

Effects of Distamycin A & Netropsin on Condensation of Mitotic Chromosomes in Early Embryos & Larval Brain Cells of *Drosophila nasuta*

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Effects of distamycin A (DA) and netropsin (NP) treatments on mitotic chromosome condensation in early embryonic and late larval brain cells of *D. nasuta* cultured *in vitro* have been compared. These A-T specific DNA ligands cause inhibition of condensation of the heterochromatin segments much more frequently in embryonic cells than in larval brain cells. Examination of colchicine induced tetraploid cells in DA treated larval brain preparations reveals that the mitotic cells, which have completed 2 cell cycles in the presence of the DNA ligand, remain as frequently unaffected as those exposed to DA for less than one cycle. Analysis of metaphase chromosomes in brain ganglia labelled with ^3H -thymidine and chased in DA containing medium reveals that the mitotic cells which were in DNA synthesis or gap (G1 or G2) period just before the DA treatment remain equally often unaffected. It appears that reduced sensitivity of mitotic chromosomes in larval brain ganglia to the condensation inhibitory effect of the A-T specific DNA ligands is related to differences in chromosome organization in early embryonic and larval brain cells.

A prefixation treatment of live mammalian cells with A-T specific DNA ligands like Hoechst 33258 (H), distamycin A (DA) or netropsin (NP) inhibits mitotic chromosome condensation, specially of the A-T rich regions¹⁻³. In *Drosophila* cells also a comparable inhibition of condensation in mitotic chromosomes has been observed when live cells are exposed to H treatment^{4,5}. However, in our earlier study⁵ we noted a significant difference in the condensation inhibitory effect of H on mitotic cells from early embryos and on those from the larval brain: while all metaphases in H treated early embryos revealed inhibition of condensation of the A-T rich heterochromatin segments, only a small proportion of the larval brain mitotic cells was affected even after long periods of H treatment. This difference was considered to be due to possible differences in chromosome organization in the 2 cell types⁵. In the present study we have further examined the effects of 2 other A-T specific DNA ligands, viz. distamycin A (DA) and netropsin (NP), on the condensation of mitotic heterochromatin in *D. nasuta*. The mechanism of binding of these 2 ligands to DNA⁶⁻⁸ is different than that of H⁹. However, our present results show that in spite of these differences, DA and NP resemble H in affecting mitotic chromosome condensation in embryonic and larval cells in a differential manner. In order to explore possible reasons for the relative lack of effects of these DNA ligands on larval brain mitotic cells, in this study we have also used ^3H -thymidine labelling in conjunction with DA treatment on larval brain ganglia to see whether cells at certain stages of cell cycle respond to the treatment in a different way than those at other stages.

Materials and Methods

A wild type strain of *Drosophila nasuta* from Varanasi has been used for these studies. Eggs were collected, and larvae were reared on standard agar-cornmeal-brown sugar-yeast food at $24^\circ \pm 1^\circ\text{C}$. The following experiments were done:

(i) *DA or NP treatment to embryonic or late third instar larval brain cells*—4-5 hr old eggs of *D. nasuta* were dechorionized and exposed to 20 $\mu\text{g/ml}$ DA or 20 $\mu\text{g/ml}$ NP for 2 hr as described earlier^{5,10}. Parallel control cultures were also maintained without any drug. Colchicine (1 $\mu\text{g/ml}$) was added to the same medium after 2 hr and 1 hr later, the medium was replaced by a 0.67% tri-sodium citrate (hypotonic) solution. After 25 min, the cells were fixed and air dried preparations were made as described earlier⁵.

Brain ganglia excised from late third instar larvae were cultured *in vitro* with DA (20 $\mu\text{g/ml}$) or NP (20 $\mu\text{g/ml}$) for 16 hr. Parallel control cultures were also maintained. Colchicine (1 $\mu\text{g/ml}$) was added in the culture for the last 4 hr. After routine hypotonic treatment air dry preparations were made¹¹. All slides were stained with Giemsa, mounted in D.P.X. and examined for condensation patterns of metaphase chromosomes.

