ASYNCHRONY OF DNA SYNTHESIS IN CHROMOSOMES OF HUMAN DIPLOID CELLS

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It has been previously demonstrated (1) that the length of the period of DNA synthesis (S period) as measured by uptake of tritiated thymidine is approximately the same in several animal cells. Since the length of this period (with a range of 6½ to 8 hours) appears to be independent of the species of the cell, the chromosome number, the amount of DNA per cell, and the generation time, it is of interest to determine the patterns of chromosome asynchrony or of intrachromosomal site asynchrony. Asynchronous replication of DNA by only one X chromosome and one arm of the other in female cells of the Chinese hamster (2) and in one X of human female cells from peripheral blood cultures has been reported (3) and confirmed (4, 5). Others (6) have observed asynchrony for the arms of the human X. Cultured cells from heteroploid lines of human origin have been studied with similar techniques (7). The work reported here was designed to determine what patterns of H^3-thymidine incorporation could be demonstrated in the chromosomes of cells of human diploid strains in long term cultivation.

MATERIALS AND METHODS

Cell strains derived from embryonic sources which have maintained their diploid characteristics in long term continuous cultivation *in vitro* were used in these experiments (8). At the time of these experiments these fibroblast-like cells were at different passage levels (18th to 35th subcultivation, 3 to 6 months) and showed no differences in growth rate; the percentage of cells incorporating H^3-thymidine was quite similar at different passages.

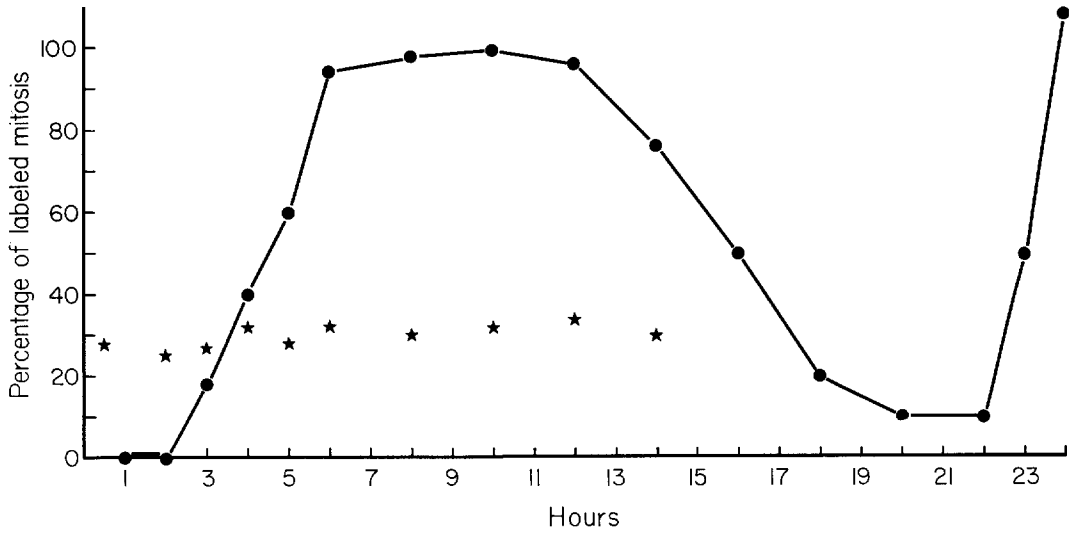

FIGURE 1

Distribution of labeled mitoses in cells of WI-26 at various periods after 20 minute pulse with tritiated thymidine. The lower curve, at a constant level (*), represents percentage of interphase nuclei.

Cells were seeded into several 1-liter Blake bottles and grown as a monolayer in medium of 10 per cent calf serum and 2× Eagle's components in Earle's balanced salt solution with antibiotics (100 units/ml Streptomycin, 100 μg/ml Penicillin).

At 36 hours when the cell sheet was almost confluent, half of the medium was removed and retained and an equal volume of fresh medium was added. All supernatant medium was removed 12 to 16 hours later and retained. The cultures were exposed for 20

minutes to tritium-labeled thymidine[1] at a final concentration of 0.3 to 0.5 μc/ml in medium composed equally of that first removed and a fresh portion. To the medium which had been removed at 12 to 16 hours excess unlabeled thymidine (100X–200X) was added, and this was placed on the cultures after two rinses. All manipulations were performed in a 37°C incubator room to minimize possible shock to the culture which might have induced phasing of cell division.

