphragm diam. 0.1 mm, field diaphragm diam. 0.2 mm, LD-Epiplan 16/0.3 condenser, 0.5 × 0.5 μm spot size and λ = 560 nm obtained with a Schott monochromator ruler. The half band width at spot of transmission was considerably small as the width of the effective light beam was equal to 0.2 mm (Zeiss Information, 1977). A predominantly monodirectional scanning motion was used. The grid points showing absorbances ≤ 0.020 were considered background and were automatically removed from the nuclear image. The nuclear and cc Feulgen-DNA values and absorbing areas were determined for nuclei of the three sets (control, H and H + hy treated) of slides. cc Feulgen-DNA values and absorbing areas were obtained through integration of the absorbances over fixed values considered cut off (c.o.) points (see Results) which were chosen after a preliminary test.

The Feulgen-DNA values and absorbing areas were plotted as frequency histograms distributed in a scale of values in geometric progression[12,13].

Netropsin, Distamycin A or Olivomycin treatment of embryonic or larval brain cells—4hr old embryos of *D. nasuta* were dechorionized and treated with 20 μg/ml Netropsin (NP) or 20 μg/ml Distamycin A (DA) or 20 μg/ml Olivomycin (OM, all 3 from SIGMA) for 2 hr as described earlier for the H-treatment[8]. Brain ganglia from late third instar larvae were excised and cultured *in vitro* in presence of NP (20 μg/ml), DA (20 μg/ml) or OM (20 μg/ml) for 16 hr. For each experiment parallel control cultures without any drug were maintained. Air dried preparations of the treated or control embryonic or brain cells were made with or without any pre-fixation hypotonic (hy) treatment (see ref 8). They were stained with 5% Giemsa and analyzed for the morphology of chromocentre (cc) and non-chromocentre regions as described earlier[8].

Reversibility of H + hy treatment induced supercondensation of cc—To test the reversibility of the H + hy effect, larval brain ganglia were first exposed to H (40 μg/ml) for 16 hr *in vitro* and then subjected to the following treatments: (a) hy (0.67% tri-Sodium Citrate) treatment for 15 min, (b) a 15 min hy treatment followed by incubation for 1 or 4 hr in the normal (isotonic) culture medium, (c) 15 min hy treatment and 4 hr isotonic medium incubation followed by a second 15 min hy treatment, (d) incubation in H-free medium for 4 hr followed by 15 min hy treatment and (e) hy treatment in the culture medium diluted 1:1 with distilled water. After these various treatments the ganglia were fixed in acetomethanol; their air dried preparations were stained with 5% Giemsa and observed for cc morphology.

Results

Feulgen-DNA cytophotometric measurements—In larval brain ganglia, cells with widely varying nuclear sizes and DNA content are present[15]. For the present analysis, only the smallest sized nuclei have been measured. Even choosing visually the smallest nuclei, nuclear Feulgen-DNA values are found distributed in at least two doubling intervals. However, the frequency of Feulgen-DNA values within the two intervals, named I and II, varies between the control

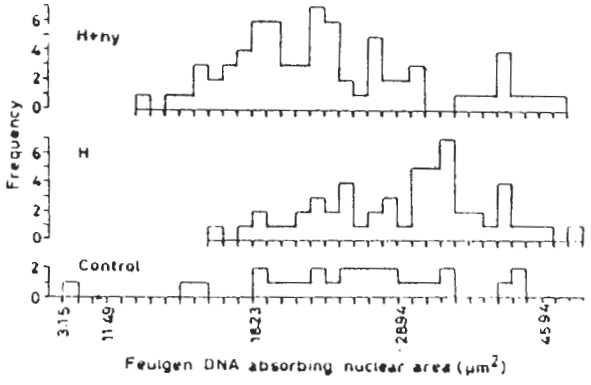

Fig. 1—Frequency distribution of Feulgen-DNA nuclear values (in arbitrary units, a.u.) of the smallest sized nuclei in Control (27 nuclei), Hoechst 33258 (H, 54 nuclei) or H + hypotonic (H + hy, 72 nuclei) treated samples