(ii) *^3H -thymidine labelling and DA treatment to late third instar brain cells*—Freshly excised brain ganglia were pulse labelled with ^3H -thymidine for 30 min (Act. 20 $\mu\text{Ci/ml}$, Sp. Act. 15.2 Ci/mM, BARC, Trombay), washed and transferred to fresh radio-isotope free medium containing 20 $\mu\text{g/ml}$ DA and 0.2 $\mu\text{g/ml}$ colchicine for 16 hr. Air dry preparations were made after prefixation hypotonic treatment. They were rinsed in absolute alcohol, coated with Ilford L4

nuclear emulsion (1:1 diluted) and exposed in dark at 4-6°C for 25-30 days. The developed autoradiograms were stained with 5% Giemsa. Labelled metaphases were observed and photographed. Slides were then degreased¹² and again observed for the morphology of heterochromatin in labelled and unlabelled metaphase cells.

Results

As described earlier⁵⁻¹¹, chromosomes of *D. nasuta* (2N=8) in control preparations are characterized by the presence of large blocks of densely stained centric heterochromatin on all chromosomes except the dot like 4th chromosome pair. A large number of diploid and many tetra- and octaploid metaphases are present in preparations of treated larval brain ganglia made after 4 hr or 16 hr long colchicine treatments (see materials and methods). Each of the metaphase plate present in preparations of DA or NP treated embryonic and larval brain cells has been examined with respect to condensation patterns of heterochromatin blocks on different chromosomes and on this basis classified into (i) *normal*, with the heterochromatin segments of all chromosomes in the plate remaining normally condensed, and (ii) *affected*, in which the condensation of heterochromatin segments of some or all chromosomes has been inhibited to varying degrees. The condensation pattern of euchromatin regions in the treated metaphases has not been taken into consideration since even in many non-treated control metaphases from embryos as well as larval brain, the euchromatin regions are seen to be condensed to varying degrees, ranging from very diffused to typically condensed types. Thus classification of euchromatin regions of treated metaphases into normal and affected types can be subjective and has been avoided.

Data on the frequencies of normal and affected metaphases in DA or NP treated early embryos and larval brain ganglia are presented in Table I. A very large number (more than a thousand) of metaphases in control preparations have been examined in this and

other studies and none of them are classifiable as the affected types. Therefore data on control are not included in the table. Data on the 2N metaphases in DA treated brain ganglia are from only the 4 hr colchicine exposed samples while for the 4N metaphases, data from 4 hr and 16 hr (³H-thymidine experiment) colchicine exposed DA treated ganglia are pooled. All the six 8N metaphases in DA treated brain ganglia have been seen in preparations of 4 hr colchicine treated series. Hyperdiploid (4N or 8N) metaphases have not been found in the NP treated ganglia samples except for three unusual 4N metaphases mentioned later. It is seen from the data in Table I that DA affects heterochromatin condensation in a greater proportion of embryonic metaphase cells than NP although in larval brain their effect is similar. However it is more interesting to note that for both the DNA ligands, the mitotic cell population in early embryos is much more sensitive than the mitotic cells in larval brains since the frequencies of affected metaphases are significantly higher ($P < 0.01$ on contingency χ^2 -test) in embryonic than in larval brain samples treated with the same drug.

Some examples of the affected metaphases in DA or NP treated cells are presented in Fig. 1. Qualitatively, inhibition of heterochromatin condensation in the affected metaphases from DA or NP treated embryonic and brain cell preparations are comparable. The degree of condensation inhibition varies between different affected cells, but in a given affected cell, the heterochromatin segments of all chromosomes are decondensed to similar degree. Condensation of the Y chromosomal heterochromatin is not affected by DA or NP treatments since even in the affected 4N or 8N metaphases, all the Y chromosomes appear normally condensed (Fig. 1 b-c). In the affected 4N or 8N metaphases, in which the euchromatin regions are diffuse, a small segment at the junction of eu- and heterochromatin on one of the arms of the metacentric 2nd chromosome remains normally condensed and appears more darkly stained (see arrows in Fig. 1c). In some rare 4N metaphases the hetero- as well as

Table I—Effect of DA and NP on the Condensation of Metaphase Chromosomes in Early Embryos and in Larval Brain Ganglia of *D. nasuta*

Cell type	Treatment	Duration (hr)	Metaphase type	No. observed	Frequency (%) of metaphases	
					Normal	Affected
Embryonic	Distamycin A	3	Diploid	138	20.29	79.71
	Netropsin	3	Diploid	121	43.80	56.20
Larval brain	Distamycin A	16	Diploid	216	63.43	36.57
			Tetraploid	140	50.71	49.29
			Octaploid	6	0.00	100.00
	Netropsin	16	Diploid	162	64.24	35.76

euchromatin regions in different chromatids appear differentially affected, since, while some chromatids are normally condensed, others are less condensed (Fig. 1d).

