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A-T Specific DNA Ligands and Hypotonic Induced Supercondensation of Chromocentre in Brain Cells of *Drosophila nasuta* Larvae in Relation to Their Synthetic Activities

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When A-T base specific DNA ligands like Hoechst 33258 (H) or Distamycin A (DA) treated brain ganglia of *D. nasuta* larvae are exposed to a pre-fixation hypotonic (hy) solution, the heterochromatic chromocentre (cc) becomes supercondensed in 40-60% interphase nuclei. In the present study, the effects of these DNA ligands on DNA and RNA synthesis in larval brain ganglia and the relationship of cc supercondensation with these synthetic activities of a cell has been examined by autoradiography. It is seen that both the frequency of replicating cells and the rate of ^{3}H-thymidine uptake are inhibited by the treatment, more so in the population of cells in which the cc is supercondensed. H treatment has no effect on the rate of ^{3}H-uridine uptake in transcribing nuclei although, compared to the control ganglia, the frequency of labelled nuclei is reduced after treatment. The cc supercondensation is shown to occur preferentially in nuclei which are active in DNA and/or RNA synthesis.

We have shown earlier[1,2] that when Hoechst 33258 (H) or other A-T specific DNA ligand treated cells of *Drosophila nasuta* are exposed to a pre-fixation hypotonic (hy) treatment, the heterochromatic chromocentre (cc) in interphase nuclei becomes supercondensed (the H + hy effect) while the euchromatin regions are somewhat more decondensed. However, all nuclei in the treated tissue do not show the effect and the frequency of cells showing supercondensed cc varies in a tissue specific manner[1-3]. In the larval brain tissue the cc becomes supercondensed in only 40 to 60% of the treated cells. In the remaining cells, the cc is as much decondensed in response to the hy treatment as in the non-treated control cells, although the A-T specific DNA ligand is bound to these cells as well. The reasons for the differential response of different cells to the H + hy treatment are not known. Synthetic activities affect chromatin organization in the nucleus and therefore, in this study, we have examined the H + hy effect in relation to DNA or RNA synthetic activities of the cell. Our results suggest a correlation between the H + hy effect and synthetic activities of the nucleus.

Material and Methods

A wild type strain of *D. nasuta* (Varanasi) has been used for these studies. Brain ganglia from late third instar larvae, either grown normally on the standard food (unsynchronized) or fed for the last 16hr on 5-fluorodeoxyuridine (FUdR) containing medium to synchronize the replication cycles in different brain cells (for details see ref. 4,5), were cultured *in vitro* and treated with Hoechst 33258 (H) or Distamycin A (DA) as described earlier[1,2]. The following experiments have been done:

Experiment 1 - ^{3}H-thymidine labelling followed by H or DA treatment—Freshly excised brain ganglia from FUdR synchronized larvae were pulse labelled with ^{3}H-thymidine (20 μCi/ml, sp. act. 15.8 Ci/mM, BARC, Trombay) for 20 min. After the pulse the ganglia were washed in radio-isotope free medium and cultured in fresh medium containing either H (40 μg/ml) or DA (20 μg/ml) or no drug (control) for 16hr. After 16hr, the ganglia were given a 35 min hypotonic (hy) treatment in 0.67% tri-sodium citrate, following which they were fixed. Their air dried preparations were autoradiographed with 1 : 1 diluted Ilford L4 nuclear emulsion. After 6-10 days of exposure in dark, the autoradiograms were developed, fixed, washed and stained with Giemsa in the usual manner.

Experiment 2 - ^{3}H-thymidine labelling along with H or DA treatment—Freshly excised brain ganglia from synchronized larvae were transferred to medium containing ^{3}H-thymidine (2 μCi/ml) and H (40 μg/ml) or DA (20 μg/ml) or no drug (control). After 16hr, the ganglia were given the hy treatment and their air dried preparations were processed for autoradiography as above. The autoradiograms were exposed for 6-10 days.

Experiment 3 - ^{3}H-thymidine labelling after H treatment—Freshly excised ganglia from unsynchronized larvae were cultured in medium containing H (40 μg/ml) or no drug (control) for 16hr following which they were pulse labelled with ^{3}H-thymidine (20 μCi/ml) for 30 min. After the hy

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treatment, the air dried preparations were autoradiographed.

