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Cell cycle and DNA content of mitotic cells in brain ganglia of *Drosophila* larvae

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Abstract. The programmes of replication of hetero- and euchromatin regions, mitotic cell cycle and the DNA content in metaphases in brain ganglia from late third instar larvae of *Drosophila melanogaster* (wild type and a tumour bearing mutant, 1(*2*)*gl,* strain) and of *Drosophila nasuta* were examined by autoradiography of [^{3}H]thymidine labelled (continuous or pulse) cells and by cytophotometry, respectively. Brain ganglia labelled continuously with [^{3}H]thymidine for 24 h *in vitro* showed a significantly high proportion of cells with incorporation of radioactivity restricted to heterochromatin only. Pulse labelling of brain ganglia from larvae of *Drosophila melanogaster* and *Drosophila nasuta* followed by chase for different time intervals showed that (i) the frequency of labelled metaphases was more than 50% within 15 to 30 min of chase and remained higher than 50% in nearly all the chase samples till 24 h, (ii) euchromatin labelled metaphases appeared with a low frequency within 1 to 4 h chase period but the heterochromatin labelled metaphases continued to be more common in the later chase samples also, (iii) single chromatid labelled second cycle metaphases were seen within 1 to 4 h after the pulse, but their frequency did not increase in the later samples. Cytophotometry of feulgen-DNA and Hoechst 33258 stained metaphases in late third instar larval brain ganglia revealed a greater variation in the DNA content of individual metaphases, although the means were close to the expected 4 C content. It appears that in relation to the known asymmetric cell divisions of neuroblast and other neural cells, the mitotically active cells in brain ganglia comprise a heterogenous population with widely varying lengths of the different phases of cell cycle; some of them may not cycle regularly and may possibly have a discontinuous S-phase.

Keywords. Metaphase chromosomes; endoreplication; replication bands; feulgen-staining; Hoechst 33258.

1. Introduction

Two kinds of replication cycles, viz., endoreplication and mitotic, occur in different cell types of *Drosophila* larvae. Among them at least four different cell types present in larval brain ganglia, only the neuroblasts and ganglion mother cells divide mitotically (Truman and Bate 1988). Many cells in larval brain ganglia endoreplicate with independent and unequal replication of the heterochromatic (H) and euchromatic (E) regions (Berendes and Keyl 1967; Lakhotia and Kumar 1978; Lakhotia 1982; Zacharias 1993). Although the dividing cells in larval brain are often used for a variety of studies involving metaphase chromosomes, a comprehensive information on cell cycle in mitotically active brain cells is lacking since in the

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few studies available (Pimpinelli *et al* 1976; Wienberg 1977; Tsuzi and Tobari 1980; Steinemann 1980; Truman and Bate 1988), the durations of the mitotic cycle and its various phases were inferred only indirectly.

Some DNA-feulgen cytophotometric studies suggested the larval brain mitotic cells to be multistranded or polynemie (Rudkin 1963; Swift 1965; Gay *et al* 1970) while others questioned this claim (Van de Flierdt 1975; Zacharias 1993). Although the debate concerning polynemie *vs* uninemic model of metaphase chromosomes in general is resolved in favour of a uninemic organization (Kavenoff and Zimm 1973; Pienta 1991), some conflicting evidences regarding unineme or polyneme structure of the mitotic cells in brain ganglia of *Drosophila* larvae persist, especially because of the occurrence of endoreplication cycles in brain ganglia. Jan and Boyes (1970) reported the presence of very large sized metaphase chromosomes in some mitotic cells in brain ganglia of *Musca domestica* larvae. Earlier studies from this laboratory (Lakhotia and Roy J K 1981; Lakhotia 1982) were also interpreted in favour of a polynemic organization of at least some mitotic cells in brain ganglia of *Drosophila* larvae. In view of these we undertook a detailed and systematic analysis of cell cycle and DNA content in mitotically active cells in larval brain ganglia of two species, viz., *D. melanogaster* and *D. nasuta*.

2. Materials and methods

2.1 *Fly stocks*

Wild type stocks of *D. melanogaster* (Oregon R^+) and *D. nasuta* (Varanasi) and the mutant *l(2)gl*^{4}*or/SM5* stock of *D. melanogaster* were reared at 24± 1°C under standard laboratory conditions. For details of genetic markers in the mutant stock, see Lindsley and Zimm (1992, also see Roy S and Lakhotia 1991) . The brain ganglia of late third instar *l(2)gl*^{4} mutant larvae show tumourous growth, high mitoic index (Gateff and Mechler 1989; Roy S and Lakhotia 1991) and highly extended metaphase chromosomes (Radhakrishnan and Sinha 1987).

Eggs from flies of each stock were collected at hourly intervals and the larvae were grown in uncrowded dishes on yeast supplemented food at 24 ± 1°C. The *l(2)gl*^{4} homozygous larvae were differentiated from the *l(2)gl*^{4} *or/SM5* heterozygotes on the basis of a prolonged larval life, sluggish movements and bloated appearance of the former.

2.2 *[*^{3}*H]thymidine incorporation in brain ganglia for studying labelling patterns of interphase nuclei*

Brain ganglia from late third instar larvae of *D. melanogaster* (wild type), *D. nasuta* and 10 days old *l(2)gl*^{4} homozygous larvae were aseptically excised in different cavity blocks containing modified Poels' medium (Sinha and Lakhotia 1980). In one set (experiment A), excised brain ganglia were immediately pulse labelled with [^{3}H]thymidine (20μCi/ml; sp. act. 15·8 Ci/mM, Bhabha Atomic Research Centre, Bombay) for 15 min. In another set (experiment B), *D. nasuta* larvae were fed on 5-fluoro-deoxyuridine (FdU, 100μg/ml food) for 16 h beginning from mid third instar stage to synchronize replicating cells at G_1–S border (Sinha and Lakhotia 1983) following which their excised brain ganglia were immediately

pulse labelled with [^{3}H]thymidine (20 μCi/ml) for 15 min as above. In a third set (experiment C), brain ganglia from *D. nasuta* larvae were first cultured *in vitro* for 24 h and then labelled with [^{3}H]thymidine (20 μCi/ml) for 15 min as above. In the fourth set (experiment D), *D. nasuta* brain ganglia were incubated in [^{3}H]thymidine medium (2 μCi/ml) for 24 h. In all cases, the labelled ganglia were immediately processed for air dry chromosome preparations (Lakhotia and Kumar 1978) and autoradiography (see below). The continued availability of [^{3}H]thymidine for the entire duration of 24 h in the set D, was confirmed by autoradiographic detection of incorporation of radioactivity in fresh ganglia incubated in the used medium after the 24 h period (data not presented).

2.3 *[^{3}H]thymidine labelling of brain ganglia for analysis of mitotic cell cycle*

Brain ganglia from late third instar larvae of *D. melanogaster, D. nasuta* and from ten days old *l*(2)*gl*^{4} homozygous larvae were aseptically dissected in the modified Poels' medium. They were freed of adhering imaginal disks and were pulse labeled with [^{3}H]thymidine (20 μCi/ml) for 10 min following which they were repeatedly washed with radioisotope free medium. Batches of labelled ganglia (~ 10/batch) were transferred to a series of cavity blocks containing isotope-free fresh medium additionally supplemented with yeast extract (2 mg/ml) and cold thymidine (10 μg/ml) for chase periods of 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 16 h, 20 h and 24 h, respectively. The labelling as well as the chase incubations were done at 24 ± 1°C. The 15 min, 30 min and 1 h samples were not exposed to colchicine, while the 2 to 24 h samples were exposed to colchicine (1 μg/ml) for the last 1 h of incubation. After the required period of incubation, air dry preparations were made as described (Lakhotia and Kumar 1978).

In another set, brain ganglia of *D. nasuta* larvae fed on FdU as above were pulse labelled with [^{3}H]thymidine within 5 min of removal from body and then chased for different durations as above. The two sets of ganglia were termed synchronized (FdU fed) and unsynchronized (normally fed), respectively.

