MITOTIC BEHAVIOR OF INDUCED CHROMOSOMAL FRAGMENTS LACKING SPINDLE ATTACHMENTS IN THE NEUROBLASTS OF THE GRASSHOPPER

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The subject of the mitotic behavior and ultimate fate of x-ray induced fragments[1] has been almost entirely overlooked, or at least neglected, by most cytologists. Usually it is dismissed with the statement that fragments will not survive mitosis. This may be due in part to the use of material unsuitable for such studies, in part to the fact that fragments behave differently in different cells and in part to the interest of the investigator in other aspects of fragmentation. At most it has been noted that fragments lag at anaphase and are often included in small accessory nuclei at telophase.

The present study is based entirely on observations of the neuroblasts of the grasshopper, *Chortophaga viridifasciata*. Embryos were irradiated with 250 r, removed from the egg at such a time that cells treated in interphase or early prophase were in their first division, smeared on glass slips, fixed and stained in aceto-carmine, and mounted in euparal.

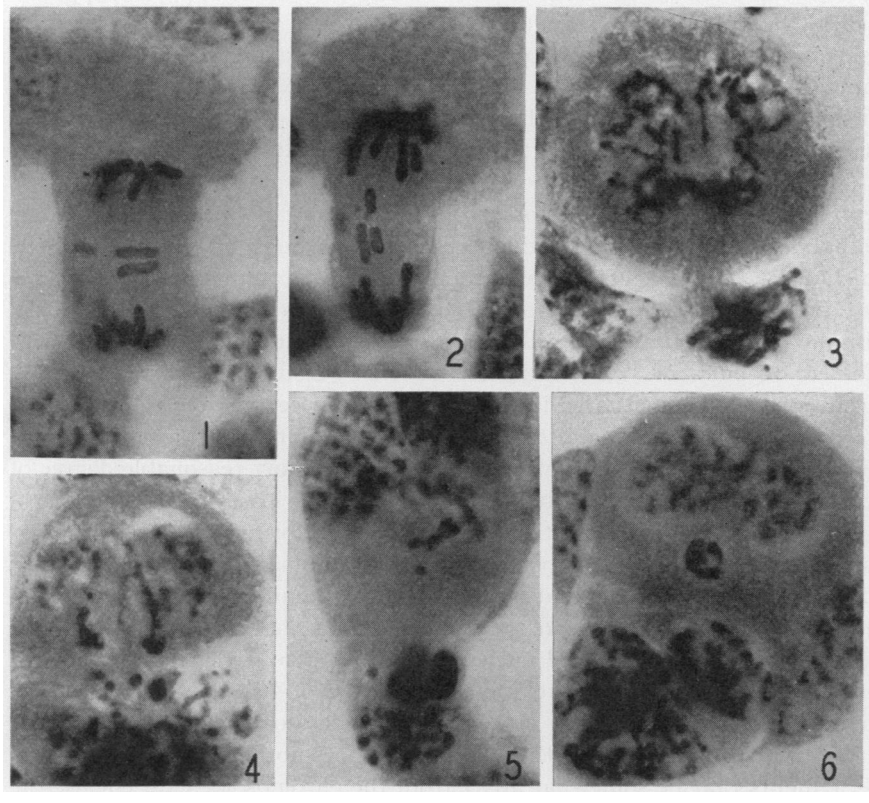
The results of this study throw light on certain factors involved in the mechanism of mitosis, because we are dealing here with chromosomal elements in which one complicating factor, namely, the spindle attachment region, is absent. Also, they indicate that the occurrence of delayed effects resulting from reattachments between chromosomes and fragments in a later mitotic cycle than that in which breakage occurred is not untenable cytologically.

Observations.—1. *Prophase.*—Fragments first become identifiable in the late prophase, when they differ from the chromosomes in that they lack the proximal heteropyknotic regions that mark the positions of spindle attachments. There is nothing in their behavior, their position in the nucleus, or their degree of condensation at this period to distinguish them from the chromosomes.

2. *Metaphase.*—The fragments lie between the cell membrane and the distal ends of the chromosomes. They are situated in the equatorial plane or at least as close to it as the distal ends of certain of the longest chromosomes, which often project slightly toward one or the other of the poles.