In some of the embryonic preparations, parts of heterochromatin regions of different chromosomes appear as highly condensed irregular masses which

may fuse with each other into one or more darkly stained bodies as observed earlier after H treatment (see Fig. 1d and e of ref.5). A comparable effect has not been seen in larval brain preparations.

Three tetraploid metaphases in NP treated brain ganglia display an unusual configuration (Fig.1e). In these, the euchromatin regions of all chromosomes

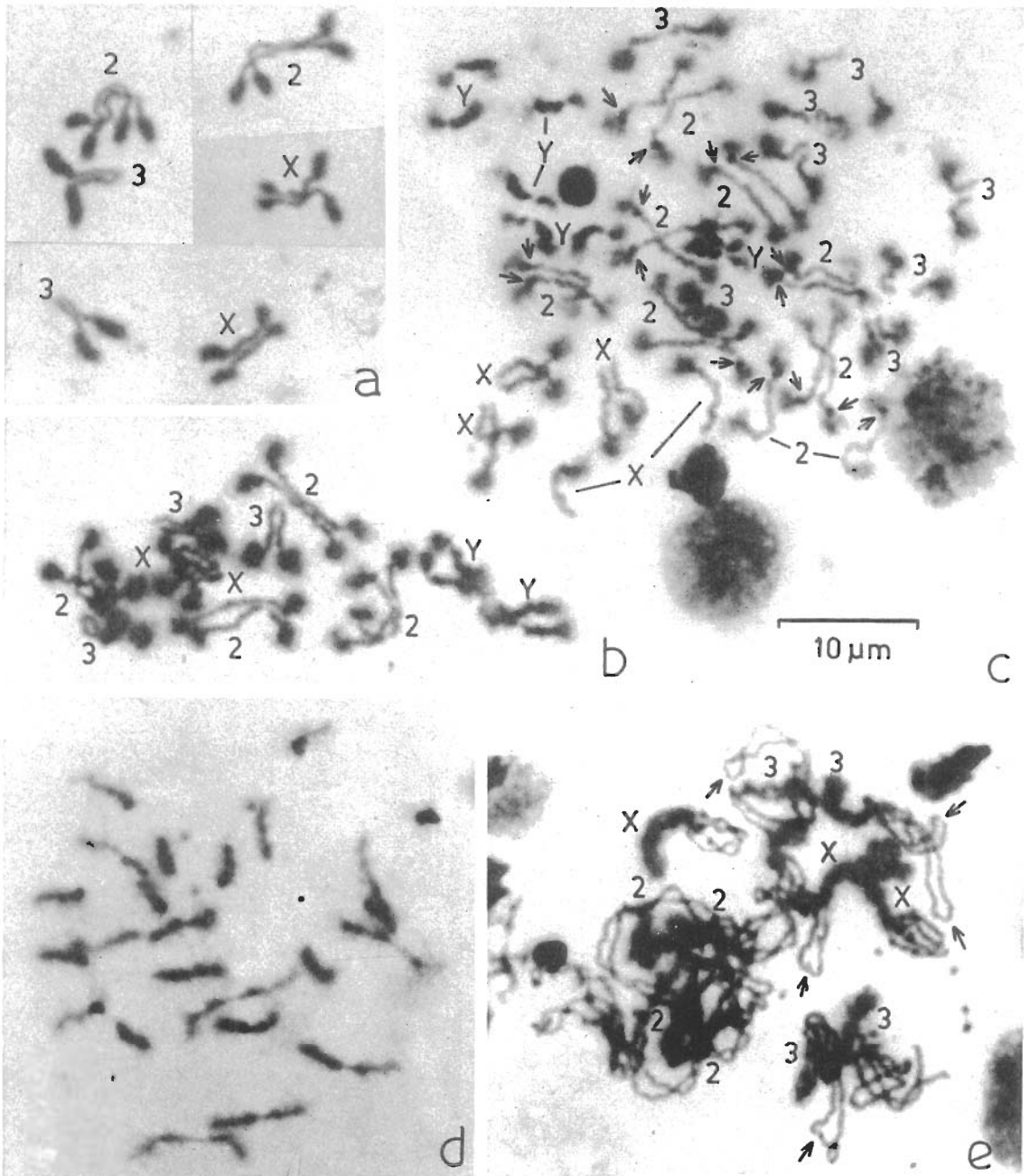


Fig. 1(a-e) — Examples of inhibition of heterochromatin condensation in DA or NP treated metaphases in brain ganglia of *D. nasuta* larvae: DA treated diploid (a), tetraploid (b) and octaploid (c) metaphases showing heterochromatin condensation inhibition in all chromosomes except the Ys in b and c; also note the darker staining segment (arrows) at the junction of eu- and heterochromatin on one arm of 2nd chromosomes in c. d - a DA treated 4N metaphase in which the condensation of different chromatids has been affected to different degrees; all chromosomes in this cell can not be unambiguously identified due to separation of sister chromatids. e - one of the three unusual "tetraploid" metaphase in NP treated ganglia - note the diplo-structures of all chromatids and the continuity of the tips (arrows) of the "sub-chromatids" in many cases. It may be noted that in this strain of *D. nasuta* the X-chromosome may be acrocentric or submetacentric type (see ref.5)

show two pairs of more or less normally condensed chromatids. Curiously, in most instances, the tips of pairs of such chromatids appear continuous (arrows in Fig. 1e). The sister heterochromatin regions of a chromosome are closely synapsed and perhaps also have a comparable tetravalent structure.

³H-thymidine labelling patterns in DA treated affected and normal larval brain metaphases—The brain ganglia have been pulse labelled for 30 min just before the 16 hr DA and colchicine treatment. Colchicine has been kept in the medium along with DA so that all the cells that enter mitotic division during

the treatment period get blocked at metaphase, and on the basis of their autoradiographic labelling patterns, they could be assigned to DNA synthetic or gap (G₂ or G₁) periods prior to the treatment. All the diploid and tetraploid metaphases have been scored for the patterns of autoradiographic labelling and for condensation of heterochromatin. The labelled metaphases have been classified into both or single chromatid labelled types and each of the classes further sub-divided into (i) only euchromatin, (ii) eu- as well as heterochromatin or (iii) only heterochromatin labelled types (Table 2). Some examples are shown in Fig. 2. The

