ULTRASTRUCTURAL STUDIES OF RADIATION-INDUCED CHROMOSOME DAMAGE

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ABSTRACT

The fine structure of radiation-induced chromosomal aberrations in *Potorous tridactylis* (rat kangaroo) cells was examined in situ by electron microscopy. The observations on the structure of terminal deletions (acentric fragments), anaphase bridges and "gaps," sidearm bridges, and specialized regions, such as the nucleolus organizer, are discussed in detail. Conclusions based on these observations are the following: (*a*) damage is physically expressed only at anaphase; (*b*) a gap region is composed of two subunits, each of which is about 800–1000 A in diameter and may correspond to a half-chromatid structure; (*c*) the ends of acentric fragments are structurally similar to normal chromosome ends, except where the break occurs in a specific region such as the secondary constriction; (*d*) at metaphase the fragment and the main portion of the chromosome move as a single unit to the equator, and the two units are disconnected only at the onset of anaphase; (*e*) sidearm bridges appear to be exchanges, involving a subchromatid unit. The interpretation of this evidence is consistent with the hypothesis that the chromosome is a multistranded structure.

INTRODUCTION

The exposure of plant and animal cells to ionizing radiation results in damage to the chromosomes, which can be detected and quantitated at the light microscope level (8, 18, 21). A closer examination of the fine structure of radiation-induced aberrations in situ with the electron microscope may influence the interpretation of the quantitative data thus far obtained and provide better insight into the structure and organization of the metazoan chromosomes during mitosis.

Recently the development of an embedding procedure for cells in vitro (4) has made it possible to locate radiation-damaged chromosomes with a phase-contrast microscope and further examine the same chromosomes with an electron microscope. Cultured cells of the rat kangaroo were used in this study because of their large size and relatively low chromosome number ($2N = 13$, ♂, and $2N = 12$, ♀).

MATERIALS AND METHODS

Cell Line and Growth Procedure

Cells derived from kidney of the rat kangaroo were used. The cell line PtK_2 (American Type Culture Collection No. CCL) was grown as a monolayer on a modified McCoy's 5a medium supplemented with 20% fetal calf serum. For the experiments, 1×10^6 cells were seeded into plastic 30-mm Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) and incubated overnight at 37°C. The dishes were removed from the incubator and exposed to X-rays. One set received 250 rad, and another set received 500 rad. Immediately following irradiation the dishes were returned to the incubator. A single dish was fixed at half-hour intervals for a total of 8 hr. Control cultures were treated in exactly the same way, except that they were not irradiated.

Irradiation Procedure

The cell monolayers in plastic Petri dishes were exposed to X-rays produced by a General Electric Maxitron X-ray unit. The conditions were 250 kev (peak), 15 ma, filtered with 0.5 mm Cu and 1.0 mm Al, giving a half-value layer of 1.26 mm Cu. The dose rate was 180 rad/min.

Electron Microscopy

Cell monolayers were fixed and embedded in situ by the method of Brinkley et al. (4). Briefly, this method consists of fixation at room temperature for 1 hr in 3% Millonig's phosphate-buffered glutaraldehyde followed by a buffer rinse and postfixation in 1% OsO_4. The monolayers were washed in distilled water and treated with 2% aqueous uranyl acetate for 20 min. Alcohol dehydration was accomplished, at room temperature, up to 90% ethanol. The cells were then further dehydrated in graded steps of hydroxypropyl methacrylate (HPMA) and embedded by covering the monolayer with a thin layer of Epon 812 mixture. After polymerization at 60°C for 48 hr, the Petri dishes were frozen in liquid nitrogen, permitting easy separation of the plastic dish from the Epon. The Epon layer was viewed cell-side-up with a phase-contrast microscope. A drop of immersion oil placed over the cells permitted observation with a 97 × immersion phase-contrast objective. Many flattened mitotic cells displaying all or most of the 13 chromosomes were observed. Cells with detectable chromosome damage were photographed, and the area of damage was cut out of the Epon sheet with a cork borer. The Epon disc was mounted cell-side-up on an Epon capsule for sectioning. Sections were cut with a diamond knife on a LKB ultratome III. Silver-to-gray sections were picked up on parlodion-coated slotted grids. With care, up to 15 serial sections could be collected on a single slotted grid.