3. *Anaphase.*—A detailed analysis of the behavior of fragments at early anaphase appears in another paper.[2] The initial separation of their

"chromatids" begins at the same time as that of the chromosomes and gives rise to fragments of three classes: V's, rings and pairs of rods (Fig. 1). At first these are situated, as at metaphase, outside the distal ends of the



FIGURES 1–6

Fragments during and at the end of the first mitosis following their production. Dosage 250 r × 990. 1—Anaphase. Two pairs of daughter fragments. Those in focus have not yet become oriented in relation to the spindle. Of the pair out of focus at left the lower member is oriented parallel to the spindle axis. 2—Anaphase. Three pairs of daughter fragments on the spindle moving toward the poles. The upper member of the large pair at left is much nearer the pole than any of the others. 3—Telophase. The darkly staining fragments are included in the cell nucleus. 4—Telophase. One or more fragments lying partly outside the cell nucleus appear joined to chromosomes inside it. 5—Telophase. Accessory nuclei in neuroblast and ganglion cell, probably containing sister fragments. 6—Interphase. Neuroblast with accessory nucleus containing dark staining fragments.

chromosomes and therefore a considerable distance from the spindle. At late anaphase, however, after the daughter chromosomes have become well

separated with an accompanying elongation of the cell, the fragments move inward between their distal ends, eventually coming in contact with the spindle. Once this occurs their "chromatids" gradually become oriented in a line with the spindle axis and begin to move toward opposite poles (Fig. 2). If the "chromatids" open out as a pair of rods, separation is complete from the first, and the daughter fragments can move unhindered toward the poles. If the fragment is V- or ring-shaped, with fusion at one or both ends of the fragment, respectively, this union may persist for a time, so that the fragment lags at the equator for a varying period.

4. *Telophase.*—The position of the cleavage plane that divides the cytosome of the original neuroblast into daughter neuroblast and daughter ganglion cell determines in which cell a given daughter fragment will be included. Unless a great many fragments with complex fusion patterns are present, sister fragments usually come to lie in different cells (Fig. 5). In one embryo studied 17 cells in late anaphase and early telophase contained 81 pairs of "chromatid" fragments. Sister "chromatids" of 51 of these pairs were situated in different daughter cells, sister "chromatids" of 8 lay in the same daughter cell, while the apportionment of the "chromatids" of 22 pairs could not be determined with any reasonable degree of certainty. Coincident with cytokinesis is the loss of stainability of chromosomes and fragments and the gradual formation of clear areas about them, the extent of which mark the limits of the future nucleus. This occurs in the fragments somewhat later than in the chromosomes. Fragments that are close to the distal ends of the chromosomes are included with them in the cell nucleus (Fig. 3). The conclusion of Mather[3] in his studies of the post-meiotic resting stage of *Tradescantia*, *Eremurus* and *Allium*, therefore, that the absence of accessory nuclei is proof that irradiation took place after the last division does not apply to my material. Fragments that lie somewhat apart from the chromosomes are enclosed in small accessory nuclei (Fig. 5). In one embryo containing 52 neuroblasts in middle and late telophase the fragments are contained in 59 accessory nuclei and in 10 of the cell nuclei. Thirty-seven per cent of the former and 40% of the latter exhibit a stainability comparable to that of normal chromosomes, while the remaining 63 and 60%, respectively, are pyknotic, resembling in this respect the more deeply staining chromosomes of the ganglion cell nucleus (Fig. 6). There are also intermediate types in which one end of the fragment lies out in a small accessory nucleus, while the other end lies within the cell nucleus and appears to have become joined to the distal end of a chromosome (Fig. 4). Of the 59 accessory nuclei and 10 cell nuclei referred to above, 30% of the former and 60% of the latter show connections between fragments and chromosomes. This suggests delayed attachment.

5. *Interphase.*—Fragments persist throughout the interphase as dark staining elements contained within either accessory nuclei (Fig. 6) or cell nuclei, depending on their final location at late telophase.

Fragments and the Mechanism of Mitosis.—Any hypotheses bearing on the mechanism of mitosis must not overlook certain parallels that are manifest in the behavior of chromosomes, which possess spindle attachments, and fragments, which lack them. The facts must not be disregarded that (1) the fragments lie in the equatorial plane at metaphase, (2) their "chromatids" begin to separate at anaphase at the same time as do those of the chromosomes, (3) their "chromatids" come into intimate contact with the spindle at middle anaphase, and (4) sister "chromatid" fragments usually move toward opposite poles behind the chromosomes and so are included at telophase in different daughter cells.