All preparations of synchronized or unsynchronized ganglia were rinsed in absolute ethanol, air dried, treated with 5% trichloroacetic acid at 4°-5°C for 10 min, washed in running water, dehydrated through ethanol grades and finally coated with nuclear emulsion for autoradiography. After exposure in dark at 4°-6°C for 15-20 days, the autoradiograms were developed, fixed, washed and stained with Giemsa in the usual way. The slides were examined for labelling of mitotic chromosomes. After recording and photographing the metaphase labelling patterns, the preparations were degrained (Bianchi *et al* 1964), restained with Giemsa and the corresponding mitotic cells rephotographed to correlate the labelling and chromosomes.

2.4 *Feulgen-DNA staining of mitotic chromosomes from larval brain ganglia and of spermatozoa from adult testes*

Excised brain ganglia of 7–8 days old wild type and 10–11 day old *l(2)gl*^{4} tumorous larvae (late 3rd instar) were incubated in Poels' modified medium (Sinha and Lakhotia 1980) for 2 h at 24 ± 1°C in the presence of colchicine (1 μg/ml) following which they were exposed to hypotonic solution (0.67% tri-sodium citrate, 35 min at 24°C), fixed in freshly prepared acetomethanol (1 : 3) for 5 min, passed through

descending grades of ethanol, washed in distilled water and subjected to acid hydrolysis (4 N HCl at 22°C for 85 min). The intact brain ganglia were washed with 0·1 N HCl, immediately stained (2 h at 22°C in dark) with 1% Schiffs reagent (Pararosaniline, Sigma), washed with bleaching solution (0·5% potassium metabisulphite, 3 times for 10 min each) and finally rinsed in distilled water. The feulgen stained ganglia were transferred to a drop of 60% acetic acid on one end of a clean slide (1 or 2 ganglia per slide) for air-dried chromosome preparations as described (Lakhotia and Kumar 1978).

Testes from 1–2 day old male flies (wild type *D. melanogaster* and *D. nasuta* or *l(2)gl*^{4} *or/SM5)* were dissected in modified Poels' medium and after a brief hypotonic treatment (10 min at 24°C), were fixed and feulgen stained as above. The feulgen stained testes were squashed in a drop of 50% acetic acid in the middle part of the slides on which air-dried preparations of feulgen stained brain ganglia of corresponding genotype/species were already made. After removal of coverslips by freezing, the slides were rinsed with ethanol and air-dried. The feulgen stained preparations of brain ganglia and testes were mounted using Nikon immersion oil (refractive index 1·51), sealed with DPX and kept in dark till cytophotometry was undertaken.

In another experiment, brain ganglia from late 3rd instar larvae of wild type *D. melanogaster* were cultured *in vitro* in the Poels' modified medium for 16–18 h at 24 ± 1°C in the presence of colchicine (0·1μg/ml) to induce polyploid metaphases. Air dried preparations of feulgen-DNA stained brain ganglia were made as above. Squash preparations of feulgen stained testes from young *D. melanogaster* flies were also made on the same slides as above. Feulgen DNA content of diploid and tetraploid (colchicine induced) metaphases from larval brain ganglia and of sperms from adult testes were measured by scanning cytophotometry (see below).

To estimate the haploid DNA content of *D. nasuta,* preparations of feulgen stained tests from *D. nasuta* and *D. melanogaster* flies were made on the same slide and the relative DNA feulgen contents of sperms of the two species were measured by scanning cytophotometry.

2.5 *Scanning cytophotometry of feulgen stained metaphases and sperms*

A Leitz MPV3 system equipped with a mirror-scanner and HP85B (Hewlett Packard) microcomputer in conjuction with the M3SC05 software (Leitz) was used. The DNA-feulgen quantities of metaphases and sperms were measured as integrated absorbance in arbitrary units (AU) at 560 nm (Interference filter no. S3546-11, Leitz) using an 100x oil immersion NPL Fluotar objective with the mirror scanner measuring diaphragm set at 600 nm and the scanning step width at 540 nm. Background absorbance for each slide was determined by scanning several blank areas to electronically correct the integrated absorbance value of the scanned object for background. Only metaphases with the full diploid chromosome complement with little overlap of different chromosomes were used for scanning. In the case of wild type brain ganglia exposed to colchicine for 16-18 h, both 2 N and 4 N metaphases were measured for feulgen-DNA absorbance.

To permit a direct comparison of relative absorbances of metaphases on different slides, the integrated absorbance values for individual metaphase plates on a slide were normalized against the mean value for the sperms (1 C) on that slide being taken as 100.

As a measure of the performance for cytophotometric estimation of relative DNA content under our conditions, a pilot study was carried out with feulgen-stained preparations of meiotic stages of male grasshopper (data not presented). In this study, the relative DNA contents of spermatids, metaphase II and metaphase I cells were within the expected ratio of 1 : 2 : 4, respectively. The observed deviation of the mean values from expected was 3% for metaphase II and 12% for metaphase I cells; these deviations were within the range noted in other cytophotometric studies (Mulligan and Rasch 1980; Zacharias 1986). Thus the conditions of feulgen DNA staining and scanning cytophotometry used in the present study were reasonably free of various possible errors.

2.6 *Plug measurement of Hoechst 33258 fluorescence of metaphases and sperms of l(2)gl^4*

Air dry preparations of unstained brain ganglia from 10 day old *l(2)gl^4* larvae were made on one end of clean slides. Squash preparations of unstained testes from young *l(2)gl^{4}or/SM5* flies were also made on the same slides. Both preparations were then stained in dark (24°C, 1 h, see Yamamoto *et al* 1990) with Hoechst 33258 (5 μM) in 10 mM Tris-HCl and 1 M NaCl (pH 7·2), washed several times in distilled water and finally mounted in 10 mM Tris-HCl and 1 M NaCl (pH 7·2). The coverslips were sealed with DPX and the preparations stored in dark at 4°C till the measurement of fluorescence (within 1 day). Fluorescence of individual metaphase plates or of sperms was measured by the plug method on Leitz MPV-3 setup using a 100 W mercury lamp, a 50x oil immersion objective and an appropriate filter block (no. B2, BP 350-410, Leitz). The fluorescence values for each object were recorded, after background correction, as arbitrary fluorescence units (AFU) at 1x gain. Mean of the AFU obtained for the sample of sperms on a given slide was taken as 100 and the values obtained for the sample of metaphases on that slide were normalized to this value.

3. Results

Interphase nuclei of *D. nasuta* display a prominent chromocentre formed by association of the blocks of heterochromatin of all chromosomes (Lakhotia and Kumar 1978). Although the nuclei of *D. melanogaster* do not display such prominent chromocentre, some dark stained heterochromatic areas can be identified.

The diploid karyotype (2N = 8) of *D. melanogaster* (wild type and the *l(2)gl^4* mutant) consists of a pair of sex chromosome (XX/XY, X being large acrocentric and Y being large submetacentric), two pairs of large metacentrics (chromosome 2 and 3) and a pair of dot like 4th chromosomes. The karyotype of *D. nasuta* (2N = 8) consists of a pair of sex chromosomes (XX/XY), a pair of large metacentrics (chromosome 2), a pair of large acrocentrics (chromosome 3) and a pair of dot like 4th chromosome; the X-chromosome in this stock is either a large acrocentric or submetacentric type (Lakhotia and Kumar 1978). In females, the two types of Xs can be present in homo- or in heterozygous condition.