At intervals following the pulse exposure, two cultures were harvested for fixation of a representative sample. Samples were taken at 4, 6, 8, and 12 hours after the thymidine pulse; as illustrated in Fig. 1, this period (4 to 12 hours) includes almost 100 per cent of labeled mitosis, equivalent to the period of DNA synthesis. Colcemid[2] was added (final volume 0.5 gamma/ml) to the medium for 15 minutes solely to disarrange the metaphase plate, not to accumulate metaphases. Cells were harvested by treatment with 0.25 per cent trypsin (Difco, 1:250) and resuspended in ½ ml of the same. Distilled water was added to yield a 1:4 hypotonic solution and after 8 minutes the cells were centrifuged and fixed in methylacetic (3:1) fixative. Fixation of the "4 hour" sample was made 4 hours and 50 minutes after the pulse and later samples were similarly spaced. A drop of fixative-suspended cells was applied to the wet surface of a clean slide and metaphase spreading accomplished by immediate ignition of the fixative on the slide. This provided the excessive spreading desired for analysis of metaphases. The flame-dried slides were rinsed in distilled water, coated with NTB3 emulsion (Kodak, Rochester, New York), and exposed in the dark for 10 to 20 days. Slides were then developed in D-19 (Kodak), treated with fixer, dried, rinsed in distilled water, and stained for 15 minutes in Giemsa blood stain. Stained slides were rinsed in distilled water and mounted permanently with a drop of Polyvinylpyrrolidone.[3]

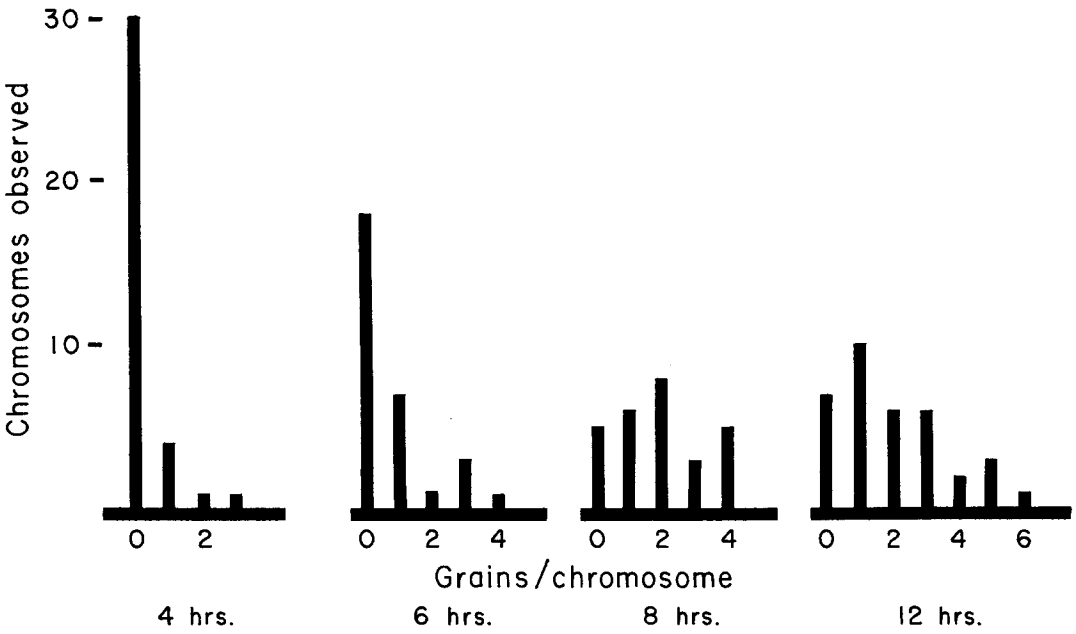

FIGURE 2

Frequency distribution of grains per chromosome for small metacentrics 19-20 at various points during the S period. Fewer grains are observed toward the end of the S period, *i.e.*, samples at 6 hours and 4 hours.

Metaphases selected for widely spread chromosomes were analyzed and photographed using oil immersion optics. Chromosomes overlying one another were disqualified from quantitative tallies of grain counts. For the larger chromosomes of the human complement (Nos. 1, 2, 3, 4, and 5) (9) grain localizations with respect to the centromere, the

[1] 1.9 C/mM, Schwarz Research, Inc., Mt. Vernon, New York.