(1) Many cytologists who have studied the mechanism of mitosis have been inclined to view the movement of the chromosomes into the equatorial plane at the end of the prophase as a force of some kind exerted by the spindle—in conjunction with the poles—on the chromosome through its spindle attachment. The regularity with which fragments, which lack spindle attachments and have no contact with the spindle, come to lie in the equatorial plane at metaphase, while it does not disprove the existence of an influence exerted by the spindle through the spindle attachment of the chromosome, nevertheless does demonstrate that other factors may be involved. To account for the orientation of the chromosomes in the equatorial plane at metaphase, Lillie[4] developed the hypothesis that the poles and chromosomes are electronegative, while the mid-region of the spindle is electropositive, the metaphase orientation resulting from the equilibrium established between these repelling and attracting forces. Darlington[5] holds that "the arrangement on the metaphase plate must be due to repulsion from the poles acting on the centromeres." Repelling forces, whatever their nature, between poles and chromosomes and between chromosomes *inter se* seem to offer the most reasonable explanation of the metaphase location of fragments. Forces must not be limited, however, to an action through the spindle and spindle attachments, since the fragments lie outside the spindle and lack spindle attachments. Their location in the peripheral part of the metaphase plate outside the other chromosomes suggests polar and interchromosomal repulsions, while their failure to lie within the spindle is doubtless due to the absence of spindle attachments. During cytokinesis vortical currents of the protoplasm are known to pass from the poles to the equatorial plane near the cell periphery, inward at the equator and poleward along the spindle. Currents at metaphase in the outer region of the cell moving toward the equator might carry fragments to the equatorial region, where they would

be held in the slower moving or stationary protoplasm among the distal ends of the chromosomes and between them and the cell membrane. It is a question, however, to what extent currents are present at metaphase. Bělař[6] stated that in the grasshopper spermatocyte they usually begin at early anaphase, though sometimes earlier or later than this. I have no evidence regarding this in the grasshopper neuroblast.

(2) Bělař,[6,7] Bleier,[8] Schaede[9] and Schrader[10] have come to the conclusion, from observations of a variety of material, that at least the initial separation of chromosomes is autonomous. The separation of the fragments, which occurs simultaneously with that of the chromosomes, demonstrates conclusively that, at least in these neuroblasts, the initial separation of "chromatids" can occur even though a spindle attachment region and a connection with the spindle are absent. This indicates, then, unless one assumes that other outer forces in the cell are the effective factors, that the forces causing chromatid separation reside within the chromosome itself, and so the act is autonomous. This evidence is not in accord with the views of Mather and Stone,[11] Darlington,[5] Upcott[12] and others, who hold that the anaphase separation of chromatids is invariably determined by the division and mutual repulsion of the spindle attachment bodies at the end of the metaphase.

(3) Fragments appear to be pushed against the spindle as a result of the decrease in equatorial diameter of the cell that accompanies its axial elongation at anaphase. This movement may be aided by protoplasmic currents passing inward at the equator. Just what connection is finally established between fragment and spindle is difficult to determine. While daughter chromosomes at anaphase have their distal ends rounded and their proximal ends pointed, as if they were continuous with a spindle fibre, both ends of daughter fragments frequently show an encircling fringe of dark-staining material, resembling, though perhaps only superficially, the ends of the anaphase meiotic chromosomes described by Schrader in *Protortonia* and by Hughes-Schrader in *Llaveia*.[13] The "Stemmkoerper" hypothesis of Bělař[6,7] seems to offer the most satisfactory explanation of such structures in fragments. When daughter fragments that have separated get among the outer fibres of a "Stemmkoerper" that is actively elongating and therefore tends to exert axial forces and lateral pressure against the sides of these elements, the edges at their ends might be pushed outward as encircling fringes. It seems improbable that any more of a connection between fragment and spindle exists than a close contact, which is effective in altering the position or shape of the fragment only in so far as there is pressure and friction between the two.

(4) Once the fragments have come in contact with the spindle, their tendency to become oriented parallel to its axis, their final separation

and their subsequent movement away from each other in the direction of the poles, may possibly be due, at least in part, to currents passing poleward. The hypothesis proposed by Schaede[9] could account for this behavior, since, according to it, poleward moving streams of protoplasm within the spindle are assumed to carry the daughter chromosomes to the poles. Vortical currents passing from the equator along the outer sides of the spindle to the poles might have a part in the poleward movements of daughter fragments not lying entirely within the spindle. Bělař,[6] who investigated the relation of these to chromosome movement, came to the conclusion, however, that the anaphase movements of the chromosomes are entirely independent of such currents. He attributed the middle and late anaphase movements of the daughter chromosomes to the elongation of the "Stemmkoerper." The poleward movement of fragments, if it is not the result of currents, supports Bělař's hypothesis at the same time that it is at variance with hypotheses positing only a pulling action of the spindle fibres; for it seems less likely that these elements, which apparently have no true attachment to the spindle, should be pulled toward the poles by contracting spindle components than that they should be pushed along because of their contacts with an elongating "Stemmkoerper." Bleier[8] attributed the whole anaphase movement of the daughter chromosomes to repelling forces of some kind originating in the chromosomes. It is true that this could account for the anaphase movements of fragments, though it fails to explain why the daughter fragments become oriented parallel to the spindle axis and delay their poleward movement until they come in contact with the spindle. The same difficulty confronts hypotheses of anaphase movement based on the presence of attracting forces between the poles and the chromosomes.