Experiment 4 - ^{3}H-uridine labelling after H treatment—16hr H treated and control brain ganglia from unsynchronized larvae were pulse labelled with ^{3}H-uridine (20 μCi/ml, sp. act. 12.7 Ci/mM, BARC, Trombay) for 30 min, exposed to the hy solution and processed for autoradiography as above. Autoradiographic exposure was for 6-10 days.

Scoring of the autoradiograms—Since it was not always easy to resolve if the cc in an autoradiographically labelled nucleus is supercondensed or not, several areas of autoradiographic slides of treated ganglia were photographed to record the labelling patterns of different nuclei. The slides were then degrained[6], restained with Giemsa and the cc morphology in each of the earlier photographed nucleus was identified. The frequency of labelled nuclei in the control and treated samples in different experiments have been compared by contingency χ^2-test.

Results

Effects of H or DA on frequency of replicating or transcribing nuclei—In ganglia pre-labelled with ^{3}H-thymidine and then exposed to H or DA for 16hr (Expt. 1), the frequencies of labelled nuclei are similar in control and treated (19.7 to 23.4%, Fig. 1). However, when ^{3}H-thymidine is made available during the 16hr H or DA treatment (Expt. 2), the frequencies of labelled cells are significantly lower ($P<0.001$) than in the corresponding control ganglia (Fig. 1). After 16hr H treatment in the third Expt. also, the frequency of replicating nuclei is significantly ($P<0.05$) lower (5.5%) than in parallel control set (7.3%, Fig. 1). It may be pointed out here that the considerably lower frequency of replicating nuclei in the control brain ganglia of Expt. 3 than in control ganglia of Expts. 1 and 2 is due to the fact that in the first two cases FUdR synchronized brain ganglia have been used while in the third case, unsynchronized ganglia have been taken. It has been shown earlier[3,4] that the frequency of replicating nuclei in FUdR synchronized brain ganglia is considerably higher than that in ganglia from normally growing larvae.

^{3}H-uridine pulsed control ganglia show about 86% labelled nuclei (Expt. 4) but in the H treated ganglia, the frequency of transcribing nuclei is significantly less (about 79%, Table 1).

Effects of 16hr H treatment on rates of ^{3}H-thymidine and ^{3}H-uridine incorporation—The numbers of silver grains present on different ^{3}H-thymidine labelled nuclei from Expt. 3 have been counted. In the H treated preparations, two different morphologies of nuclei, i.e. nuclei with supercondensed or normal cc have been distinguished. Among the different small and large sized nuclei present in brain ganglia[7], only the smaller sized nuclei have been considered for this analysis. The data are presented in Fig. 2a. For

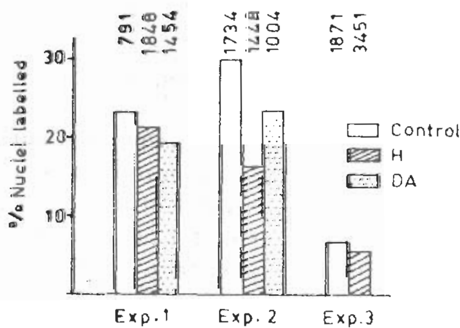

Fig. 1—Frequencies of ^{3}H-thymidine labelled interphase cells in brain ganglia of *D. nasuta* larvae cultured *in vitro* in absence (control) or in presence of Hoechst 33258 (H) or Distamycin A (DA). The results from experiments 1, 2 and 3 are shown separately (for details see material and methods). The numbers of nuclei scored in each case are indicated above the respective bars