3.1 *[^{3}H]thymidine labelling patterns in interphase nuclei*

Unsynchronized or synchronized larval brain ganglia pulse (15 min) or continuously (24 h) labelled with [^{3}H]thymidine were examined for the patterns of autoradiographic

labelling of interphase nuclei in random areas of slides. As described earlier (Lakhotia and Roy J K 1995), three distinct patterns of labelling viz., *H label* (only the heterochromatin labelled), *E label* (the heterochromatic chromocentre unlabelled but the euchromatin labelled) and *H + E label* (the hetero- as well as euchromatin regions labelled) were seen (figure 1). The data on the frequencies of the three different labelling patterns among the labelled nuclei are presented in table 1. In experiment A (pulse labelling of freshly dissected brain ganglia),

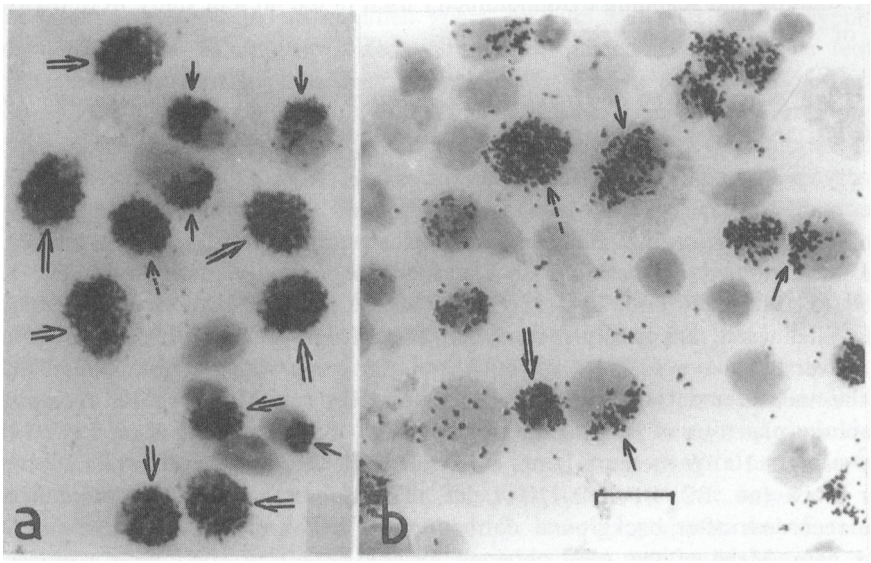

Figure 1. Patterns of [^{3}H]thymidine labelling of interphase nuclei from late third instar larval brain ganglia of *D. nasuta* **(a)** and *D. melanogaster* **(b)** pulse labelled for 10 min. →, *H* labelled; ⇒, *E* labelled and – – –>, *H+E* labelled nuclei. The bar represents 10μm.

Table 1. [^{3}H]thymidine labelling patterns of interphase nuclei from late third instar brain ganglia of *D. melanogaster* (wild type), *D. nasuta* and of the homozygous *l*(2)*gl*^{4} mutants of *D. melanogaster*

Expt	Labelling protocol	Types of larvae	No. of nuclei observed	Nuclei labelled (%)	Different labelling patterns (% among labelled) *H*	*H+E*	*E*
A	15 min [^{3}H]thymidine pulse at 0 h fixation	*D. melanogaster* (wild type)	392	22·5	65·5	28·5	6·0
		l(2)gl^4	153	28·5	56·0	39·0	5·0
		D. nasuta	1276	26·7	53·8	27·4	18·8
B	16 h FdU fed larvae pulse labelled with [^{3}H]thymidine (15 min) followed by fixation	*D. nasuta*	3402	35·1	23·4	6·3	68·6
C	24 h radio-isotope free incubation followed by [^{3}H]thymidine pulse for 15 min	*D. nasuta*	868	24·9	69·4	13·9	16·7
D	[^{3}H]thymidine 24 h chronic	*D. nasuta*	1447	35·4	37·7	58·2	4·1

H labelling pattern accounted for more than 50% of labelled nuclei in all the three samples, the *H* + *E* class accounted for 27-39% while *E* labelled cells were less frequent (see table 1). In experiment B (pulse labelling of FdU synchronized brain ganglia), the *E* labelled cells were frequent (68·6%), while *H* labelled and *H+E* labelled classes were less common (23·4% and 6·3% respectively). The total labelling frequency in brain ganglia of *D. nasuta* which were pulse labelled for 15 min after *in vitro* culture for 24 h (experiment C) was closely similar to that seen for *D. nasuta* in experiment A; the *H* labelled cells were also the most frequent in set C as in set A. Thus the 24 h *in vitro* culture did not affect replication in different nuclei.

The frequency of labelled nuclei in ganglia exposed to [^{3}H]thymidine continuously for 24 h (experiment D) was higher than in pulse labelled ganglia. As expected with the continued availability of [^{3}H]thymidine, the frequency of fully labeled nuclei *(H* + *E* labelled) was high (58·2%, see table 1) after 24 h chronic labelling. However, it is noteworthy that even after continuous labelling for 24 h, a significant proportion of nuclei still displayed a restricted incorporation of [^{3}H]thymidine in only the *H* or *E* regions. Since the experimental check mentioned in §2.2 confirmed continued availability of [^{3}H]thymidine to end of the 24 h labelling period in experiment D, the autoradiographic labelling in these brain ganglia reflects the cumulative uptake of the radioisotope in nuclei that engaged in DNA synthesis during the 24 h period.

3.2 *Analysis of cell cycle parameters*

The frequencies of labelled mitoses at different time intervals (15 min to 24 h) after a 10 min pulse of [^{3}H]thymidine to unsynchronized (*D. melanogaster, D. nasuta* and *l(2)gl^4)* and synchronized (*D. nasuta*) brain ganglia were calculated to define the mitotic cell cycle parameters. The data are presented in figure 2a-c Most of the mitotic figures were typical metaphases but in some (figure 3), the sister chromatids were separate (anaphases). Both types are referred to as metaphases in the following.

Labelled metaphases were already seen 15 min (*D. nasuta*) or 30 min (*D. melanogaster* wild type and *l(2)g1^4* mutant) after the pulse with frequencies as high as 52% in unsynchronized and 30% in synchronized ganglia of *D. nasuta* (see figure 2a). In later samples of *D. nasuta* also, the labelled mitoses frequencies were always higher than 50%. Preparations of *D. melanogaster* wild type brain ganglia also showed a similar distribution of frequencies of labelled metaphases (figure 2b) except that in the 16 h chase sample the frequency of labelled metaphases declined to 6%. In the brain ganglia of *l(2)gl^4* mutants too, the labelled metaphase frequency did not decline below 40% in any later chase samples (figure 2c). Thus for any of the four cases viz., wild type and *l(2)gl^4* of *D. melanogaster, D. nasuta* unsynchronized and synchronized brain ganglia cells, typical labelled mitoses curves could not be derived from these data.

Single chromatid labelled metaphases (figure 3) were seen in chase samples beginning from 3 h (in *D. melanogaster.* figure 2b-c) or 4 h (in *D. nasuta,* figure 2a). However, their frequencies (expressed as % of all labelled metaphases seen in a given sample) generally remained below 10% of labelled metaphases in most of the chase samples of the *l(2)gl^4* mutant and wild type *D. melanogaster* and below 30–40% in *D. nasuta.*