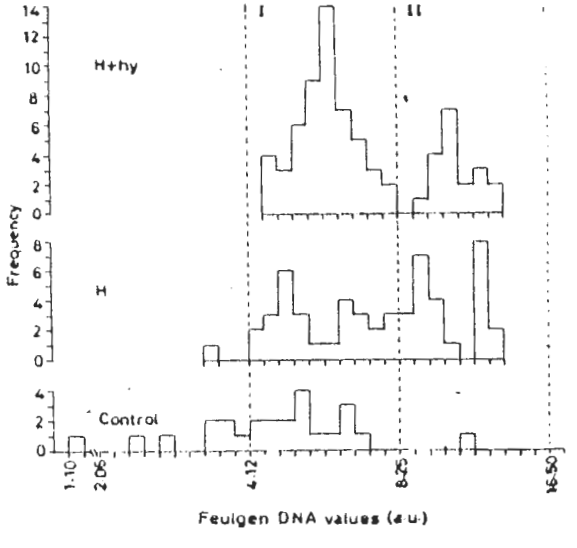

Fig. 2—Frequency distribution of Feulgen-DNA absorbing nuclear areas in control, H or H + hy treated samples

and treated samples: in H as well as H + hy treated samples, the frequency of nuclei in interval II is higher than in the control sample (Fig.1). The distribution of the nuclear Feulgen-DNA absorbing areas of H and H + hy treated nuclei appear shifted to larger and smaller values, respectively, as compared with the control (Fig.2). However, when comparing the mean nuclear absorbing areas with '*t*' - test, a statistically significant difference at $P < 0.01$ level is detected only for the II × H + hy comparison.

The cc in nuclei of control preparations even visually appear less stained than those of the treated preparations. When considering the absorbance c.o. point 0.200, most of the cc of the control appear underrepresented as their absorbances are smaller than 0.200 but higher than 0.100. On the other hand, the relative frequency of cc Feulgen-DNA values of the treated preparations differ within certain intervals (Table 1). In addition, when choosing a c.o. point 0.300, the relative frequency of cc Feulgen-DNA values is larger in H + hy treated preparations (Table 1).

The frequency with which the cc areas can be discriminated is also a function of the treatment condition and the c.o. point chosen (Table 2). Considering the c.o. point 0.200, this frequency is practically the same for H and H + hy treated nuclei. However, when using the relatively larger c.o. point (0.300) this frequency is larger in the H + hy treated sample. On the other hand, when using a c.o. point 0.200, cc with a larger area are more frequent in H treated nuclei as compared with H + hy treated ones (Table 3). These results show that the cc heterochromatin becomes supercondensed in H + hy treated cells (see Discussion).

Effects of Netropsin, Distamycin A or Olivomycin treatments on embryonic or larval brain cells—Non-hy-treated control interphase nuclei from embryos or larval brains of *D. nasuta* show a prominent cc (Fig. 3a, also see ref 8,14). After hy treatment, the cc in control embryonic or larval brain nuclei becomes diffuse (Fig.3b). As a result of the NP + hy or DA + hy treatments, interphase nuclei from embryonic or larval brain cells show supercondensation of cc (Fig. 3c,d) as seen earlier with II + hy treatment[8]. The frequency with which the treated nuclei show supercondensation of cc, varies with the cell type and with the drug used (see Table 4), being more common in embryonic than in larval brain nuclei. The non-chromocentre (ncc) euchromatic regions also appear homogeneous in the drug + hy treated than in control or only drug treated samples (Fig.3). The areas of cc and ncc regions in the control and NP or DA treated cells show differences similar to those noted earlier[8] for the H-treatment, i.e. the cc area in non-hy exposed control and the drug treated cells is similar, while in the drug + hy treated cells, the cc area is much smaller (detailed data not presented). OM does not cause any such effect since only in very rare nuclei the cc is seen to be supercondensed (Table 4 and Fig.3e).

Reversibility of H + hy induced supercondensation of cc in larval brain cells—When 16 hr H-treated larval brain ganglia are exposed for 15 min either to 0.67% tri-sodium citrate (the usual hy solution) or to 1:1 diluted culture medium, the morphology of the nuclei shows typical changes as seen after a complete 35 min hy treatment, i.e. the cc becomes supercondensed,

Table 2 – Frequency of Detectable Chromocentre (cc) in Feulgen Stained Nuclei of Larval Brain Cells of *D. nasuta* as a Function of the Absorbance c.o. Point and Treatment Used

Treatments	No. of nuclei measured	Frequency (%) of detectable cc		
		c.o. = 0.100	c.o. = 0.200	c.o. = 0.300
Control	27	100.00	37.04	0.00
H	54		96.30	48.15
H + hy	72		100.00	88.89