Unstained or uranyl acetate-lead citrate–stained sections were viewed at 75 kV on an Hitachi HU-11C electron microscope.

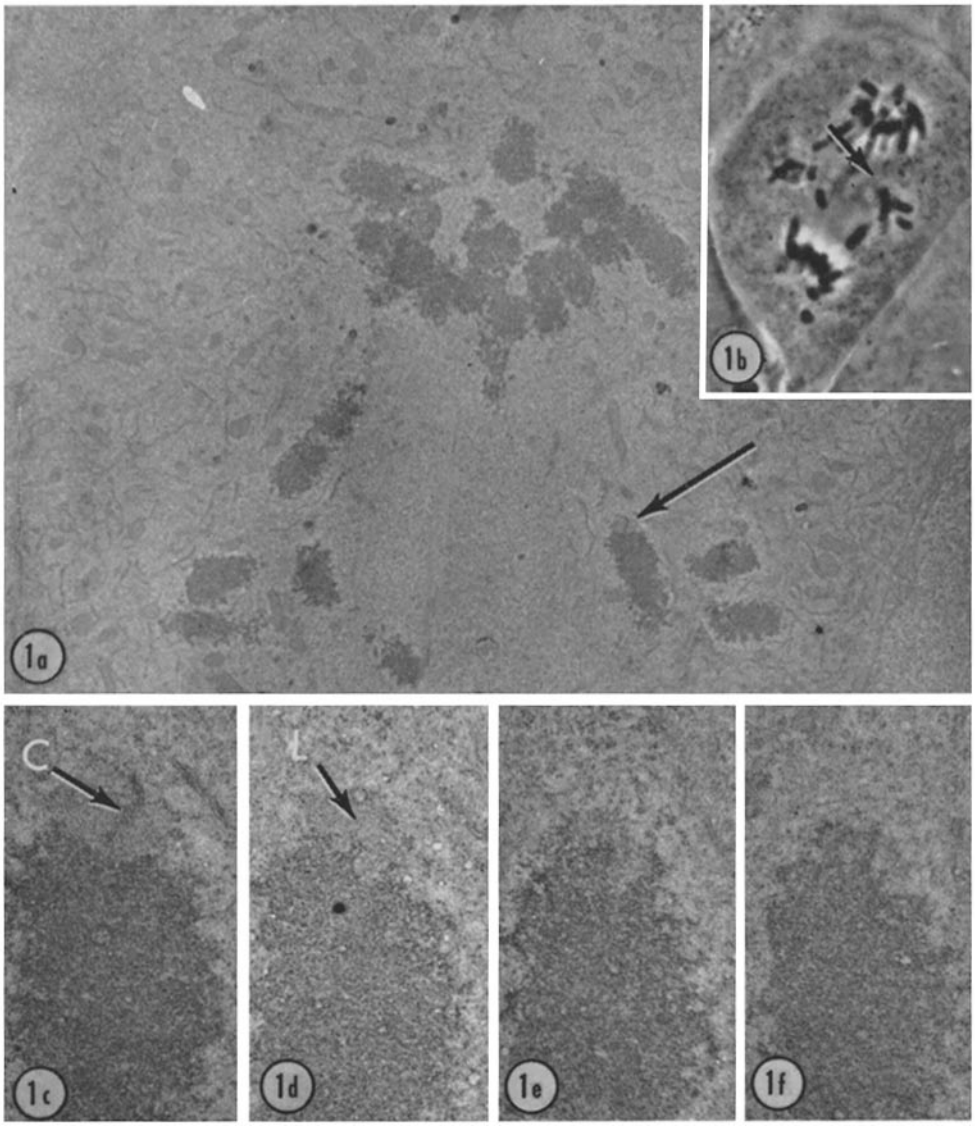
RESULTS AND DISCUSSION

The method just described has permitted the study of only a limited number of aberrations from the complete spectrum of chromosomal anomalies induced by ionizing radiation. Since we examined cell monolayers in situ, it was difficult, if not impossible, to detect aberrations such as translocations, inversions, or interstitial deletions. For this reason, our observations have been limited to the following anomalies.