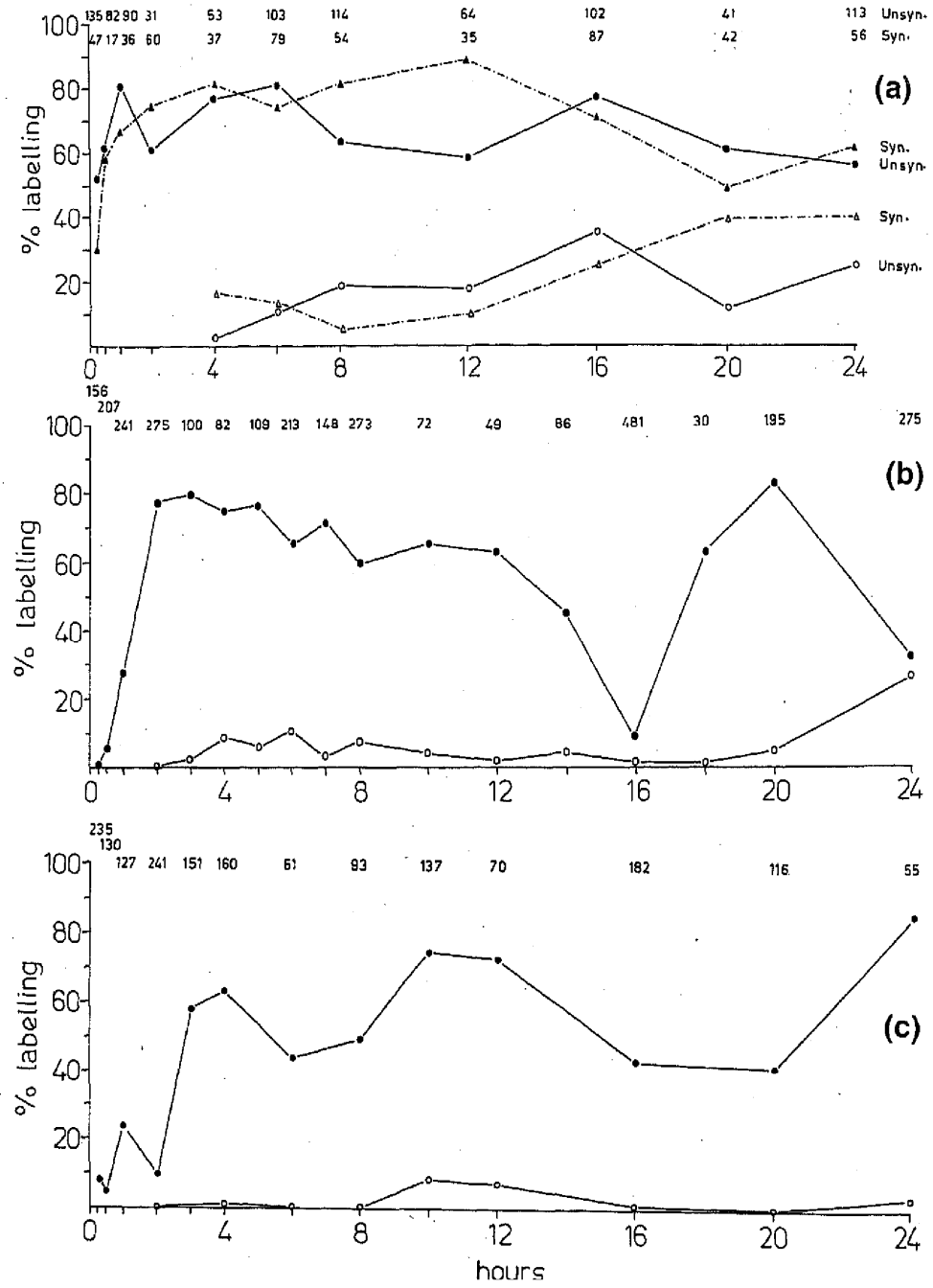

Figure 2. Frequencies (%) of labelled mitoses(● or ▲) in brain ganglia from late third instar larvae of *D. nasuta* **(a),** *D. melanogaster* **(b)** and *l(2)gl*^{4} **(c)** at different time periods after a 10 min [^{3}H]thymidine pulse. The frequencies of metaphases showing labelling of single chromatids only (calculated as % among the total labelled metaphases in a given sample) are also shown (O or Δ). The numbers on top indicate the total number of metaphases examined for each sample. In **(a)** Unsyn. and Syn. refer to unsynchronized and synchronized ganglia, respectively (see text).

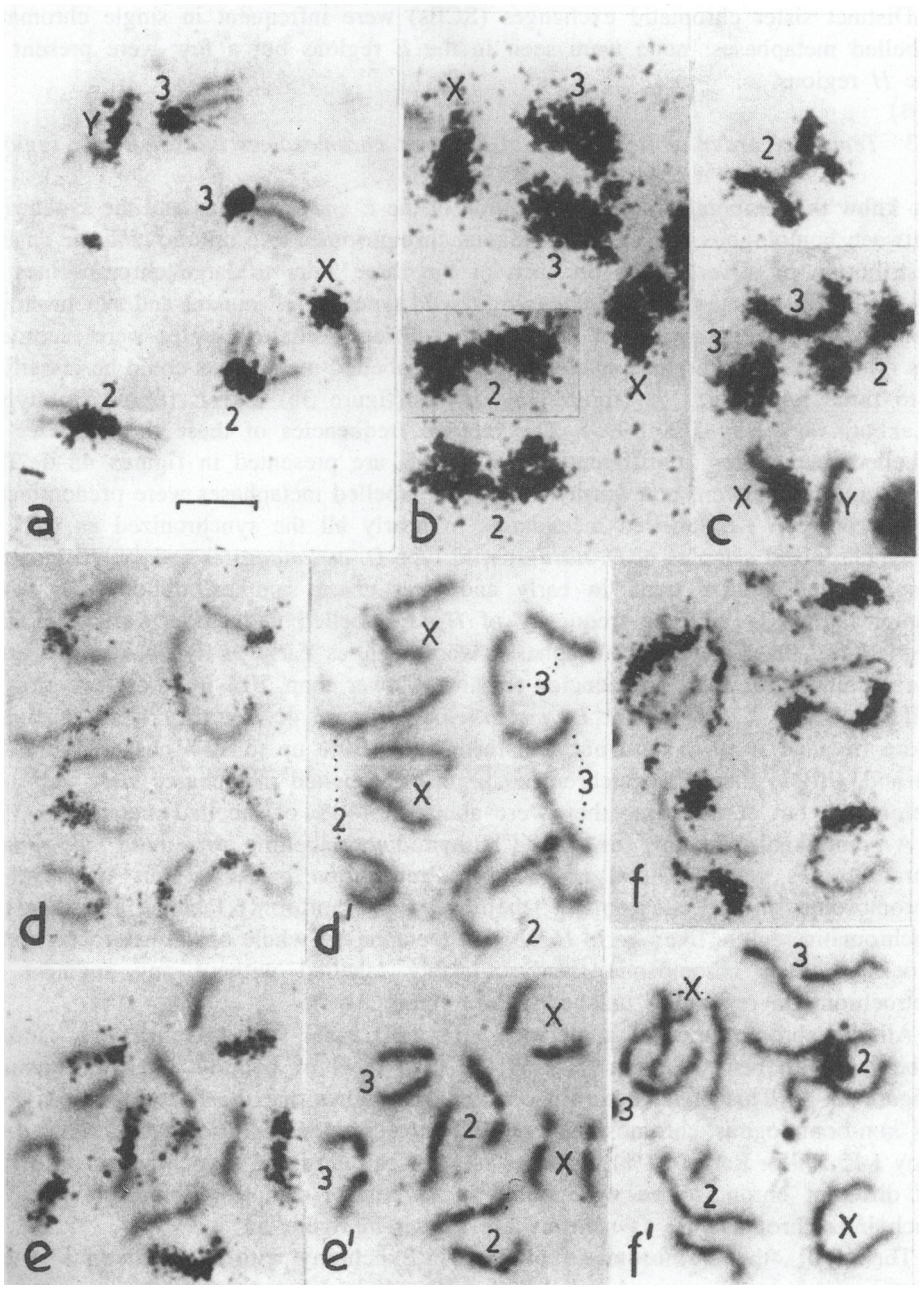

Figure 3. Autoradiograms of nutotic chromosomes from 10 min [^{3}H]thymidine pulse labelled brain ganglia of late third instar larvae of *D. nasuta* chased for various lengths of time showing label on heterochromatin **(a, d),** hetero + euchromatin **(b, e)** or euchromatin **(c, f)** regions of both **(a, b, c)** or single **(d, e, f)** chromatids of each chromosome. The sister chromatids are separate in **d, c, f;** to facilitate their identification, degrained chromosomes **(d', e', f')** are shown adjacent to the respective autoradiogram; the widely separated sister chromatids of a chromosome in d' and f' are indicated by dotted line. Bar represents 5 µm.

Distinct sister chromatid exchanges (SCEs) were infrequent in single chromatid labelled metaphases: none were seen in the *E* regions but a few were present in the *H* regions.