Table 3 – Chromocentre (cc) Feulgen-DNA Absorbing Areas in Control and Treated Larval Brain Cells of *D. nasuta* at Absorbance c.o. Point = 0.200

Treatments	Frequency (%) of cc absorbing area			
	$<0.25\ \mu m^2$	$0.25\text{-}1.0\ \mu m^2$	$1.0\text{-}2.0\ \mu m^2$	$>2.0\ \mu m^2$
Control	62.96	33.33	3.70	0.00
H	3.70	42.59	42.59	11.11
H + hy	0.00	72.22	19.44	8.33

Table 1—Frequency (%) of Chromocentre Feulgen-DNA Values within Chosen Intervals in Larval Brain Cells of *D. nasuta* as a Function of the Absorbance c.o. Point and the Treatment Used

Treatments	c.o. = 0.200					c.o. = 0.300				
	< 0.200	0.200-0.400	0.400-1.030	1.030-2.060	2.060-4.730	< 0.300	0.300-0.400	0.400-0.860	0.860-1.720	1.720-3.590
Control	62.96	18.52	14.81	3.70	0.00		—	—	—	—
H	3.70	11.11	25.93	37.04	22.22	51.85	11.11	11.11	20.37	5.56
H + hy	0.00	0.00	40.28	41.67	18.06	11.11	33.33	31.94	22.22	6.94

while the nce euchromatin regions become more diffused and uniformly stained (Figs. 4a,b and also ref 8). When 16 hr H + 15 min hy treated ganglia are returned to normal culture medium (without H) and fixed after 1 hr or 4 hr of incubation in the isotonic solution, the nuclear morphology no more shows the H + hy effect, instead, the condensation patterns of cc and nce regions are comparable to those seen in control nuclei (Figs, 4c,d). Traces of H + hy effect are still visible in 1 hr isotonic solution incubated ganglia but those incubated for 4 hr do not show any trace of H + hy effect. When 16 hr H-treated ganglia are transferred to control medium (without H) for 4 hr and then given a 15 min hy treatment and fixed, the characteristic H + hy effect is still present (Fig.4e). Similarly, when 16 hr H + 15 min hy treated ganglia are returned for 4 hr to the control culture medium and re-exposed to 15 min hy treatment, the cc supercondensation and diffusion of euchromatin areas become immediately obvious (Fig. 4f).

Table 4 — Effect of Pre-fixation Netropsin, Distamycin A or Olivomycin and Hypotonic Treatments on Supercondensation of Chromocentre (cc) in Interphase Nuclei from Embryonic and Larval Brain Cells of *D. nasuta*

Cell type	Treatments	Duration (hr)	No. of nuclei observed	% nuclei showing supercondensed cc
Embryonic	Control	2	816	0.00
	Netropsin	2	1282	52.18
	Distamycin A	2	967	69.80
	Olivomycin	2	981	3.59
Larval brain	Control	16	1896	0.00
	Netropsin	16	1392	44.97
	Distamycin A	16	1093	64.32
	Olivomycin	16	1510	5.43