[2] Courtesy of Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[3] Courtesy of Antara Chemicals, Philadelphia.

TABLE I

Average Grain Count per Chromosome in WI-26 Cells for Chromosomes 1, 2, 3, 4–5

Chromosome No.	Time of sample after H^3-thymidine pulse			
	4 hrs.	6 hrs.	8 hrs.	12 hrs.
1	5.5 ± 3.3 (17)*	6.0 ± 4.5 (23)	7.5 ± 3.4 (17)	6.4 ± 3.0 (21)
2	4.8 ± 3.9 (16)	4.6 ± 3.4 (17)	6.7 ± 3.3 (17)	6.8 ± 2.1 (21)
3	5.4 ± 3.6 (11)	3.4 ± 2.3 (14)	6.8 ± 3.4 (10)	5.1 ± 3.2 (12)
4–5	4.3 ± 4.0 (42)	4.8 ± 3.9 (40)	6.8 ± 3.2 (33)	4.9 ± 1.9 (41)

* Number of cells contributing to datum given in parentheses.

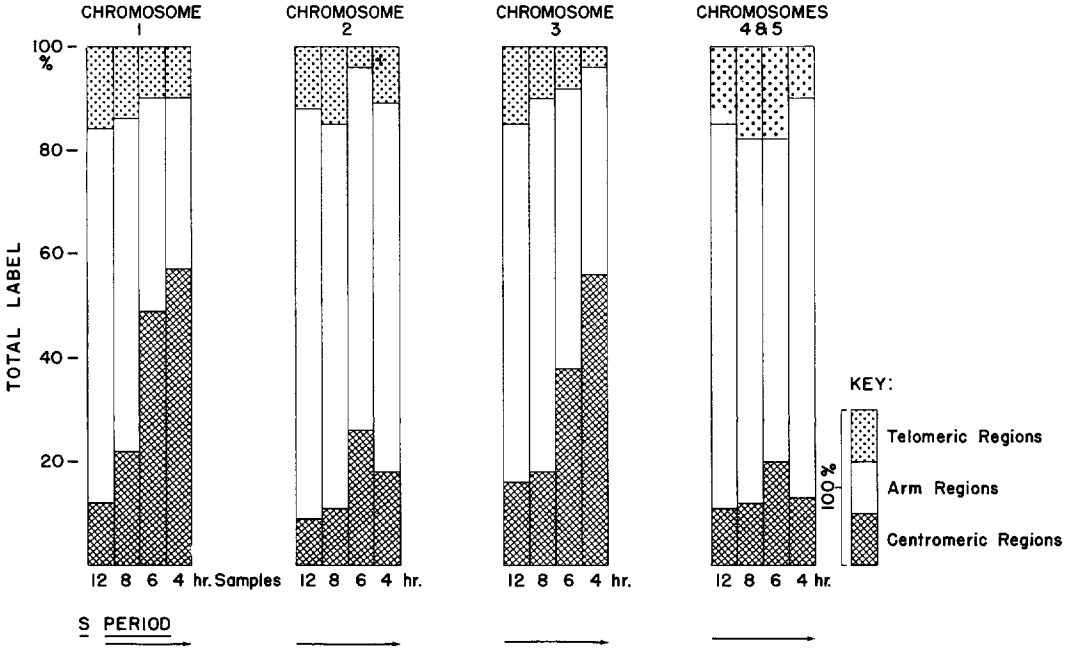

FIGURE 3

Comparison by chromosomal region of the grain distribution for each of the longest chromosomes in WI-26 cells after thymidine pulse treatment.

telomeres, and the arms were tabulated. Limits for these regions were arbitrarily taken as two grain diameters to either side of the centromere and the same distance from each telomere.

RESULTS AND DISCUSSION

DNA Synthesis Cycle

The period of DNA synthesis, the S period, in these cells was very similar to that reported for other mammalian cells (1). In Fig. 1, the variation with time of the percentage of labeled mitoses is illustrated. On the basis of this profile it can be calculated that the generation time of these cells is approximately 18 hours; the S period lasts between 7 and 7½ hours; while 3 to 6 hours are spent in G2 (premitotic rest). It can also be seen that the percentage of interphase nuclei labeled at any time during the experiment was very close to 30 per cent with no increase in the later period, indicating that an actual pulse labeling of 20 minutes was achieved. The fact that only 30 per cent of the cells were labeled, a proportion lower than expected for a generation time of 18 hours and an S period of 7 to 7½ hours, indicates that not all the cells in the culture were capable of DNA replication. This was confirmed in other experiments in which it was found that the non-duplicating

portion of the cell population amounted to 20 to 25 per cent.