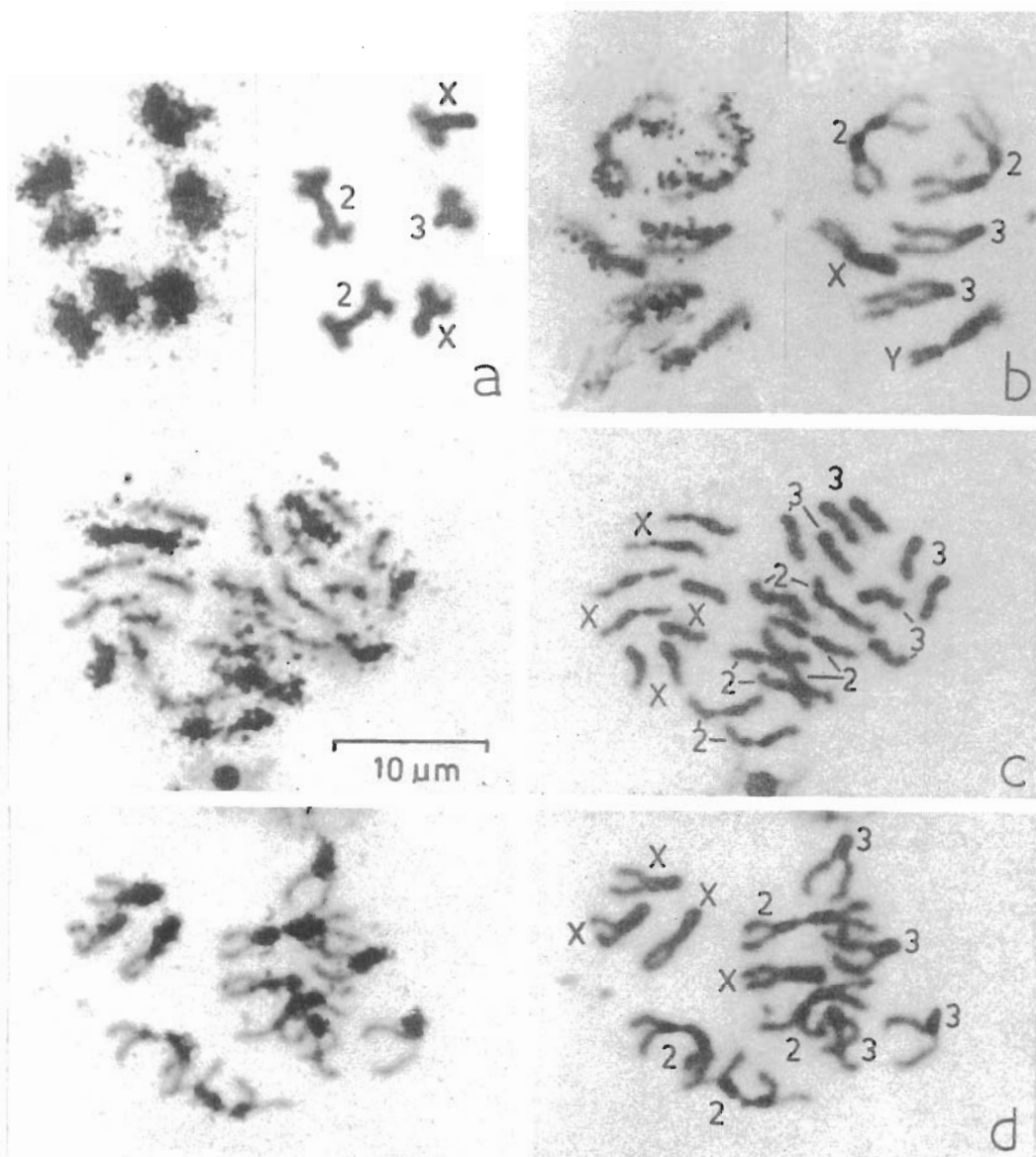


Fig. 2(a-d)—Autoradiographs of 2N or 4N metaphases from larval brain ganglia pulse labelled with ³H-thymidine and chased for 16 hr in medium containing DA and colchicine. In each case the degrafted metaphases are shown adjacent to respective autoradiograms. a - An affected diploid metaphase with euchromatin of both chromatids of all chromosomes labelled (one homolog of 3rd chromosome is not seen); b - a normally condensed diploid metaphase with labelling of euchromatin regions of one chromatid of each chromosome; c - a normally condensed tetraploid metaphase (all sister chromatids separated) with labelling (eu + heterochromatin) of one chromatid of each chromosome; d - a normally condensed tetraploid metaphase with heterochromatin of both chromatids of all chromosomes labelled.

Table 2—³H-Thymidine Labelling and Heterochromatin Condensation Patterns in 16 hr DA Treated Larval Brain Ganglia

[Figures in parentheses indicate per cent values in the given group]

Metaphase type	Type of labelling	Total no. observed	No. of affected and normal metaphases in different labelling classes						Total	
			Euchromatin		Eu + heterochromatin		Heterochromatin		Aff.	Nor.
			Aff.	Nor.	Aff.	Nor.	Aff.	Nor.		
Diploid	Both chromatids labelled	128	19	—	15	23	38	33	72	56
	Single chromatid labelled	41	1	2	8	10	8	12	17	24
	Unlabelled	101	—	—	—	—	—	—	64	37
									(56.25)	(43.75)
									(41.46)	(58.54)
									(63.37)	(36.63)
Tetraploid	Both chromatids labelled	2	—	—	—	—	—	2	—	2
	Single chromatid labelled	34	—	6	10	6	4	8	14	20