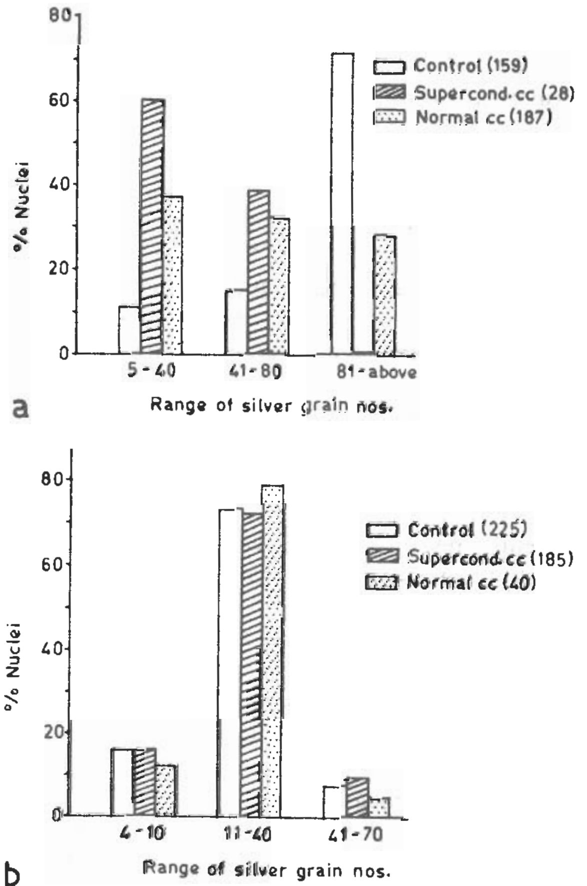

Fig. 2—Frequencies of different grain densities on larval brain cells pulse labelled with ^{3}H-thymidine (a) or ^{3}H-uridine (b) after 16hr *in vitro* culture in absence (control) or in presence of Hoechst 33258 (H). The H treated cells have been classified into supercondensed or normal cc categories. The numbers of nuclei scored for each sample are indicated in parentheses

comparison, the ^{3}H-thymidine labelling density of a nucleus has been categorized as low (5-40 silver grains/nucleus), medium (41-80 silver grains/nucleus) or high (more than 80 silver grains/nucleus). In control ganglia, a majority of nuclei are high labelled but in H treated ganglia the labelling is lesser (Fig. 2a). Interestingly, a majority of nuclei with supercondensed cc are low labelled and none are heavily labelled (Fig. 2a). These results thus show that, after 16hr H treatment, the rate of ^{3}H-thymidine uptake is significantly decreased, more so in cells which show supercondensed cc.

A comparison of the rate of ^{3}H-uridine uptake in control and H treated ganglia (Expt. 4, Fig. 2b) reveals that the H treatment has no effect on the rate of transcription in the labelled cells. Moreover, treated and labelled nuclei with supercondensed cc incorporate as much ^{3}H-uridine as those with normal cc (Fig. 2b).

H+hy effect in replicating and non-replicating brain cells—In the H or DA treated brain ganglia of the four experiments, the frequency of cells with supercondensed cc (the H + hy effect) ranges from 48-61%. However, the distribution of nuclei with the H + hy effect in relation to their being labelled or unlabelled, varies in the different experiments (Fig. 3). In ganglia prelabelled with ^{3}H-thymidine and exposed to H or DA (Expt. 1), 88% (H) to 95% (DA) of the nuclei that