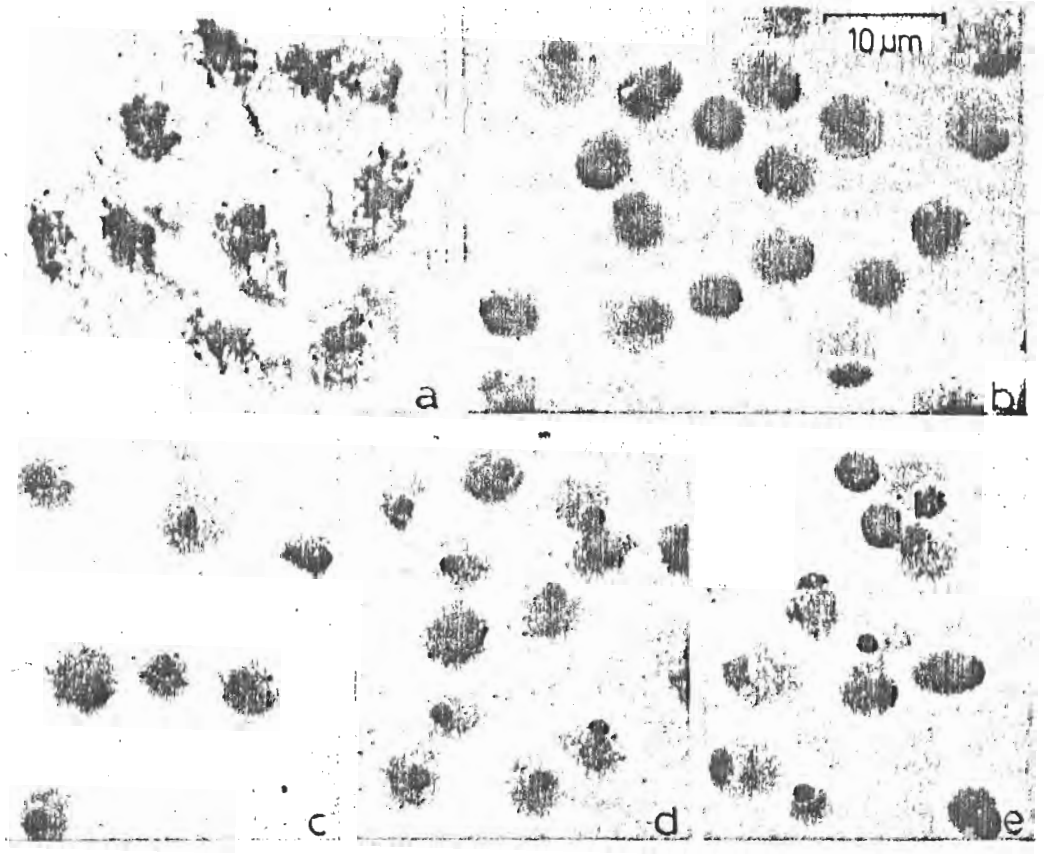

Fig. 3—Giemsa stained interphase nuclei from larval brains of *D. nasuta* [Control nuclei without (a) or with (b) a pre-fixation hypotonic exposure - note the partial or complete diffusion of chromocentre (cc) in (b). Pre-fixation hypotonic treated nuclei from ganglia exposed for 16 hr to NP (c) or DA (d) or OM (e). Note the supercondensation of cc in most nuclei in c and d but only in rare cases in e]