Asynchrony of Chromosomes

In each of the samples taken at different times after the pulse there was a range of 150 to 250 grains per metaphase observed. In those cells in the upper range of total grains all chromosomes showed some degree of labeling. In the pulse experiment with the male strain WI-26 the impression was obtained that autosomal pairs Nos. 19 and 20 were precocious in their uptake of H^3-thymidine with respect to all other chromosomes. From those metaphases which permitted identification of these small metacentrics, counts of grains per chromosome at different points in the S period (Fig. 2) indicated heavier labeling in samples from the earlier portion of the S period (8 hours, 12 hours). However, the numbers available were small and the same could not be demonstrated in a similar experiment with the female strain.

An early labeling pattern for chromosome 17 was mentioned by German (4), and some instances possibly supporting this were observed in our material. Evidence of late labeling for chromosomes 21 and/or 22 as was reported by Morishima *et al.* (5) was never observed. Confirming earlier reports (3–6) occasionally one chromosome of the 6-X-12 group was observed in female strain WI-25 to be heavily labeled in samples representative of the later part of the S period (4 hours). No such distinctly labeled chromosomes were seen in any of the metaphases of male WI-26 cells. In the female strain it is notable that metaphases with such a characteristically heavily labeled metacentric were only rarely seen. The same rarity of such instances was noted in cells from one of the 4 females studied by German (4).

Total grain counts for chromosomes 1, 2, 3, and 4–5, which are easily identified, are presented from male strain WI-26 (Table I) and are regarded as generally similar with respect to the different times sampled during the S period. No attempts were made to compare grain counts between homologous chromosomes *within the same cell*, since cells in which all members of pairs 1, 2, 3 could be analyzed were insufficient in number.

It appears that during the entire period of DNA synthesis, 7 to 7½ hours, some portions of all the chromosomes are engaged in replication. The one clear exception is the heterochromatic X, in accord with earlier works, (3–5) and a possible

TABLE II

Average Number of Grains According to Chromosome Regions of Largest Chromosomes in Male Strain WI-26

Time after H^3-thymidine pulse	Centromeric regions	Arm regions	Telomeric regions
	Chromosome 1		
hrs.			
4	3.18	2.12	0.24
	57%	38%	5%
6	2.96	2.78	0.24
	49%	46%	5%
8	1.65	4.82	1.00
	22%	64%	14%
12	0.76	4.42	1.00
	12%	72%	16%
	Chromosome 2		
4	0.88	3.37	0.50
	19%	70%	11%
6	1.18	3.24	0.18
	26%	72%	4%
8	0.76	4.94	1.00
	11%	74%	15%
12	0.62	5.38	0.76
	9%	79%	12%
	Chromosome 3		
4	3.00	2.18	0.18
	56%	40%	4%
6	1.29	1.71	0.29
	39%	52%	9%
8	1.20	4.70	0.60
	18%	72%	10%
12	0.83	3.67	0.75
	16%	69%	15%
	Chromosomes 4–5		
4	0.56	3.30	0.40
	13%	77%	10%
6	0.92	2.83	0.77
	20%	62%	18%
8	0.84	4.76	1.15
	12%	70%	18%
12	0.54	3.68	0.71
	11%	74%	15%

