flow of materials through the frequently occurring sieve elements with extremely thick walls, especially those whose lumen is almost occluded.

Summary.—Sieve elements that have reached the stage of development commonly assumed to be mature and functional show a wide range of variation in thickness of walls. Some species have more or less thin homogeneous walls, others have a distinct inner thickening—the so-called nacreous thickening—which varies from a barely perceptible layer to one that almost occludes the lumen of the cell. In the present study 45 species of a total of 142 species of dicotyledons had the nacreous layer. Thus the thickening is not an unusual feature and must be taken into account in formulating concepts of translocation.

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INFLUENCE OF THE NUCLEOLUS ON MITOSIS AS REVEALED BY ULTRAVIOLET MICROBEAM IRRADIATION

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Nucleoli are found in most plant and animal nuclei. Their morphological characteristics and cyclic changes during mitosis have been studied extensively. Although evidence is accumulating that indicates a relatively high turnover rate of ribonucleic acid (RNA) and protein in the nucleolus,[1-3] the specific role of this structure in the cell's activity is still in the realm of speculation.

Incidental observations by several workers have suggested a relation between nucleoli and mitotic activity of cells. McClintock[4] noted in maize that microspores with abnormal amounts and distributions of the nucleolar organizing body showed slower rates of nuclear division than did spores with unaltered chromosomes; unfortunately, the nucleolar effects were complicated by changes in number and sequence of genes. Philp and Huskins[5] and McLeish[6] observed that absence of nucleolar organizers in microspores and root tip cells is accompanied by lack of division of these cells. La Cour,[7] McLeish,[8] and Darlington and Haque[9] found that micronuclei in plant cells survive and undergo mitotic changes only if they contain one or more nucleolar organizers.

An excellent tool for determining whether a relation does in fact exist between the nucleolus and mitotic activity is the ultraviolet microbeam as developed by Uretz

and colleagues[10–13] for selective irradiation of cell structures. In the present study the ultraviolet microbeam was used to irradiate the nucleolus and non-nucleolar portions of the nucleus of the grasshopper neuroblast in vitro. The cells were then individually followed by microscopic observation to determine their subsequent mitotic activity. It was found that exposure of only one nucleolus to ultraviolet radiation at stages from late telophase to the middle of middle prophase usually results in immediate and permanent cessation of mitosis. This observation strongly suggests that the nucleolus is directly involved in the mitotic process.

MATERIALS AND METHODS

The neuroblast of the embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) is excellent material for the present study. It is a large cell, about 25 μ in diameter. The various stages of mitosis are highly resolvable and can be readily identified in the living neuroblast. The cell can be separated from the embryo and cultured so as to provide the good optical specimen required for the ultraviolet microbeam apparatus. In the mature living condition, the neuroblast contains two refractile, highly irregularly shaped nucleoli approximately 3 μ in diameter. The nucleoli assume this form in late telophase and keep it until late prophase, when they become smoother in outline and disappear in very late prophase. The nucleoli are formed at a subterminal position on one of the longest pairs of chromosomes and consequently lie in the apolar region of the nucleus. They have never been observed to come in contact with the nuclear membrane or to fuse, even though occasionally they are quite close to each other.

Separation of neuroblasts from 13- and 14-day-old embryos was effected by the trypsin-hyaluronidase technique of St. Amand and Tipton[14] and Roberts[15] with a few minor exceptions. Since Shaw's culture medium[16] promotes good growth of neuroblasts for several days, it was used for making the culture preparations. After 30 minutes in the enzyme solution, the embryos were rinsed with three changes (3 minutes each) of Shaw's medium minus calcium and transferred to complete Shaw's medium for dissociation with a pipette. Culture preparations were then made by spreading thinly on a fused quartz cover glass (0.18–0.20 mm. thick) a small drop of the suspension of cells and tissue fragments. The cover glass was sealed over a flat-bottomed depression slide with mineral oil and fixed in position with melted paraffin. The cultures, even those observed for several weeks, were never opened.

The microbeam apparatus has been described in detail by Uretz and Perry.[12] Use is made of a reflecting objective (0.56 N.A.) that allows one to view the specimen and simultaneously to focus onto a particular part of the cell a demagnified image of a pinhole on which there is a real image of an ultraviolet source. The source consists of a General Electric AH-6 lamp and the filter of an aqueous solution of nickel sulfate and cobalt sulfate.[17] The energy flux of biologically effective radiation per unit area was approximately 0.01 erg μ^{-2} sec $^{-1}$ at the focal point of the microbeam. The beam at this point was a little less than 3 μ in diameter. No effort was made to determine the possible influence of visible light after ultraviolet irradiation. Uniform irradiation and observation procedures were, however, followed for all cells.

Cells were irradiated and studied at room temperature (24° ± 2° C.), observa-

tions being made with either bright-field illumination or phase-contrast microscopy. A few cells were observed at 32° C., but this temperature and higher ones proved to be detrimental to the cultures. Since, in general, completely isolated cells ceased dividing several hours after being separated from the embryo, only neuroblasts in a tissue fragment (one cell thick)[18] were used.