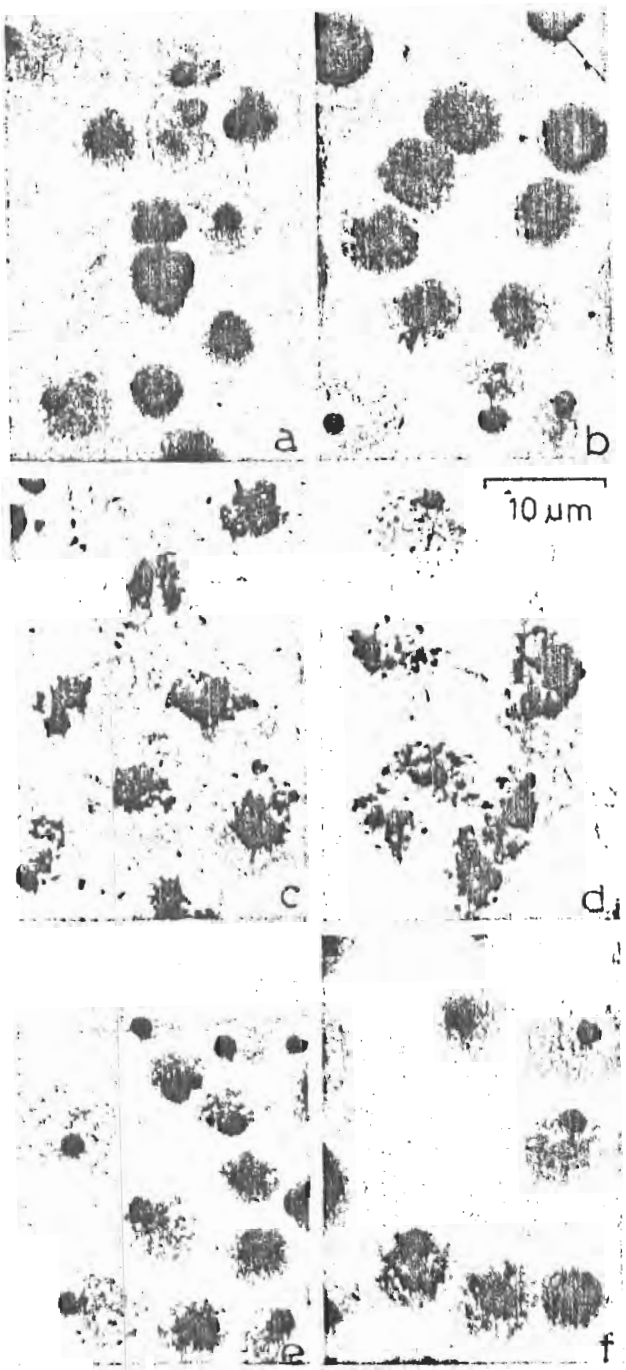

Fig. 4—Giemsa stained interphase nuclei from 16 hr Hoechst 33258 (H) exposed brain ganglia subjected to following pre-fixation treatments: a-15 min 0.67% tri-sodium citrate (hypotonic); b-15 min 1:1 diluted culture medium; c,d-15 min hypotonic and then 1 hr (c) or 4 hr (d) H-free culture medium; e-4 hr H-free culture medium and then 15 min hypotonic and f-15 min hypotonic followed by 4 hr H-free culture medium and a second 15 min hypotonic treatment. Note the general similarity of nuclear morphology in c and d with that in control nuclei in Fig.3a

Discussion

In our earlier study on the effect of H-treatment on *Drosophila* cells[8], we suggested the more intense Giemsa staining and the decrease in area of the cc in H + hy treated cells to be due to a supercondensation of interphase heterochromatin in such treated cells. Our present Feulgen-DNA cytophotometric measurements confirm this.

The Feulgen-DNA values for nuclear as well as cc areas are, in general, higher in H or H + hy treated cells than in controls. Since the larvae used for these different samples were of similar age and since we have restricted our analysis to the smallest sized nuclei, the differences in Feulgen-DNA values in these samples are unlikely to be due to differences in the Feulgen-DNA content. These are more likely to be due to differences towards the Feulgen hydrolysis related to chromatin compactness. In this context, one or more of the following hypotheses may be considered: (a) For the hydrolysis time of 80 min, the inevitable loss of some DNA aldehydes concomitant to DNA depurination during Feulgen reaction occurred relatively late or was not very significant in the treated preparations; (b) the depurination rate in the presence of the acid was faster in the treated preparation; (c) as grid points exhibiting absorbances smaller than 0.020 were cleaned from the nuclear image and they were more frequent in the control, their removal could account for a certain decrease in the total Feulgen-DNA values. Examining some preparations subjected to a longer acid hydrolysis (2 hr), Feulgen-DNA values of the control and H-treated preparations were found to decrease significantly, whereas those of the H + hy treated cells did not change or decreased only slightly (detailed data not presented). Therefore, the hypothesis (b) is discarded. The hypotheses (a) and (c) thus appear to satisfactorily explain the differences in frequencies of the nuclear and cc Feulgen-DNA values in control and H or H + hy treated samples.

The nuclear Feulgen-DNA absorbing areas in the three sets of nuclei appear to be not strictly comparable with our earlier measurements of the nuclear areas in control and H or H + hy treated larval brain cells[8], since in H as well as H + hy treated cells, the nuclear area was greater than in control (non-hy treated) cells (see (Table 3 of ref 8), while the present data on nuclear Feulgen-DNA absorbing areas in control and H or H + hy treated samples show a different relationship (Fig.2). This is presumably due to the effect of hydrolytic treatment preceding the Feulgen reaction[16] and also to non-inclusion of the area covered by grid points having absorbance $\leqslant 0.020$ in the computation of integrated nuclear Feulgen-DNA absorbing areas. These small absorbances were discarded to avoid a systematic error[17]. Apparently, the more diffused

appearance of the euchromatin regions in H + hy treated cells causes more grid points to have absorbances ≤0.020. This agrees with our earlier suggestion that the H-treatment causes a certain degree of decondensation of euchromatin regions of interphase nuclei.

As mentioned above, the higher cc Feulgen-DNA absorbance values in H-treated than in control cells are presumably due to a greater Feulgen binding *per se* in H-treated nuclei. Since in our earlier study[8], we had found comparable cc areas in control and H-treated cells, it appears that the condensation pattern of interphase heterochromatin is not much altered by H-treatment alone, but H + hy treatment drastically alters this. The higher relative cc Feulgen-DNA values in H + hy than in H-treated cells (Tables 1,2) and the higher frequency of cc with a larger absorbing area in H than in H + hy treated cells when using 0.200 as the c.o. point (Table 3) imply a greater Feulgen-DNA content in a per unit cc area in H + hy treated than in H-treated cells. In other words, this shows that the cc is supercondensed in H + hy treated cells.