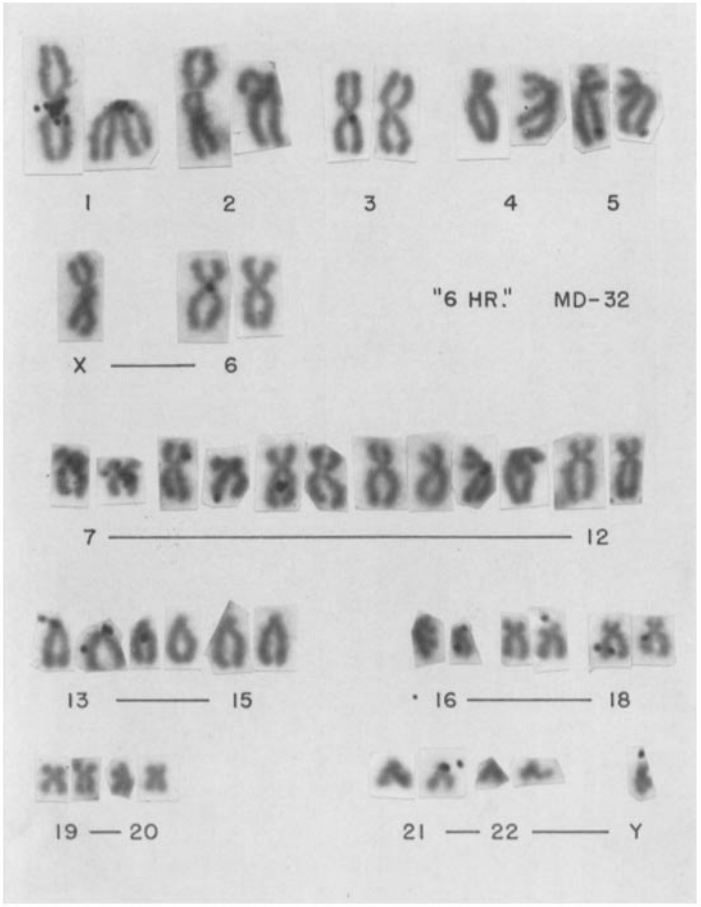

FIGURE 4

Karyotype of chromosomes of metaphase from male strain WI-26. Sample taken 6 hours following 20′ pulse with tritiated thymidine (Experiment MD-32) showing excess of label over centromeres of both homologues of chromosome 1. × 1600.

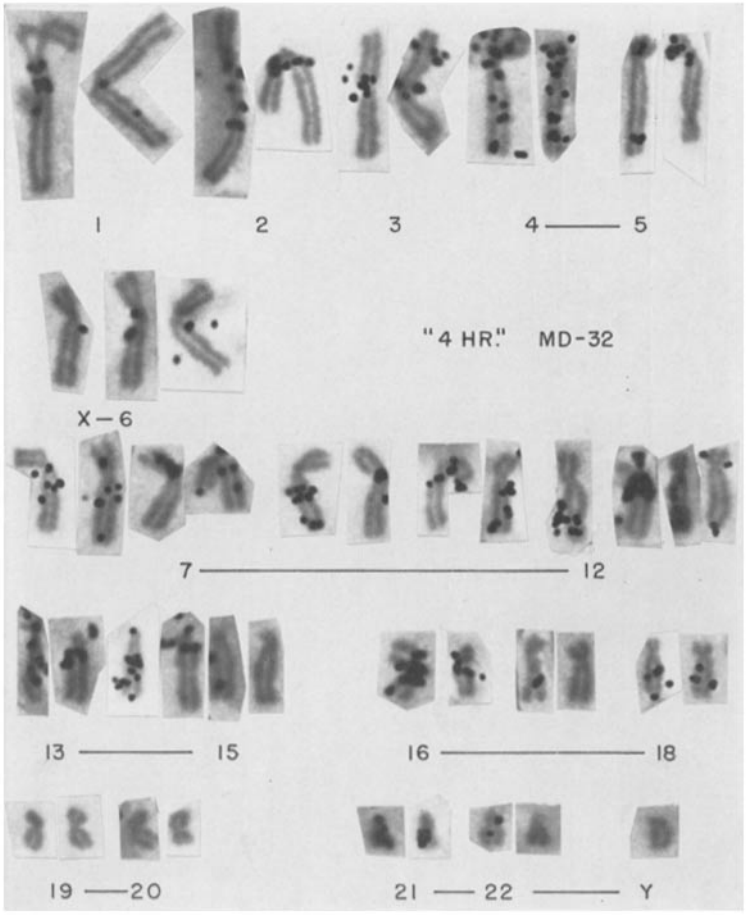

FIGURE 5

Karyotype of metaphase cell from same material (MD-32) showing late labeling in respect to the S period of centromeric regions of a No. 1 and both No. 3 chromosomes. × 1600.

exception in pairs 19 and 20. Morishima *et al.* (5) have observed late labeling by *one* pair of this group (Nos. 19 and 20). Morphologically, however, pair 19 is indistinguishable from pair 20. Other reports (4, 5) of generalizations concerning asynchrony of particular chromosomes, Nos. 21 and 17, may prove to be substantiated with more detailed studies. However, to consider the entire chromosome as a replicating entity (except for the heterochromatic X) obscures the behavior of specific sites of synthesis such as the centromeric regions of Nos. 1 and 3 demonstrated in this work.