The main difference in the behavior of chromosomes with attachments and fragments without them is that the former move toward the poles more rapidly, and their points of attachment to the spindle lie at all times in a plane at right angles to the spindle axis, while the daughter fragments move toward the poles more slowly and with less regularity, and never arrive at a point as near the poles as the normal chromosomes. The conclusion seems justifiable, therefore, that in these cells, at least, the functions of the kinetochores are primarily to make uniform the orientation of the chromosomes in the equatorial plane at metaphase and to synchronize the middle and late anaphase separation of daughter chromosomes in order to insure their equal apportionment to the daughter cells, and not to effect their initial anaphase separation.

Delayed Effects.—In another paper[2] I have demonstrated that broken ends of chromosomes possessing spindle attachments can be transmitted, through the formation and breaking of chromatin bridges, to the second

cell generation following their production. It has been shown in the present paper that daughter fragments may be included at telophase in the cell nucleus with the chromosomes. McClintock[14] has demonstrated in *Zea* that broken ends of chromosomes retain their tendency to fuse and undergo unions from generation to generation. If the same is true of fragments, all the chromosomal conditions necessary for the occurrence of delayed attachment, as postulated by Stadler,[15] are fulfilled. In the present paper cells have been described that exhibit what are probably delayed attachments between fragments and chromosomes (Fig. 4).

I have no direct evidence regarding the behavior or fate of fragments beyond the mitotic cycle in which they appear. If, failing to become attached to a chromosome during that interphase, they fail to survive the next division, delayed effects of this kind will be limited to the chromosomes of the second cell generation after irradiation. If they may persist, undergo division and be included in the cell nucleus at the end of the second division after their formation, there is no reason why they may not survive through further cell generations, with the possibility at any time of delayed attachment with chromosomes at their broken ends. Some cells show sister fragments passing into different daughter cells seven days after irradiation, and it seems likely that these are undergoing their second division after irradiation.

Helwig[16] found a sufficient number of different chromosomal alterations represented in the secondary spermatogonia of single cysts, all the cells of which are descended from a single irradiated primary spermatogonium, to conclude that "fragmentation of the chromatin does not necessarily occur at the time of irradiation, but may be delayed." Up to the present time there has been no positive demonstration of delayed fragmentation except in some relatively rare cases of the breaking of a chromatin bridge in two places, so that a fragment lags at the cell equator. In this study and another,[2] however, I have found, in the persistence from one cell generation to the next of chromosomes and fragments with broken ends, a cytological basis for the occurrence of delayed attachments. The suggestion seems justifiable, therefore, that the delayed effects observed by Helwig might be interpreted in this way.

Summary.—The mitotic behavior of x-ray induced fragments lacking spindle attachments parallels that of the unaltered chromosomes of the grasshopper neuroblast in several respects. Sister "chromatids" of fragments separate at anaphase and are usually included in different daughter cells at telophase. Not infrequently they are included in the newly formed cell nucleus. This behavior has a bearing on certain hypotheses of the mechanism of mitosis and on the question of delayed reattachments following fragmentation.

[1] Throughout this paper the term *fragment* is used to designate a portion of a chromosome resulting from one or more breaks in an original chromosome and having no spindle attachment. The term *chromosome* is applied to an original chromosome or the part of an original chromosome containing a spindle attachment.

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A TITANOTHERE FROM THE TYPE SESPE OF CALIFORNIA

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Introduction.—In previous papers,[1] published mostly in these PROCEEDINGS, have appeared some of the paleontological results of explorations conducted in the Sespe deposits of Southern California. While the field efforts south of the Santa Clara Valley, Ventura County, were rewarded by rather startling results, no small amount of irritation was felt because of failure to find fossil vertebrate remains in the Sespe at the type locality north of the Santa Clara Valley. For, as is now known, the Sespe is not of same age throughout its stratigraphic thickness or at the several localities where fossil mammals have been found in it. It is, in fact, a series of beds that range in age from at least the upper Eocene to apparently the lower Miocene. Thus it seems especially important to determine by means of vertebrate evidence the age relationships of the type Sespe on Sespe Creek to that portion of the Sespe whose age is already established south of the Santa Clara Valley.

With this problem in mind the rugged terrain north of the Ojai Valley,