The supercondensation of interphase heterochromatin caused by H + hy treatment of *Drosophila* cells is unusual since the generally known effect of H is the inhibition of pre-mitotic chromatin condensation[1-3,8]. Therefore it is significant that NP and DA, when combined with hy treatment, also cause an identical change in the condensation pattern of interphase chromatin in larval and embryonic cells of *D. nasuta*. Like H, NP and DA also inhibit the mitotic chromosome condensation in mammalian cells[4-7] and also in *D. nasuta* cells[18]. In contrast with these, OM has little or no effect on mitotic chromosome condensation (our unpublished observations) and in accordance with the above correlation, OM has been found to have no effect on interphase heterochromatin. Since H, NP and DA all specifically bind to A-T rich DNA segments while OM binds to G-C rich regions[9,10], it appears likely that their inhibitory effect on mitotic chromosome condensation and the reverse effect on interphase heterochromatin condensation in *Drosophila* cells are related to the A-T specificity of these three DNA ligands. It remains to be seen, if these agents also affect the heterochromatin in mammalian or other interphase cells in a comparable manner.

The molecular basis of supercondensation of cc brought about by exposure of these A-T specific drug treated cells to a hy solution is not understood. Persistence of the H + hy effect in cells chased for 4 hr in H-free medium suggests that the alterations in chromatin condensation after the hy treatment are due to the H remaining bound to cellular DNA. Our earlier observation on the extreme rapidity of this effect[8] and the present findings on its reversibility, suggest that the A-T specific drug binding to DNA alters the nucleoprotein interaction in such a way that even a brief hy treatment causes rapid but reversible reorganization, rather than a loss of some chromatin constituents. We do not know if some heterochromatin specific proteins[19-21] are also involved in this supercondensation of cc. This needs further analysis. It will also be interesting to examine the nucleosomal and supranucleosomal organization and histone modifications[22] in the H or H + hy treated cells of *Drosophila*.

Acknowledgement

This work was supported by UGC, New Delhi, under its Programme of Special Assistance to the Department of Zoology, B.H.U.

References

1 Hilwig I & Gropp A, *Expl Cell Res*, **81** (1973) 474.
2 Marcus M, Nielsen K, Goitein R & Gropp A, *Expl Cell Res*, **122** (1979) 191.
3 Marcus M, Goitein R & Gropp A, *Hum Genet*, **51** (1979) 99.
4 Thust R & Ronne M, *Hereditas*, **93** (1980) 321.
5 Thust R & Ronne M, *Hereditas*, **94** (1981) 209.
6 Prantera G, DiCastro M, Marchetti E & Rocchi A, *Expl Cell Res*, **109** (1979) 459.
7 Schmid M, Poppen A & Engel W, *Cytogenet Cell Genet*, **30** (1981) 211.
8 Lakhotia S C & Roy J K, *Expl Cell Res*, **132** (1981) 423.
9 Jorgenson K F, van de Sande J H & Lin C C, *Chromosoma*, **68** (1978) 287.
10 Berman H M, Neidle S, Zimmer C & Thrum H, *Biochim biophys Acta*, **561** (1979) 124.
11 Mello M L S & Vidal B C, *Chromosoma*, **81** (1980) 419.
12 Hintzche E B, *Experientia*, **1** (1954) 103.
13 Palkovits M & Fischer J, *Karyometric investigations* (Budapest: Akad. Kiads) 1968.
14 Lakhotia S C & Kumar M, *Cytobios*, **21** (1978) 79.
15 Lakhotia S C & Kumar M, *Indian J exp Biol*, **18** (1980) 1066.
16 Mello M L S & Zanardi V A, *Nucleus*, **19** (1976) 1.
17 Ewing G W, *Instrumental Methods of Chemical Analysis* (McGraw Hill, New York) 1969.
18 Roy J K, *The V All India Cell Biology Conference* (Bangalore) 1981, 39 (Abstract).
19 Alfageme C R, Rudkin G T & Cohen L H, *Chromosoma*, **78** (1980) 1.
20 Comings D E, Harris D C, Okada T A & Holmquist G, *Expl Cell Res*, **105** (1977) 349.
21 Blumenfeld M, Orf J W, Sina B J, Kreber R A, Callahan M A & Synder L A, *Cold Spr Harb Symp Quant Biol*, **42** (1977) 273.
22 Kanungo M S, *Biochemistry of ageing* (Academic Press, New York) 1980.