	Unlabelled	8	—	—	—	—	—	—	6	2
									(41.18)	(58.82)
									(75.00)	(25.00)

Aff. = metaphases with inhibition of heterochromatin condensation; Nor. = normally condensed metaphases.

On contingency χ^2 -tests, the differences in the proportions of affected and normal metaphases in the different labelled and unlabelled classes are found to be non significant ($P < 0.05$).

detailed results presented in Table 2 reveal that the incidence of DA induced inhibition of heterochromatin condensation is generally comparable in unlabelled and the differently labelled 2N and 4N metaphase classes.

Discussion

The present observation on the effects of prefixation treatment of live cells of *D. nasuta* with DA or NP on the condensation of A-T rich heterochromatin in mitotic chromosomes are in general agreement with our previous results with H⁵. Like H, both DA and NP inhibit premitotic condensation of heterochromatin. However there are some differences in the condensation inhibitory action of the three DNA ligands on *D. nasuta* chromosomes. With DA and NP we have seen that the condensation of Y-chromosomal heterochromatin is not inhibited in the affected metaphases while with H the Y chromosome condensation was found⁵ to be as much inhibited as of the other chromosomal heterochromatin. This difference in the effects of these three DNA ligands is perhaps related to heterogeneity in the constitution of heterochromatin of Y and other chromosomes of *D. nasuta*¹¹ and to the differences in the modes of binding of these ligands to A-T rich DNA sequences⁶⁻⁹.