Asynchrony of Chromosomal Regions

The pattern of label distribution for the five largest chromosomes, which were reasonably divisible into centromeric, arm, and telomeric regions, is presented in histogram form (Fig. 3, data of Table II). The centromeric regions of chromosomes 1 and 3 exhibit labeling with the same pattern; *i.e.*, both centromeres appear to have been involved in the later portion of the S period (Figs. 4 to 6). Chromosome 2 definitely showed no such regional pattern nor did chromosomes 4 and 5. These observations were confirmed in the female strain WI-25.

For chromosome 1 any conclusions are complicated by the fact that a conspicuous secondary constriction exists immediately adjacent to the primary constriction so that the microscopic localization of grains as between these two loci is not feasible. By the use of appropriate cytological techniques the appearance of secondary constrictions may be enhanced in such metaphase preparations (10). No obvious secondary constriction is observable near the centromere of No. 3, however, so that its late labeling pattern may possibly be specifically attributable to its primary constriction. Obvious secondary constrictions may be observed adjacent to the centromeres of chromosomes 1, 9, and 16 (Fig. 7); however, the identification of chromosomes 9 and 16 after development of the radiographic film was not sufficiently reliable to permit grain analysis. One entire X in female cells and most of the Y chromosomes appear negatively heteropyknotic as a result of these treatments (10). In other organisms a distinction between heterochromatin and euchromatin as to pattern of DNA synthesis has been observed (11). It will be of interest to determine whether the diffuse, puff-like secondary constrictions (Fig. 6) on chromosomes 9 and 16 do follow the pattern of late labeling such as occurs in No. 1, in the entire heterochromatic X, and possibly in the Y. The method of photographing a cell before and again after emulsion development (4) may permit reliable enough identifications of these specialized chromosomal regions.

It seems reasonable to conclude that the actual period of synthesis for any specific site, as these centromeric regions represent, is only some fraction of the entire period of DNA synthesis of 7 to 7½ hours. If all centromeric regions of chromosomes are to be regarded as heterochromatic, our results would preclude any generalization with respect to asynchrony and heterochromatin since centromeric regions in general do not follow the late labeling pattern of those of Nos. 1 and 3.

SUMMARY

With the use of pulse exposures to a medium containing H^3-thymidine, observations were made of chromosomal replication in diploid human cells of fibroblastic type in long term cultivation. A generation time for these cells was computed to be 18 hours and the S period to be 7 to 7½ hours. A portion (20 to 25 per cent) of the population was not engaged in replication. Unequivocal late labeling of the presumed X was observed in a female strain. No other entire chromosome showed a similar obviously disparate distribution of label. It is suggested that members of pairs 19 and 20 are early labeling; however, this was not confirmed in a second pulse experiment. Over-all grain counts for entire chromosomes 1, 2, 3, 4, and 5 at different times during the S period did not reveal significant differences. Considerations of specific subdivisions of these longest chromosomes did reveal definite late labeling for the centromeric regions of chromosomes Nos. 1 and 3 but not for Nos. 2, 4, and 5.

This work was supported in part by United States Public Health Service research grant C-4534 from the National Cancer Institute.

Dr. Defendi is a Leukemia Society Scholar.

Received for publication, August 4, 1962.