3.3 *Temporal order of replication of different chromosomes and their sub regions*

To know the temporal order of replication of the *E* and *H* regions and the synchrony between homologous and non-homologous chromosomes in a mitotic cell, the spatial distribution of silver grains on each of the three pairs of large chromosomes in all well spread plates of *D. melanogaster* wild type, *l(2)gl*^{4} mutant and synchronized and asynchronized samples of *D, nasuta* in different chase samples were recorded. As in the case of interphase nuclei above, the labelled metaphases could be classified into three types, viz., *H* (figure 3a), *H* + *E* (figure 3b) and *E* (figure 3c) types (Lakhotia and Roy J K 1995). The relative frequencies of these three classes of labelled metaphases at different chase periods are presented in figures 4a-d. The following points were noteworthy: (i) The *H* labelled metaphases were predominant, followed by *H* + *E* labelled metaphases in nearly all the synchronized as well as unsynchronized samples of *D. nasuta;* wild type *D. melanogaster* and *l(2)gl*^{4} mutants also showed similar trend in early and later chase samples, although in some samples viz., 3-12 h, the frequency of *H* + *E* labelled metaphase was marginally higher, (ii) the *E* labelled metaphases were seen as early as 1–2 h samples after the labelling but their frequencies remained lower than 10% in most samples of wild type and *l(2)gl*^{4} mutant *D. melanogaster* (figure 4c, d); they were relatively more frequent in *D. nasuta* but remained below 30% up to 12 h chase (figure 4a, b) and, (iii) in the FdU synchronized brains, *E* labelled metaphases were not seen before 4h but at that time they were abundant (40% of labelled metaphases).

A remarkable feature of the [^{3}H]thymidine labelling of these metaphase chromosomes was the total absence of "replication bands". Thus whenever a chromosome showed euchromatin labelling, it was uniformly labelled all along the euchromatin region; likewise in *H* labelled metaphases, whole of the heterochromatin block of each chromosome was uniformly labelled without any segment of hetrochromatin remaining unlabelled (see figure 3a-f).

All the chromosomes of a metaphase in most cases showed remarkably similar labelling with respect to *H, E* or *H* + *E* type label of both or single chromatids (figure 3a-f). This high degree of concordance shows that the homologous as well as non-homologous chromosomes replicate synchronously (also see Lakhotia and Roy J K 1995). Rare (< 1%) instances of inter-chromosomal asynchrony in labeling of different chromosomes were noted in *D. nasuta* samples. Some examples of such inter-chromosomal asynchrony are shown in figure 5.

The small 4th chromosomes replicated in synchrony with euchromatin of other chromosomes and remained unlabelled in the *H* labelled metaphases.

3.4 *Haploid DNA content of D. nasuta*

Since the haploid DNA content of *D. nasuta* was not known, sperms of *D. melanogaster* and *D. nasuta* were examined for their relative feulgen-DNA absorbance. Taking mean of the values for *D. melanogaster* sperms (1 C) to be 100 AU, the mean of the DNA-feulgen absorbance values for *D. nasuta* sperms was found to

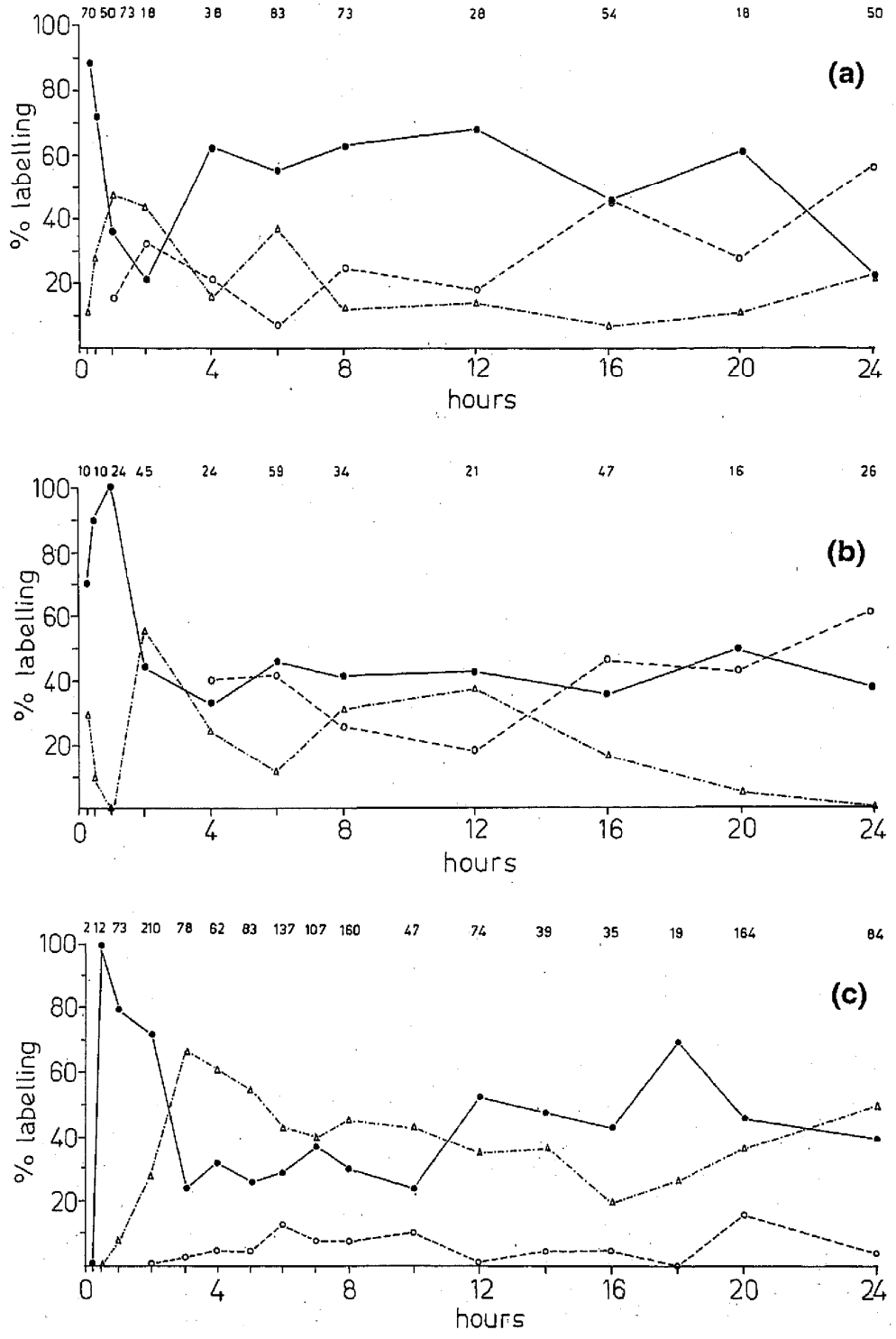
70 50 73 18 38 83 73 28 54 18 50
(a)
% labelling
hours
10 10 24 45 24 59 34 21 47 16 26
(b)
% labelling
hours
2 12 73 210 78 62 83 137 107 160 47 74 39 35 19 164 84
(c)
% labelling
hours

Figure 4. a, b, c.

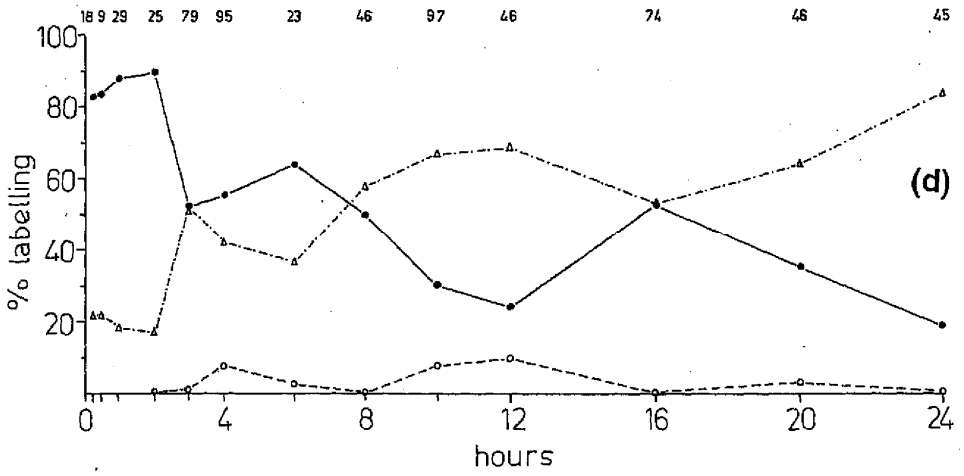

Figure 4. Frequencies (% among labelled) of *H* (●), *H+E* (Δ) or *E* (O) labeled metaphases in different samples of chase after a 10 min [^{3}H]thymidine pulse in unsynchronized (a) and synchronized (b) late third instar larval brain banglia of *D. nasuta,* unsynchronized larval brain ganglia of *D. melanogaster* (c) and *l(2)gl^4 (d).* The numbers of labeled metaphases analysed for each sample are indicated on top.

be 156 AU. Since the haploid DNA content of *D. melanogaster* is 0·18 pg (Rasch 1970), the haploid DNA content of *D. nasuta* on this basis was estimated to be 0·28pg(l·56×0·18(M8pg).