Terminal Deletions (Acentric Fragments)

Anaphase cells examined 6–8 hr following irradiation with either 250 or 500 rad exhibited numerous chromosomal fragments near the cell equator. These broken segments were assumed to be acentric fragments resulting from chromosome and chromatid breaks and terminal deletions. Fig. 1 shows an anaphase cell with numerous acentric fragments still near the metaphase plate. The broken ends appear to be structurally identical with normal chromosome ends, except where the breaks occur in a specific region such as the secondary constriction. The latter has a distinct ultrastructural morphology, which has been described in a previous study (12) (Fig. 6). The arrows in Figs. 1 *a* and *b* point to a chromosomal fragment showing a portion of the secondary constriction on the long arm of the X chromosome. The electron-opaque axial core (Fig. 1 *c*) of the nucleolar organizer (12, 6; see Figs. 5, 6) can be seen in the electron micrographs. Serial sections through the same chromosome show the axial core in only one section. The less-dense fibrous material (*L*) which surrounds the core can be seen in each of the four serial sections (Figs. 1 *c–f*).

The axial core consists of a bundle of 50–80-A fibrils. The less-dense fibrous elements were of similar dimensions. It was particularly evident in electron micrographs like those shown in Figs. 1 *a* and *b* that acentric fragments arrived on the metaphase plate during prometaphase but failed to move to the poles in anaphase. It is generally believed that acentric fragments fail to move in a directed manner in anaphase because of the absence of a kinetochore. In our study numerous fragments were examined; all were truly acentric and lacked an associated kinetochore or spindle tubule of any kind. Since prometaphase movement and alignment (metakinesis) apparently required kinetochore function, it is difficult to explain how fragments lacking a kinetochore arrive at the equator. It is possible that a physical connection exists between the fragment and the rest of the chromosome, such that they move as in a single unit during prometaphase. Thus, complete severage probably occurs at the end of metaphase or at the onset of anaphase. We have not been able to detect breaks in prophase, which supports our contention that a break is physically expressed only at anaphase in cells fixed in situ. Spreading of chromosomes by squashing, smearing, or air drying might permit the break to be seen at or before metaphase.



FIGURES 1 *a–f* Acentric fragments in anaphase cell fixed 6 hr after irradiation with 250 rad. Fig. 1 *a* is a survey micrograph showing fragment of X chromosome (arrow) broken through the nucleolus organizer. × 7,400. Fig. 1 *b* is a phase-contrast micrograph of the same cell as in 1 *a*. Arrow points to X chromosome fragment. × 1,400. Figs. 1 *c–f* are four serial sections through the X chromosome fragment seen in 1 *a* and *b*. The dense axial core (*C*) is clearly seen in 1 *c*. Subsequent sections (1 *d–f*) show the less-dense fibrous component (*L*) of the nucleolus organizer. Compare with intact chromosome shown in Figs. 5–7. × 30,000.

Anaphase Bridges and "Gaps"

When daughter chromatids separate at anaphase a structural exchange either between the two daughters (intrachange) or between daughters of two different chromosomes (interchange) will usually result in the formation of an anaphase bridge. These anaphase bridges may be of three types.

(*a*) The first type are "false bridges" or a "stickiness" due to adherence of surface material, such as ribosomes or persistent nucleoli (11).

(*b*) The second type are *chromosome bridges* which result from sister chromatid exchanges due to isolocus breaks and terminal deletions. The kinetochore-bearing halves rejoin, and when they move to opposite poles in anaphase a bridge results. The terminal fragments will be acentric and are usually lost from the daughter nuclei. A similar bridge may form due to an exchange between the kinetochore-bearing arms of two different chromosomes.

(*c*) The last type, *sidearm bridges*, are reported to involve an exchange of fibers at the subchromatid level (8).

Subchromatid exchanges have been the subject of considerable controversy, and some investigators believe sidearm bridges to be "pseudochiasmata" or "masked" chromatid exchanges (17). The phenomenon can be produced by irradiating cells in late G_2 (post-DNA synthesis period) or the beginning of prophase in either mitosis or meiosis.

Fig. 2 *b* shows a phase-contrast micrograph of an anaphase bridge in a rat kangaroo cell fixed 8 hr after a dose of 250 rad; arrows *1* and *2* indicate gaps or "achromatic" areas in the bridge. An electron micrograph of the same cell is shown in Fig. 2 *a*; arrows *1* and *2* point to the same gaps seen in Fig. 2 *b*. When the gap indicated by arrow *1* was examined under higher magnification, fibrils of varying thickness and staining capacity were observed (Fig. 3). The finest fibrils resolvable in our preparations measured 50–80 A in diameter (*A* fibrils), the next thickest fibrils measured 200–250 A (*B* fibrils), and the still coarser fibrils measured 500–600 A in diameter (*C* fibrils). The fibrils of the last category were similar to those of the normal chromosome arms.