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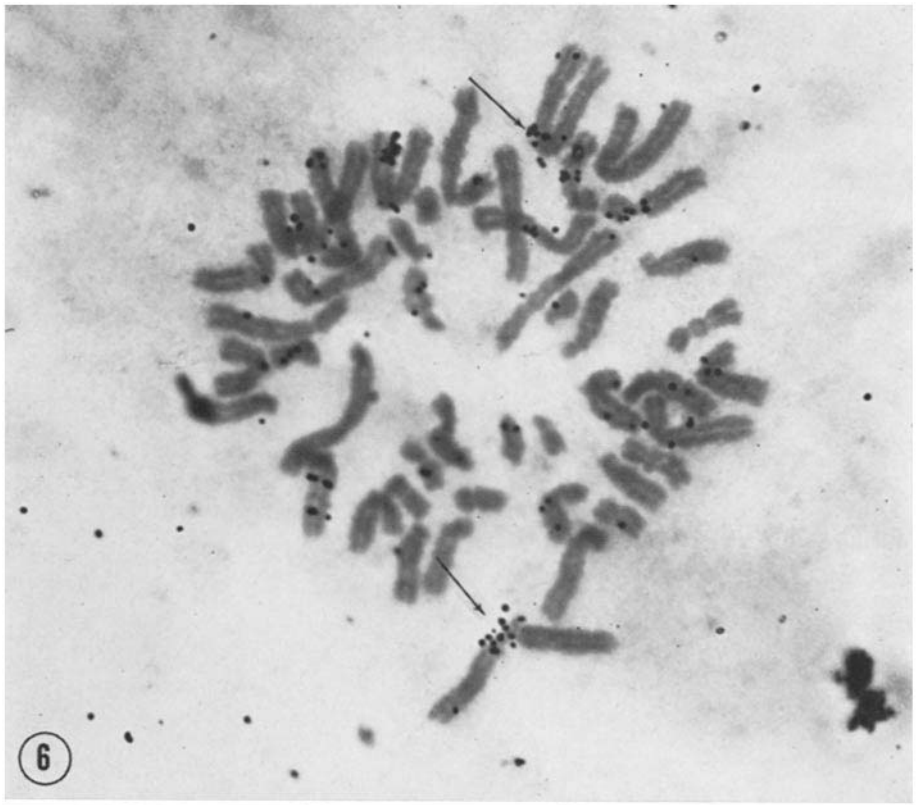
FIGURE 6

Metaphase cell (MD-32) taken 6 hours following pulse treatment showing concentration of label over centromeric regions of both No. 1 homologues (arrows). × 1250.

FIGURE 7

Example of appearance of puff-like secondary constrictions induced by flaming technique for drying of preparation. The constrictions appear as diffuse, pale regions (arrows). No emulsion has been applied. Note the two longest (No. 1) chromosomes, a medium metacentric which is No. 9, and a small metacentric which is No. 16 (arrows). × 1200.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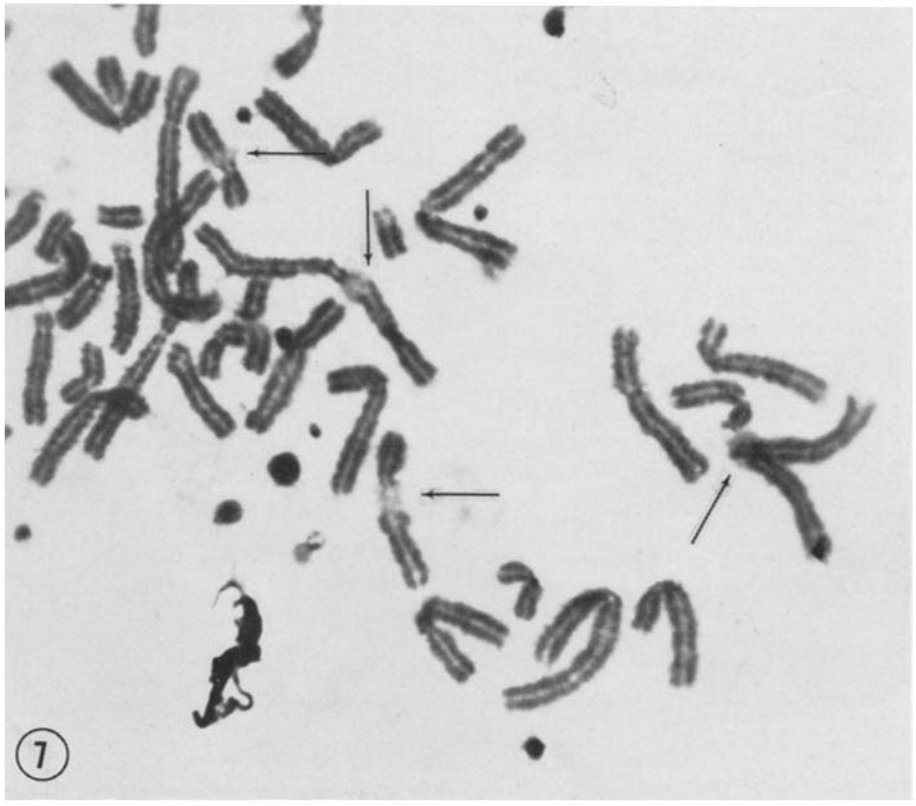
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