3.5 *Relative feulgen-DNA content of metaphase cells in brain ganglia of D. melanogaster* (*wild type and l(2)gl^4) and D. nasuta larvae*

Table 2 presents the normalized data on the relative feulgen-DNA values as determined by scanning cytophotometry of sperms from adult testes and metaphase cells from larval brains.

The data in table 2 reveal that the range of variation in the feulgen-DNA absorbance values (reflected in the range as well as coefficient of variance, see table 2) was higher for metaphase samples than for the corresponding sperm samples measured at the same time. However, in all cases the mean feulgen-DNA absorbance values of the metaphases (2 N) in *D. melanogaster* (wild type and *l(2)gl^4*) as well as in *D. nasuta* were close to 4 C (400 AU) level; the mean feulgen-DNA absorbance for the colchicine - induced 4 N metaphases was 919·50 AU, slightly higher than 8C (800 AU) level.

3.6 *Estimation of the relative DNA contents of brain metaphases and sperms of l(2)gl^4 using the flurochrome Hoechst 33258*

The data on plug measurements of such preparations (table 2) revealed that when stained with Hoechst 33258 in the presence of 1 M NaCl, the mean fluorescence value for metaphase sample (426 AFU) was close to the 4 C level (400 AFU), although the metaphase values were spread over a wider range than the values for sperms.

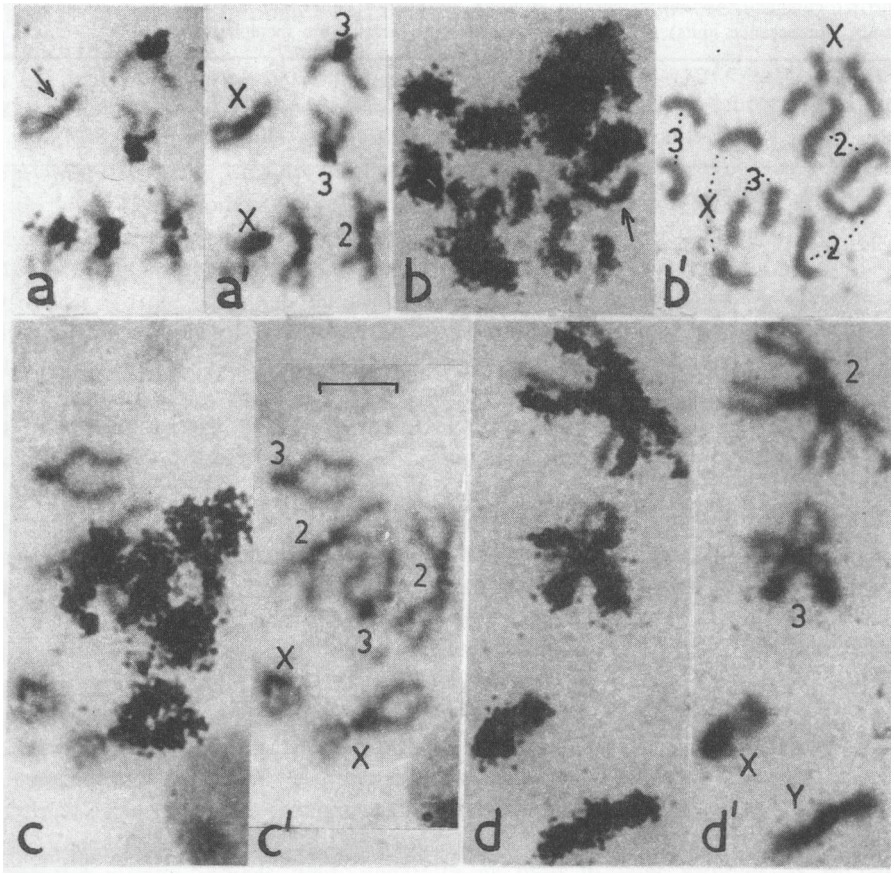

Figure 5. Examples of metaphases from unsynchronized brain ganglia of *D. nusuta* showing unusual patterns of autoradiographic labelling Giemsa stained chromosomes (after degraining) are shown **(a'-d')** adjacent to the autoradiograms **(a-d) , (a)** *H* regions of all except one submetacentric type X (arrow) are labelled: **(b)** all chromosomes show labelling of *H +E* regions of both chromatids except one homologue of second chromosome in which one chromatid is totally unlabelled (arrow); **(c)** metaphase with some chromosomes unlabelled and some labelled on single or both chromatids and **(d)** metaphase with *H* regions of both sister chromatids and *E* regions of only one sister chromatid of all chromosomes labelled. Bar represents 5 μm.

4. Discussion

The brain ganglia in *Drosophila* larvae comprise of cells with varying replication programmes. Besides the cells that do not engage in DNA synthesis, there are those that follow endoreplication cycles and those that traverse through mitosis. A major objective of the present study was to analyse cell cycle in the sub-population of cells that divides mitotically in larval brain ganglia by labelling the S-phase cells with [^{3}H]thymidine and chasing the label in individual chromosomes at metaphase stage.

Our data on the [^{3}H]thymidine labelling of metaphases in brain ganglia of *Drosophila* larvae present unusual and intriguing features which defy simple

Table 2. Feulgen-DNA absorbance (in arbitrary absorbance units) and Hoechst 33258 fluorescence (in arbitrary fluorescence units) of *Drosophila* sperms and larval brain metaphases.

Staining and species	Cells	N	Mean (normalized)	Range		SD	Coef. var. (%)
				Min.	Max.		
Feulgen-DNA staining							
D. melanogaster (wild type)	Sperms	104	100·00	66·30	127·07	9·94	9·94
	2 N Meta.	100	417·59	264·29	641·45	91·17	21·80
D. melanogaster (wild type — colchicine treated)	Sperms	42	100·00	82·64	113·25	7·47	7·47
	2 N Meta.	44	436·13	337·47	624·49	57·47	13·17
	4 N Meta.	20	919·50	673·18	1069·80	166·24	18·07
D. melanogaster ($l(2)gl^4$)	Sperms	52	100·00	81·47	116·35	8·70	8·70
	2 N Meta.	57	420·09	316·81	572·25	55·75	13·27
D. nasuta (wild type)	Sperms	24	100·00	76·58	111·64	8·35	8·35
	2 N Meta.	88	399·62	306·31	518·02	42·19	10·56
Hoechst 33258 with 1 M NaCl							
D. melanogaster ($l(2)gl^4$)	Sperm	20	100·00	80·00	120·00	11·61	11·61
	2 N Meta.	20	426·50	290·00	530·00	63·47	14·88

N, Total number; SD, standard deviation; Coef. var., co-efficient of variance; Meta. metaphases.

explanations. A labelled mitoses curve in a typical [^{3}H]thymidine pulse-chase experiment (Howard and Pelc 1953) is expected to show a gradually rising peak followed by a trough and a second peak of smaller size. Further with a typically cycling population of cells, the frequency of metaphases with single chromatid labelling (2nd cycle) and of those with some single chromatid labelled and some unlabelled chromosomes (3rd or later cycle) will increase with increasing periods of chase. Compared to these expected patterns, the labelled mitoses curves obtained in the present study (figure 2) with wild type and $l(2)gl^4$ mutants of *D. melanogaster* and unsynchronized as well as synchronized ganglia of *D. nasuta* were very different.