Further stretching of the chromosomal bridge produces even more attenuated gaps like that indicated by arrow *2* in Figs. 4 *a*, *b*. A higher magnification of the same region is shown in Fig. 4 *c*. As in Fig. 2 *b*, the stretched areas frequently appeared bipartite, consisting of two morphologically similar units. In one case, this was confirmed by serial sections. Each unit in Figs. 4 *b* and *c* measured 800–1000 A in diameter. Much coarser units were seen in chromosomes that were less attenuated. The double nature of anaphase chromosomes has been widely reported in the light microscope literature (1, 2, 14), but the preparations shown here are, to our knowledge, the only examples of doubleness that have been observed by direct means with the electron microscope. Whether each unit could correspond to a half-chromatid must await further investigation. The most convincing evidence that chromatids are double comes from the study of sidearm bridges (8).

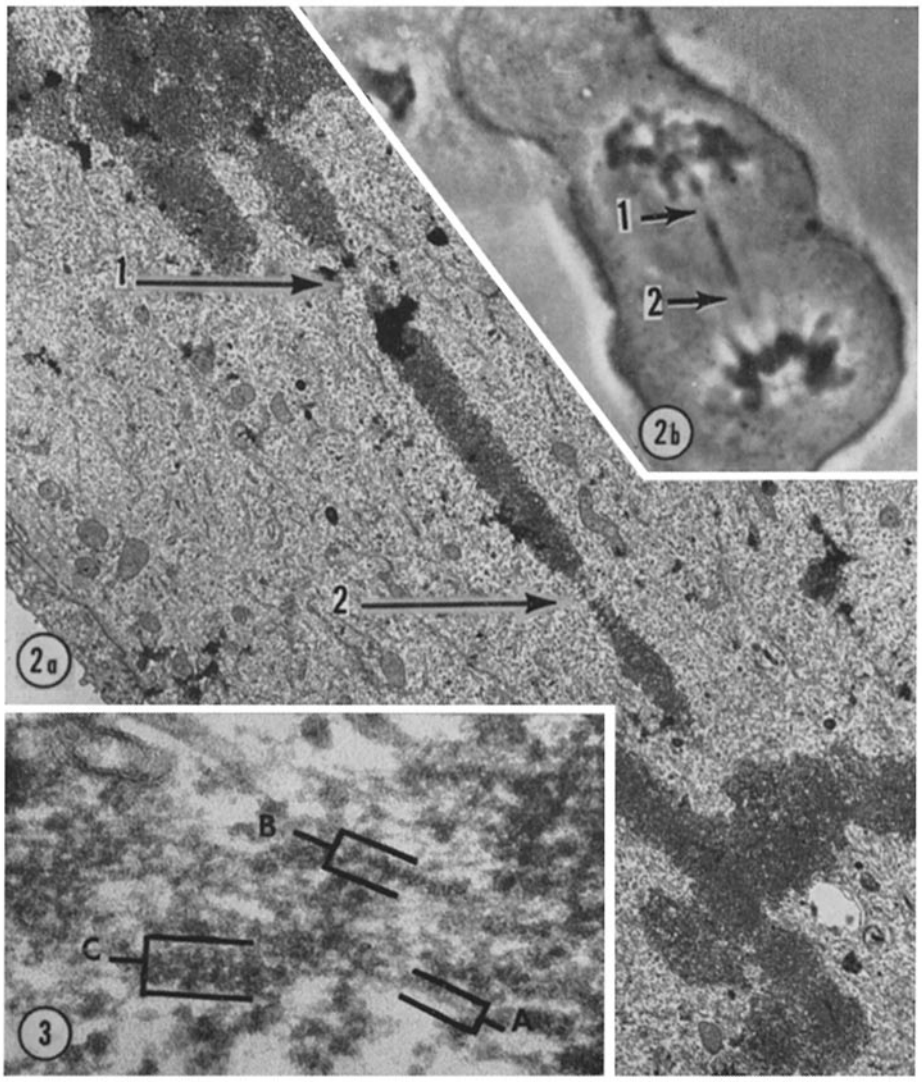
Sidearm Bridges

The ultrastructure of half-chromatid bridges or of sidearm bridges has recently been investigated in our laboratory and is the subject of a separate article (3).