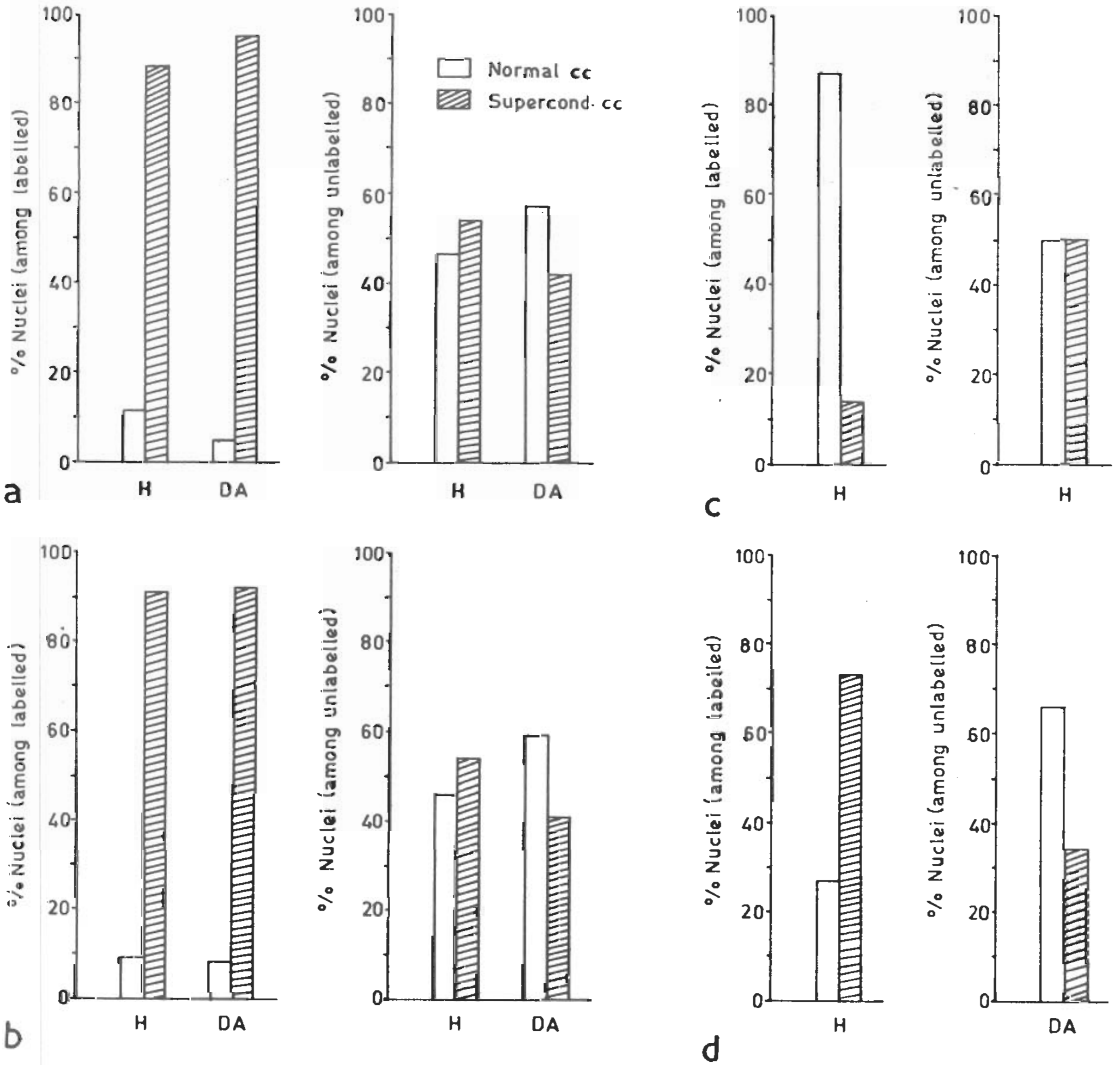

Fig. 3—Frequencies of labelled or unlabelled vs. normal (▭) or supercondensed (▨) cc in *D. nasuta* brain cells cultured in presence of Hoechst 33258 (H) or Distamycin A (DA). In a, b, c brain ganglia were labelled with ^{3}H-thymidine according to experiment protocols 1, 2 and 3, respectively, while in d the cells were labelled with ^{3}H-uridine (Expt. 4; for details see material and methods). In each case, the frequencies of two categories of cc are shown separately among labelled or unlabelled nuclei

were replicating prior to the treatment (i.e. those labelled with ^{3}H-thymidine) show supercondensed cc (Fig. 3a). On the other hand, among the unlabelled class, the proportion of nuclei with supercondensed or normal cc is equal (see Fig. 3a). Similarly, in ganglia treated with H or Da and simultaneously labelled with ^{3}H-thymidine (Expt. 2), about 90% of the cells that incorporated ^{3}H-thymidine during the 16hr treatment period show a supercondensed cc (Figs 3b, 4a). Again, as in Expt. 1, among the large number of non-replicating (unlabelled) cells, the frequencies of cells with a supercondensed cc and of those with normal cc remain nearly equal (Fig. 3b).