The sequential appearance of *H, H + E* and *E* labelled metaphases in that order in the early chase samples in all sets suggests that as in other cell types, in the mitotic cells of *Drosophila* also the euhcromatin regions replicated in early S while the heterochromatin regions were late replicating (Lakhotia and Roy J K 1995). The high frequency of labelled metaphases in the 15 min sample in *D. nasuta* suggested that in a significant proportion of mitotic cells, the G_2 period was extremely brief or nonexistent. The appearance of *H + E* labelled metaphases in the 15 min sample, the increase in their frequency in 30 min and 1 h unsynchronized samples and the appearance of *E* labelled metaphases in the 1 h unsynchronized sample further indicated that the time required to traverse through the S and G_2 periods was very brief in many of the brain mitotic cells of *D. nasuta.* The presence of single chromatid labelled metaphases in the early samples showed that some

cells at least progressed through a cell cycle in less than 2 h. With bromodeoxyuridine (BrdU)-Giemsa staining also, some second cycle metaphases were seen within 1–2 h of BrdU labelling (Lakhotia *et al* 1979). In *D. melanogaster* wild type and in *l(2)gl*^{4} mutants also, the G_2 period may not be very long since labelled metaphases were seen with significant frequency within 1 and 3 h, respectively.

Surprisingly however, results of the later chase samples were not as expected with the brief G_2 period or short cell cycle duration suggested by the early chase samples. It is intriguing that more than 50% of metaphases continued to appear labelled in all (*D. nasuta*) or most chase samples (wild type and *l(2)gl*^{4} mutant) of *D. melanogaster*. The sudden dip in the frequency of labelled metaphases in the 16 h chase sample of wild type *D. melanogaster* seems to be some artifactual variation since in the later chase samples the labelled metaphases were more frequent and among them, the second cycle metaphases were not more common as expected if the dip in the 16 h sample reflected completion of one cycle. The labeled mitoses curve for *l(2)gl*^{4} brain ganglia showed more than one peak, as if these cells had very short cell cycle duration and had completed three cycle in the 24 h period. However, this curve too did not reflect typical cell cycle since the second and subsequent peaks of labelling frequency were progressively higher (see figure 2c) while the frequency of second cycle metaphases did not increase in the later chase samples. Thus in spite of the G_2 and S-periods appearing to be brief, a decline in the frequency of labelled metaphases corresponding to G_1 period was not found. More intriguing was the fact that the frequency of single chromatid labeled metaphases remained lower than both sister chromatids labelled types even in the later chase samples. With BrdU-Giemsa staining also, the 2nd and later cycle metaphases were infrequent (unpublished observations).

Paucity of the 2nd cycle metaphases in later samples was not due to poor mitotic activity under the *in vitro* conditions used since firstly, the metaphase labelling as well as the mitotic indices were generally as high in later samples as in early samples, secondly the frequency of replicating nuclei in brain ganglia cultured *in vitro* for 24 h prior to [^{3}H]thymidine pulse was as high as in freshly excised ganglia and finally, a high frequency of tetraploid or even higher ploidy metaphases were found in brain ganglia exposed *in vitro* to colchicine for up to 16 h (also see Lakhotia 1982).

Continued availability of [^{3}H]thymidine during the chase periods may vitiate results. However, [^{3}H]thymidine was no longer available during the chase periods as determined by independent control experiments (data not presented). Therefore, we believe that the unusual labelled mitoses curves obtained in our study were not experimental artifacts but reflected some other biological features of cell divisions in brain ganglia of *Drosophila* larvae.

Compared to our failure to define the mitotic cell cycle parameters in brain ganglia of *D. nasuta* or *D. melanogaster,* Steinemann (1980) presented a computer-simulated typical labelled mitoses curve for brain ganglia of *D. virilis* larvae. While species differences may exist, methodological and other factors appear more important in this context. A basic presumption underlying Steinemann's estimate was that the larval brain ganglia were constituted of an exponentially proliferating population of cells. This presumption, however, is not valid since as noted earlier, brain ganglia in *Drosophila* larvae consist of a variety of cells, only some of which divide mitotically while others either do not synthesize DNA or

go through endoreplication cycles. Thus extrapolation of the interphase labelling frequency in such a mixed population of cycling and non-cycling cells to assess the frequency of mitotically active cells,as done by steinemann is not valid Further, in Steinemann's (1980) experiment protocol, the [^{3}H]thymidine labeled ganglia were chilled on ice prior to the chase in radioisotope-free medium. Chilling may modify the progression of cells to mitosis in an unknown manner and this could be one of the factors responsible for the frequency of labelled metaphases in his later chase samples being much lower than in corresponding samples in our study. Moreover, in several of Steinemann's chase samples the total number of metaphases examined was less than 30 or even 10 (see table 1 in Steinemann 1980). Such small samples can lead to unpredictable variations in labelling frequencies. Thus we believe that Steinemann's estimates of the different parameters of mitotic cell cycle in larval brain ganglia derived through computer-simulation- were not valid. In an earlier study on mitotic cell cycle in brain ganglia of another dipteran larvae, *Musca domestica,* Jan and Boyes (1970) also failed to estimate durations of the various phases.

A possible reason for the lack of G_1 -dip in the frequency of labelled mitoses curves in brain ganglia could be highly variable G_2 and G_1 periods so that the labelled mitoses curves do not show the expected peaks and troughs. White and Kankel (1978), Truman and Bate (1988) and Furst and Mahowald (1985) suggested a very brief (~ 1 h) cell cycle time for the neuroblasts while the ganglion mother cells and other dividing cells were suggested to have longer interphase period. In agreement with this, it was found that the tumorous brain ganglia of *l(2)gl*^{4} larvae, which lack neuroblasts (Roy S and Lakhotia 1991), also showed a significantly lower frequency of labelled metaphases till 3–4 h chase period. It is known that neuroblasts constitute the major class of mitotically dividing cells in larval brain ganglia which go through a series of symmetric and asymmetric division to produce neuroblasts, ganglion mother cells, pre-ganglion cells and ganglion cells (Truman and Bate 1988). This pattern of cell divisions in which progeny cells cycle differently or do not cycle after a certain stage, may explain several aspects of the unusual labelled mitoses curves obtained in our study. Earlier studies (White and Kankel 1978; Truman and Bate 1988) suggested that depending upon spatial location of neuroblasts in brain, the interval between their birth and subsequent entry into the next S-phase varies. Our studies further suggest that the interval between their entry into the S-phase and progression to mitosis may also vary. This variability in the durations of the G_1, S and G_2 phases of neuroblasts in combination with their asymmetric divisions that generate progeny cells with limited division capacity, would result in a persistent high frequency of labelled metaphases in early as well as later chase intervals.

The persistence of a high frequency of *H* labelled metaphases in nearly all chase samples is unusual but correlates with the preponderance of *H* labelled interphase nuclei. This correlation, however, did not exist for the FdU -synchronized ganglia since while in interphase nuclei *E* labelling was most frequent (table 1), in metaphases, the *H* labelled class was the most frequent. The *H* labelled metaphases remained frequent (20-30%) even after 20 to 60 h continuous labelling with [^{3}H]thymidine (Lakhotia 1982). Thus it appears that brain cells which are in exclusive *H* labelling phase enter mitosis more frequently than others. Two possibilities, not necessarily mutually exclusive, arise from these observations. In

view of the independent endoreplication of eu- and heterochromatin in some brain cells (Berendes and Keyl 1967; Lakhotia and Kumar 1980; Zacharias 1993), it is possible that some of the mitotically dividing cells may also complete their S-phase in instalments such that replication of *H* and *E* regions is separated by a non-replicating phase of variable duration. A second possibility is that heterochromatin replication is slow and takes a variably long period; earlier DNA fibre autoradiographic studies (Lakhotia and Sinha 1983; Lakhotia and Tiwari 1985) indeed suggested a slow rate of fork migration in replicons associated with *H* regions of *Drosophila* genome. The first possibility also finds support in the results with FdU-synchronized ganglia. FdU blocks cells at G_1-S border (Taylor 1977) which explains the increased frequency of *E* labelled interphase nuclei in brain ganglia of *D. nasuta* after release from FdU (table 1). However, the persistence of *H* labelled interphase and metaphase cells, even after FdU feeding for longer periods of time or at higher concentration (Lakhotia 1982), leads us to suggest that many of the brain cells are programmed to replicate their *H* regions only so that upon release from the FdU-block, they initiated their S-period with *H* replication rather than the conventional *E* replication; subsequent progression of these cells to mitosis would explain the high frequency of *H* labelled metaphases seen in the various chase samples.