Heddle and Bodycote (10) have reported that rat kangaroo cells irradiated at the beginning of mmitosis exhibited a high frequency of sidear bridges, as revealed by light microscopy. In our experiments, these aberrations were readily detectable at anaphase because of the fact that separating daughter chromosome arms appeared connected by an invisible strand. Frequently, the "connection" was several microns away from the end of the arms, such that the arms were abruptly bent at the site of an invisible bridge. When the same cells were serially sectioned and examined with the electron microscope, a fiber approximately one-half the diameter of the chromosome arm was evident in every case. These observations lend morphological support to the theory that sidearm bridges are, in fact, exchanged, involving a subchromatid unit. We interpret these observations to be consistent with a bi- or multineme chromosome model. Alternate interpretations, however, are not entirely ruled out by this study. Thus, sidearm bridges could form if each chromatid consisted of a single folded-fiber, as was proposed by DuPraw (7). Such an interpretation appears unlikely when the data concerning chromosome replication following the induction of sidearm bridges is considered (see discussion by Brinkley and Humphrey, reference 3).

Damage to Specialized Regions

The discovery of acentric fragments and broken chromosome arms involving a portion of the nucleolus organizer (secondary constriction) has already been mentioned. In some cells, like the one shown in Fig. 5, the secondary constriction region was greatly attenuated as the sister X chromosomes were drawn to opposite poles. This cell was fixed 30 min following treatment with 250 rad of irradiation. The contralateral attenuation of both secondary constrictions is readily apparent.