It is significant that among the nuclei replicating after H treatment (Expt. 3) only about 13% show a supercondensed cc (Fig. 3c) but as in the previous two cases, among the unlabelled cells, the proportion of nuclei with supercondensed or normal cc is equal.

H+hy effect in transcribing nuclei—When H treated cells are pulse labelled with ^{3}H-uridine (Expt. 4), about 76% of the transcribing nuclei have a supercondensed cc, while among the unlabelled class, only 34% nuclei show a supercondensed cc (Figs 3d and 4b). Analysis of the same data in a different way (Table 1) reveals that the class of unlabelled nuclei with a supercondensed cc constitutes about 7% of the total population and interestingly, the difference in the total labelling frequency between the control and H treated ganglia is also about 7%.

Discussion

Our observations reveal a significant inhibitory effect of H on DNA replication since after a 16hr H treatment, not only the frequency of replicating nuclei shows a decline but the rate of ^{3}H-thymidine incorporation in the treated nuclei is also drastically

Table 1—Effect of 16 hr H Treatment on ^{3}H-uridine Labelling of Larval Brain Cells in Relation to the H + hy Effect

Experiment	No. of nuclei observed	% labelling	% nuclei			
			Labelled		Unlabelled	
			Normal cc	Supercond. cc	Normal cc	Supercond. cc
Control	3482	85.6	85.6	0.0	14.4	0.0
H	2889	78.5*	21.3	57.3	14.1	7.3

*The decline (7%) in the labelling frequency in H treated cells is significant ($P<0.01$).

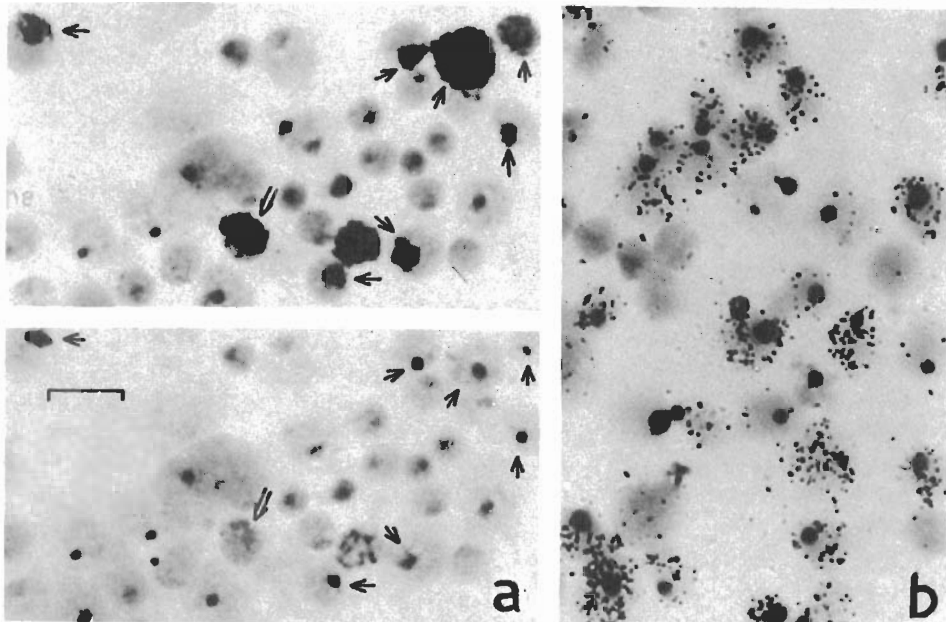

Fig. 4—Photomicrographs of ^{3}H-thymidine (a) or ^{3}H-uridine (b) labelled brain cells of *D. nasuta*. (a) Brain ganglia labelled with ^{3}H-thymidine for 16 hr in presence of Hoechst 33258 (Expt. 2); the upper half is autoradiogram and the lower half shows the same field after removal of silver grains; labelled nuclei with supercondensed cc (→) or with normal cc (⇒) are indicated. (b) Brain ganglia pulse labelled with ^{3}H-uridine after 16 hr treatment with Hoechst 33258. In both instances, most of the labelled nuclei show a supercondensed cc; moreover, in b nearly all unlabelled nuclei have a normal (diffused) cc morphology. Bar indicates 10 μm