Replication of different homologous as well as non-homologous chromosomes is highly synchronous since in nearly all cases all chromosomes of a nucleus displayed identical [^{3}H]thymidine incorporation pattern (also see Lakhotia and Roy J K 1995). In the context of such a high inter-chromosomal synchrony, the patterns of autoradiographic labelling in the mitotic cells of *D. nasuta* shown in figure 5b-d are puzzling. Although rare, such cases deserve notice. The anaphase in figure 5b should be from a first cycle post-pulse cell since both chromatids of most chromosomes are labelled; the total absence of label on one sister chromatid of one of the chromosome 2 is, therefore, unexpected. The presence of chromosomes showing autoradiographic labelling characteristic of first cycle (both sister chromatids of a chromosome labelled), second cycle (only one sister chromatid labelled) and zero or third cycle (both sister chromatids unlabelled) in the same metaphase plate (figure 5c) is also unusual. Another intriguing example is the cell in figure 5d with both chromatids of all chromosomes labelled in *H* regions but only one sister chromatid labelled in *E* regions. Although autoradiographic artifacts cannot be ruled out, the unambiguous and specific labelling in such cases leads us to think that these unusual patterns of labelling are not artifactual.

The structural organization of metaphase chromosomes in brain ganglia of *Drosophila* larvae needs to be considered in context of the present results. Several earlier DNA cytophotometric studies suggested the mitotic chromosomes in brain ganglia of *Drosophila* larvae to be polynemic (Rudkin 1963; Gay *et al* 1970). Our earlier studies (Lakhotia and Roy J K 1981, 1883) on the effects of some DNA ligands also suggested the mitotic chromosomes in larval brain ganglia to be differently organized than those in embryonic cells. In view of these and some other observations, Lakhotia (1982) suggested that some of the endoreplicating cells in larval brain ganglia may enter mitotic division cycle with polynemic chromosomes. Almost all of the intriguing observations of this study (e.g., the preponderance of both chromatid labelled metaphases even in the later chase samples and the examples shown in figure 5) are compatible with a polynemic organization of at least some mitotic cells in larval brain ganglia. However, a variety of other experimental

evidence has established the metaphase chromosomes in *Drosophila* and in other eukaryotes to be uninemic (Kavenoff and Zimm 1973; Van de Flierdt 1975; Zacharias 1993). Nevertheless, in view of our earlier (Lakhotia and Roy J K 1981, 1983; Lakhotia 1982) and the present results, we cannot rule out the possibility that some of the cells that divide mitotically in brain ganglia do so with bi- or polynemic chromosomes.

Comparison of the mean feulgen-DNA absorption and Hoechst 33258 fluorescence values showed that the means of relative DNA contents of the metaphases in all the sets of measurements were as expected with uninemy. No diploid metaphases were encountered in our sample that displayed DNA content close to 8 C or higher, although the feulgen-DNA values for several of them was about 6 C (also see, Zacharias 1993). Although the means of the observed DNA values were as expected with uninemy, a comparison of the minimum and maximum values and the co-efficients of variance for each set of measurement presented in table 2 revealed that the feulgen-DNA or the Hoechst 33258 fluorescence values for metaphases tended to vary over a wider range than for sperms. A greater scatter in the DNA values for individual metaphase ceils could be due to one or more of the following reasons (Goldstein 1981): (i) overlapping of chromatids and/or incomplete metaphase plates, (ii) residual distributional error, (iii) some inherent limitations of the instrument or (iv) a real difference in the DNA content in different metaphase plates. The reason (i) above was eliminated by careful selection of metaphases that did not suffer from these limitations. The MPV-3 system has some inherent limitations since its mirror scanning device scans with the entire area of the object fully illuminated; therefore, some instrumental error in absorbance was possible with the instrument used. However, the instrument or the residual distribution error do not apply to fluorescence measurements. Yet, the co-efficient of variance for Hoechst 33258 fluorescence DNA values (AFU) for diploid metaphases remained nearly as high as with feulgen absorbance measurements. Furthermore since the co-efficients of variance for grasshopper meiotic chromosomes varied between only 7 to 8% (data not presented but see §2), the higher values of co-efficients of variance for *Drosophila* brain metaphases (see table 2) do not appear to be an artifact but may have a biological basis. A possible biological factor that may generate the greater variance in the brain metaphase data could be a suggestion by Karpen and Spradling (1990) that several families of transposable elements present in heterochromatic regions of *Drosophila* genome are randomly eliminated in somatic cells. If this were to happen in mitotic cells in larval brain, metaphases may be expected to display DNA content significantly less than the 4 C value. Indeed many metaphases in our samples of different genotypes/species did reveal DNA values considerably lower than the 4 C level (e.g., see values under the column " min " in table 2). The above detailed analysis of mitotic cells in brain ganglia of *Drosophila* revealed several unusual features which are not incompatible with a bi- or multinemic organization of chromosomes in some, but not necessarily all, of the dividing cells; in addition, these results also suggest that the S-phase in some dividing cells may be discontinuous with a non-replicating period intervening between replication of euchromatin and heterochromatin, a feature characteristic of endo-replicating cells with disproportionate amounts of eu- and heterochromatin (Lakhotia 1982; Raman and Lakhotia 1990). More recently, Glaser *et al* (1993) have suggested that the well known under-representation of heterochromatin in endoreplicating nuclei of

Drosophila is due to physical elimination of these sequences rather than their under-replication. Integrating the present results with the suggestion of Glaser *et al* (1993), we may speculate that a combination of polynemy and somatic elimination of certain DNA sequences may generate DNA values that were higher than the 4 C level but not in multiples of the 2C level. Therefore, it remains possible that the metaphases that displayed DNA content significantly higher than the 4C level (e.g. 6 C) could actually be bi- or multi-nemic but due to loss of some sequences, the final DNA value was not 8 C or higher multiples. Thus unlike the conclusions of previous studies that all mitotic chromosomes in brain ganglia were either polynemic or uninemic, the present results suggest existence of cells with both kinds of metaphase chromosome organization in larval brain ganglia. It may be mentioned in this context that a variety of genetic and developmental factors are known to influence endoreplication cycles (Spradling and Orr-Weaver 1987; Raman and Lakhotia 1990) and therefore, the development of polynemy in dividing cells may also be influenced by as yet unknown genetic and epigenetic variables. Such variations may also have contributed to the conflict in results of earlier studies on this aspect. Further molecular and genetic studies will help resolve this issue better.

The total absence of "replication bands" in metaphase chromosomes of *Drosophila* is interesting. The "replication bands" arise due to the concerted activation of origin points in adjacent replicons (replicon clusters) with replication in nearby "clusters" being initiated at different time points in a programmed manner (Craig and Bickmore 1993). Compared to the uniform incorporation of [^{3}H]thymidine by the entire *E or H* regions of all metaphase chromosomes of a labelled brain cell, the polytene chromosomes show a remarkably elaborate and distinct temporal order of replication of different band/interband regions along their length (reviewed by Raman and Lakhotia 1990). In view of the presence of "replication bands" in polytene chromosomes, it is possible that the small size of metaphase chromosomes of *Drosophila* does not permit their resolution. However, earlier DNA fibre autoradiographic studies with polytene and mitotic cells of *Drosophila* (Lakhotia and Sinha 1983; Lakhotia and Tiwari 1985) clearly showed that "replicon clusters" were absent in polytene as well as mitotic cells of *Drosophila.* Thus the failure to detect "replication bands" in mitotic chromosomes of *Drosophila* was not due to lack of resolution but due to a near total synchrony in activation of different replicons in the euchromatin or heterochromatin regions of different chromosomes The presence of "replication bands" in polytene cells in spite of the absence of "replicon clusters" was explained by Lakhotia and Sinha (1983) to be due to asynchrony in replication of the multitudes of sister chromatids in polytene nuclei. The absence of "replication bands" in mitotic cells of *Drosophila* has implications for differences in higher order organization of metaphase chromosomes in different groups (see Raman and Lakhotia 1990).

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