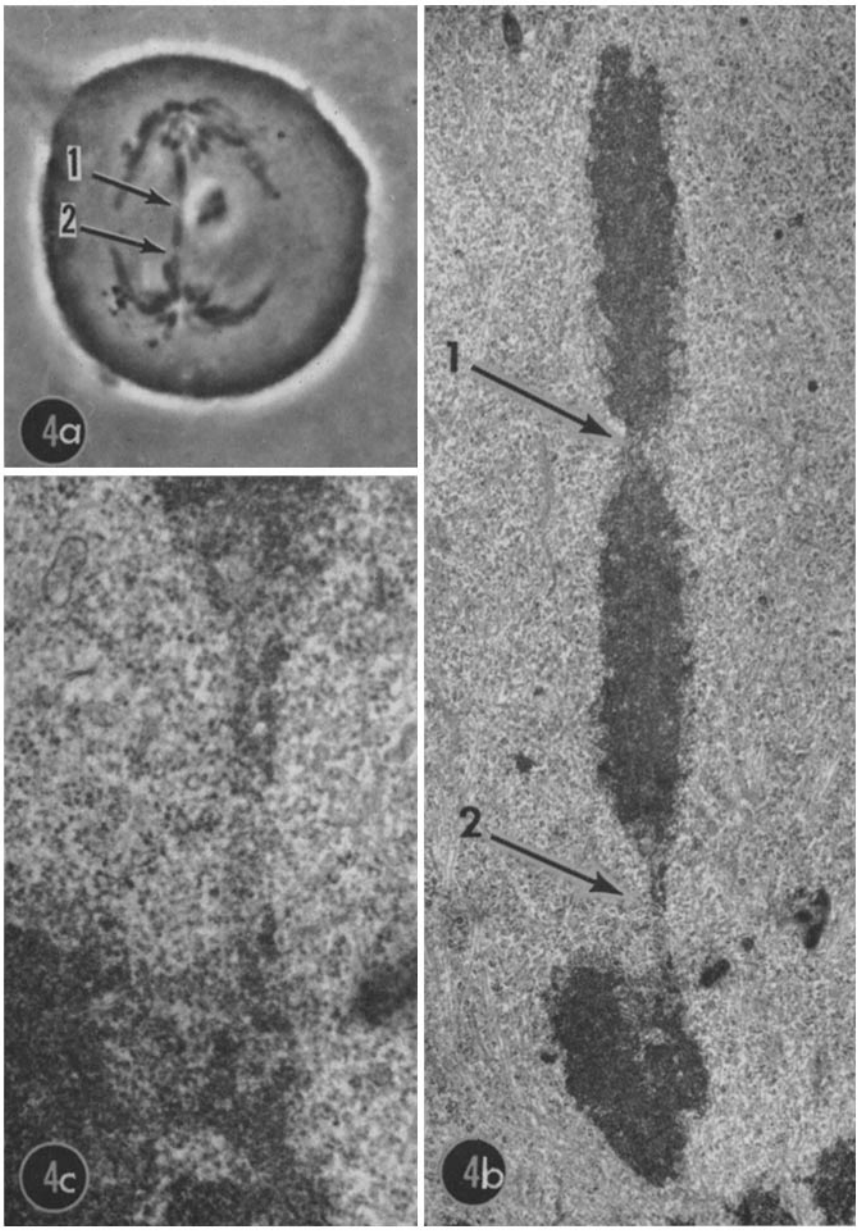


FIGURES 2 *a* and *b* Phase-contrast and electron micrographs of an anaphase chromosome bridge in a cell fixed 8 hr following irradiation with 500 rad. Fig. 2 *a* is an electron micrograph showing two gaps or achromatic regions (arrows *1* and *2*). × 9,000. Fig. 2 *b* is a phase-contrast micrograph of the same anaphase cell as in 2 *a*. The gaps appear as unstained regions in the chromosome (arrows *1* and *2*). × 1,600.

FIGURE 3 High magnification of the gap indicated by arrow *2* in Figs. 2 *a* and *b*. The *A* fibrils measured 50–80 A, *B* fibrils 200–250 A, and *C* fibrils 500–600 A. × 137,000.

Fig. 6, a higher magnification of the lower chromosome in Fig. 5, shows the details of the axial core (*C*) and fibrous material (*L*) of the secondary constriction. Since this cell was irradiated in G_2 or in the beginning of prophase, it is likely that the secondary constrictions are being stretched by a minute bridge (sidearm) that was too small to be detected either by light microscopy or by electron microscopy thin-section analysis. For comparison, a nonirradiated anaphase chromosome bearing a secondary constriction is shown in Fig. 7.

Specialized chromosome regions, such as the



FIGURES 4 *a–c* Anaphase bridge in cell fixed 6 hr after irradiation with 250 rad. Fig. 4 *a* is a phase-contrast micrograph showing two achromatic gaps (arrows *1* and *2*). × 1,400. Fig. 4 *b* is an electron micrograph of the bridge shown in 4 *a*. Gaps appear as stretched regions. The chromosome appears double in the gap indicated by arrow *2*. × 20,000. Fig. 4 *c* is a higher magnification of the area indicated by arrow *2* in Figs. 4 *a* and *b*. Note the doubled appearance of the fibers passing through this region. × 70,000.

nucleolar organizers (secondary constrictions) and centromeres, are generally susceptible to chromosome-breaking agents other than X-rays. Viruses such as SV_{40} (16), polyoma (22), and herpes simplex (9, 20) are reported to induce a high frequency of chromatid and chromosome breaks in the secondary constrictions. Chemicals such as 5-bromodeoxyuridine (BUdR) (13) and hydroxyl-