reduced. In this respect our results are in agreement with those of Hirschberg *et al.*[8] in CHO cells and of Dubey and Raman[9] in *Bandicota* cells. However, a novel aspect of our observation is that only a very small proportion of nuclei which show a supercondensed cc after the 16hr H treatment are replicating (Fig. 3c) and moreover, the rate of ^{3}H-thymidine incorporation is also much more inhibited in the nuclei with supercondensed cc (Fig. 2a). These observations indicate that although the A-T specific ligand binds to all nuclei, only a certain population of the drug bound cells would show a supercondensed cc upon subsequent hy treatment (see below) and DNA replication is preferentially more inhibited in this population of cells.

The effects of H treatment on ^{3}H-uridine incorporation in larval brain cells of *D. nasuta* differ from those in mammalian cells - Hirschberg *et al.*[8] presumed inhibition of RNA synthesis in H treated CHO cells while Dubey and Raman[9] have found a significant reduction in the rate of ^{3}H-uridine incorporation in H treated *Bandicota* cells. In the present material on the other hand, the rate of ^{3}H-uridine incorporation in transcribing nuclei is not affected by the H treatment, although the frequency of transcriptionally active nuclei is somewhat reduced.

It may be pointed out here that although we have not specifically examined the effects of DA on rates of ^{3}H-thymidine or ^{3}H-uridine incorporation in the treated cells, we presume these to be comparable to those of H treatment in view of the following: (i) the effects of H and DA on condensation of heterochromatin in metaphase and interphase nuclei of *D. nasuta* are closely comparable[1,2,10] and (ii) our present results show that with respect to supercondensation of cc vis-a-vis the replicating status of the nucleus, the H and DA are very similar.

Our analysis of the occurrence of supercondensed cc in relation to the synthetic activities (replication or transcription) reveals that the population of cells which shows the H + hy effect is not a random one, instead this effect occurs preferentially in cells which are or have been active in RNA and/or DNA synthesis. This is evidenced by the presence of supercondensed cc in more than 70% of those nuclei which have been active in ^{3}H-thymidine or ^{3}H-uridine incorporation prior to or during the treatment. It is to be noted that the frequency of replicating nuclei in the third instar larval brain ganglia is low but that of transcriptionally active nuclei is much higher (about 80%) and thus, a majority of the non-replicating nuclei would still be active in RNA synthesis. This appears to explain the presence of supercondensed cc in about 50% of the nuclei which were not replicating prior to, during or after the H treatment (Figs 3a-d). This interpretation is supported by our observation that the frequency of the class of H treated cells which does not show ^{3}H-uridine incorporation but has a supercondensed cc, is equivalent to the difference in the frequencies of total transcribing nuclei in control and H treated ganglia. Thus the supercondensation of cc in nuclei which are inactive in RNA synthesis after the 16hr H treatment appears to be due to these nuclei having been active in RNA synthesis at some stage during the period of H treatment. In the light of these results, it appears to us that the tissue specific differences noted by us[1-3] in the frequency of cells showing the H + hy effect are related to differences in the population of cells that are synthetically active in the different tissue types. The reasons for the lack of H + hy effect in a small proportion of cells which are or have been active in DNA and/or RNA synthesis are, however, not clear.

It is interesting to note that in the synthetically active nuclei, the cc itself may not always be involved in RNA or DNA synthesis and yet it is the cc which shows the H + hy effect. In ^{3}H-uridine labelled preparations the cc is mostly unlabelled (Fig. 4b), although in some nuclei it appears well labelled. With ^{3}H-thymidine also, the cc remains unlabelled during the 16hr treatment in a certain fraction of the replicating cell population. Yet these nuclei with unlabelled (with ^{3}H-uridine or ^{3}H-thymidine) cc are seen to have a supercondensed cc as frequently as those in which the cc is labelled (detailed data not presented). This indicates the existance of long ranging forces in nuclear organization so that synthetic activity elsewhere in the nucleus also brings about changes in the cc organization and these prime the cc to get supercondensed after the H + hy treatment. The nature of these changes remains to be understood.

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