With respect to the frequencies of affected metaphases in the treated embryonic cells while H was found to cause condensation inhibition in 100% metaphases⁵, DA and NP cause inhibition in only about 80% and 56% metaphases, respectively (see

Table 1). The above mentioned differences in the binding properties of these three DNA ligands are perhaps also responsible for the differences in the frequencies of the affected metaphases.

In spite of these differences in their effects, all the three DNA ligands share an important feature, i.e. the frequency of affected metaphases is significantly higher in the treated early embryonic cells than in larval brain ganglia. In relation to our earlier observations with H⁵, we had considered it unlikely that the less frequent effect in larval brain metaphases is due to factors like low permeability of H in larval brain cells or a higher threshold level of the drug requirement to cause condensation inhibition. We believe that the same arguments⁵ also apply to our present results with DA and NP.

Mitotic chromosome condensation in cells exposed to the A-T specific DNA ligands very late in G2 period is not affected¹³. In another study (MS in preparation), we have found that the G2 period in mitotically active larval brain cells is considerably variable and thus the failure of the A-T ligands to cause condensation inhibition in a large proportion of brain metaphases could possibly be attributed to cells which were blocked in G2 beyond the sensitive period and which enter mitosis after variable time intervals. However, the present observations on tetraploid and ³H-thymidine labelled metaphases show that this is not so since cells which traversed through the entire G2 period for one or even more cycles in the presence of the A-T ligands have been found to remain unaffected as frequently as

those which might have been blocked in G2 period beyond the so-called sensitive stage. The colchicine induced tetraploid metaphases would have traversed through at least one complete cell cycle in the presence of DA and yet, the frequency of affected 4N cells is as low as that in diploid metaphases. Observations on brain ganglia prelabelled with ^3H -thymidine and chased in DA + colchicine medium also show that cells which were at different phases of the S-period immediately prior to DA treatment remain as frequently unaffected as the unlabelled metaphases (G2 or G1 cells).

Inhibition of condensation in all the both chromatids euchromatin labelled metaphases (see Fig.2a and Table 2) may lead one to suspect that early S may be the sensitive stage. However, all the euchromatin labelled 4N metaphases and about 67% of the single chromatid euchromatin labelled diploid metaphases (Fig.2b) in the same population show normal condensation. Both these categories of cells would be in second cycle after the early S label incorporation. Thus our data do not provide any evidence for early S being the sensitive stage for the condensation inhibitory action of these DNA ligands. Our data further show that cells with a shorter cell cycle (single chromatid labelled 2N and 4N metaphases) and those with a longer cell cycle period (both chromatids labelled 2N metaphases) do not differ in their sensitivity to DA.

The three metaphases classified as 4N (Fig.1e) in the NP treated brain ganglia preparations are interesting. In view of the reported induction of endoreplication by the H treatment in mammalian cells^{14,15} it is possible that these three mitotic cells also endoreduplicated after their tetraploidization due to colchicine, so that each chromosome appears as diplochromosome. However, the distinct continuity of the tips of the "sub-chromatids" and the close synapsis of sister and grand sister heterochromatin regions of these "diplochromosomes" are most unusual and perhaps reflect certain unknown features of chromosome organization in larval brain ganglia. The presence of ^3H -thymidine labelling on both chromatids of all chromosomes in at least two of the tetraploid metaphases (Fig.2d) is also unusual since normally only one chromatid of each chromosome is expected to be labelled in these colchicine arrested second cycle metaphases¹⁶. We

presume that this type of labelling is related to the metaphase chromosomes in brain ganglia of *Drosophila* larvae being bi- or polynemic^{5,17-19}.

In view of the above discussion, it appears to us that the absence of the condensation inhibitory effect in a majority of the A-T ligand treated larval brain metaphases is not due to a sensitive stage not being exposed to the drug but to a refractoriness of many of the mitotically dividing brain cells to such effects. Since the undifferentiated embryonic cells of the same species are readily affected, the refractoriness of the larval brain metaphase cells is likely to be related to some changes in architecture and chromatin packing which may be associated with somatic cell differentiation^{5,19}.

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