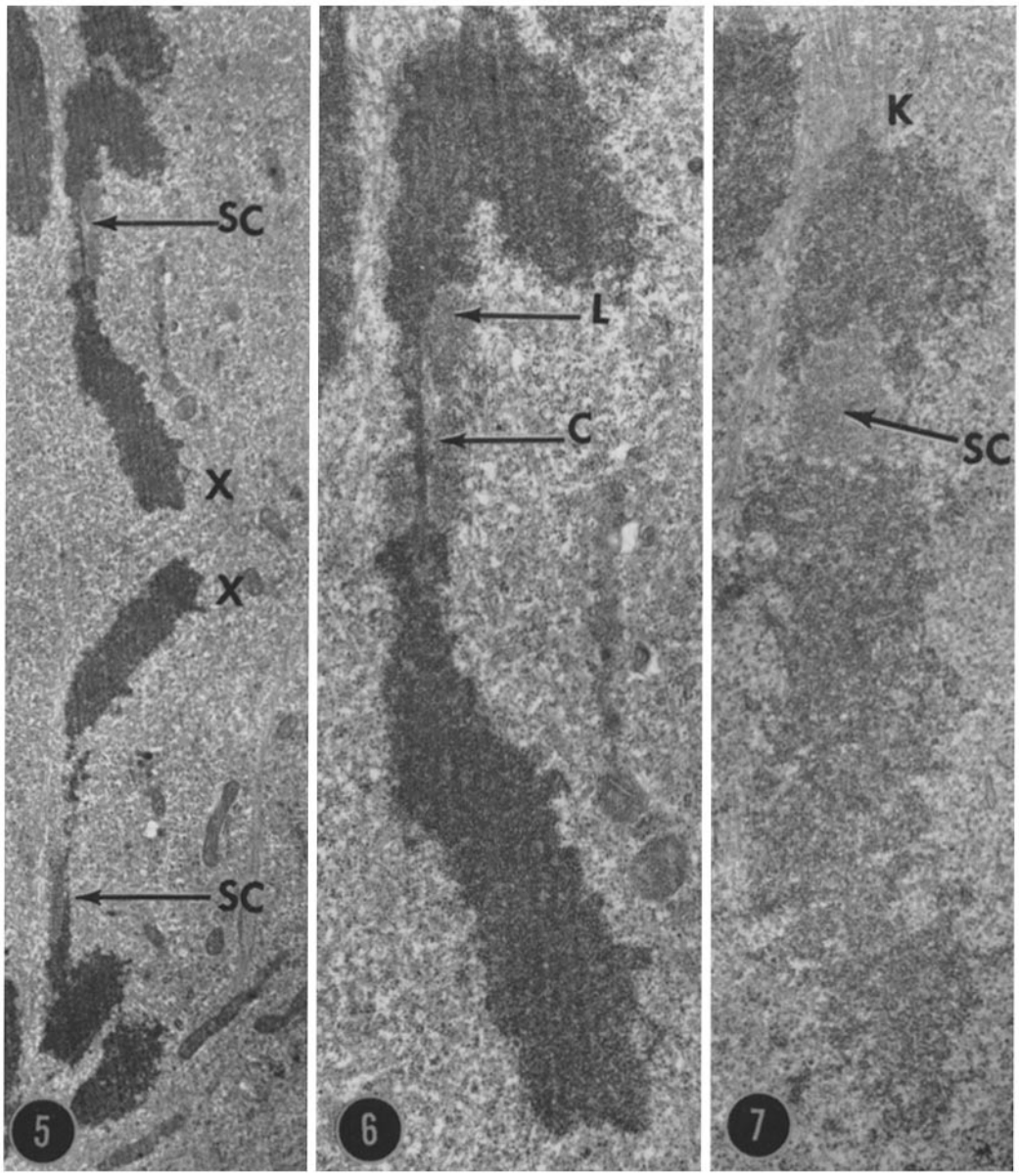


FIGURE 5 Sister X chromosomes being pulled to opposite poles in anaphase. Both secondary constrictions (*SC*) appear to be greatly attenuated. × 12,000.

FIGURE 6 Higher magnification of one of the chromosomes in Fig. 5. The axial core (*C*) and the less-dense fibrous material (*L*) are particularly evident. × 25,000.

FIGURE 7 Comparable secondary constriction (*SC*) of X chromosome in *unirradiated* anaphase cell. Kinetochore (*K*). × 26,000.

amine (19) are reported to induce damage to both the secondary constriction and the centromere.

Previous electron microscope investigations (5, 12) have shown the nucleolus organizer and centromere to be structurally decondensed regions of the chromosome. It is reasonable, therefore, that partial or complete damage to decondensed regions may be more likely to produce a detectable aberration than damage to a condensed region of the chromosome.

Also, it is interesting to note that a break through the nucleolus organizer (Figs. 1 *a–f*) does not greatly alter the fine structure of that region. That is, although the break occurs in the organizer region, both the dense axial core and the less-dense fibrous material are still present in each fragment.

Similar observations have also been made of breaks through the secondary constriction induced by mitomycin C (B. R. Brinkley and M. Shaw, data in preparation). The experiments of McClintock (15) have provided evidence that a partially deleted nucleolus organizer induced by X-rays is also capable of continued synthesis of nucleolar material.

The authors would like to thank Mr. Joiner Cartwright, Mrs. B. A. Sedita, and Miss Patricia Murphy for technical assistance.

This work was supported in part by the United States Public Health Service grants Nos. CA-04484 and GM-15887.

Received for publication 25 February 1969, and in revised form 29 April 1969.

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