Three cells fairly close to one another and in the same stage of division were selected: in one cell one nucleolus was irradiated; in another cell a non-nucleolar portion of the nucleus was irradiated; and the third cell served as a control. In cells selected for irradiation, both nucleoli were visible and were lying more or less at opposite sides of the nucleus (the usual condition). A cell was usually exposed to the ultraviolet microbeam for 3 seconds. A few cells, especially those in very late prophase, were exposed for 4, 6, 12, 30, or 60 seconds. Irradiated cells were observed until they reached prometaphase[19] or metaphase, or for at least 24 hours; observations on some cells that failed to divide extended for as long as 3 or 4 days.[20] It was not always possible to follow specific cells for longer than 24 hours because of increase in number of cells and their movement in the cultures. Nucleolar-irradiated cells that divided were not followed into the subsequent mitosis. If the unirradiated cell failed to divide, which was a relatively rare event, data on the irradiated cells were discarded.

Data were collected on 81 cells irradiated mainly in the middle, late, and very late prophase stages, designated "MP," "LP," and "VLP," respectively. These stages represent a period during which the nucleolus successively is in mature form, undergoes change before disappearing, and disappears (just after beginning of VLP). A few cells were irradiated in stages from late telophase through early prophase; they are herein designated as "premiddle prophase cells" (pre-MP).

On the basis of chromosome diameter, middle prophase can be subdivided into three parts, MPa, -b, and -c; and late prophase into two parts, LPa and LPb. The nucleus of a living neuroblast at the beginning of MP is uniformly filled with thin chromosome threads. By the beginning of LP, the chromosomes are much thicker, and the two chromatids of each are visible; the cross-sections of about seven chromosomes can be counted at one-fourth the circumference of an optical section of the nucleus. At VLP, the chromosomes are considerably shortened and are about the length of prometaphase chromosomes.

OBSERVATIONS AND CONCLUSIONS

"Permanent" Mitotic Inhibition.—Ultraviolet microbeam irradiation of one nucleolus for 3 seconds is sufficient to prevent further division of practically all the neuroblasts irradiated in stages from late telophase through MPa and of about half the cells irradiated in MPb and MPc (Table 1). Since non-nucleolar irradiation of any mitotic stage usually does not prevent division (Table 1), failure of nucleolar-irradiated cells to divide can be attributed to an effect on the nucleolus even though less intense parts of the beam are obviously passing through some of the nucleus and cytoplasm. Irradiation of one or both nucleoli in LP and VLP cells does not usually prevent division of the cell.

These data can best be interpreted to mean that (1) in stages from late telophase through MPa, the nucleolus is directly involved in the mitotic process—this period represents about 58 per cent of the total mitotic cycle, including interphase; (2)

there is a transition period during MPb and MPc in which the nucleolus is losing its ability to influence mitosis—thus some of the cells irradiated in this period divide and some do not; and (3) by LP, the nucleolus is no longer functioning with respect to the mitotic process.

The nucleolar-irradiated cells that fail to divide do not "die" immediately.[21] In some experiments, cells were observed for as long as 3 or 4 days, during which time the non-nucleolar-irradiated and unirradiated cells continued dividing, whereas the nucleolar-irradiated cells underwent very little change, with the occasional exception of a slight chromosome reversion (to be described).

Six cells irradiated in a non-nucleolar region at stages pre-MP and MP did not divide (Table 1). Failure of division may possibly have been caused by proximity of the microspot to the nucleolus. In four of the cells, the exact position of the microspot with respect to the nucleolus unfortunately was not recorded. In two of

TABLE 1

EFFECT OF NUCLEOLAR AND NON-NUCLEOLAR ULTRAVIOLET MICROBEAM IRRADIATION ON ABILITY OF NEUROBLASTS TO DIVIDE

MITOTIC STAGE AT IRRADIATION	NUCLEAR PART IRRADIATED* Nucleolus +	Nucleolus −	Non-nucleolus +	Non-nucleolus −	TOTAL CELLS
Premiddle prophase, pre-MP	0	4	2	1	7
Middle prophase MPa	1	5	5	1	12
Middle prophase MPb	3	3	5	3	14
Middle prophase MPc	3	4	4	1	12
Late prophase LPa	5	1	6	0	12
Late prophase LPb	5	0	7	0	12
Very late prophase, VLP	1	0	11	0	12
Total cells irradiated					81

* Cells exposed for 3 seconds except for several very late prophase cells (see text). Plus sign indicates cells that divided; minus sign indicates cells that did not divide.

TABLE 2

EFFECT OF ULTRAVIOLET MICROBEAM IRRADIATION ON MITOTIC RATE OF MIDDLE AND LATE PROPHASE CELLS*

MITOTIC STAGES	AVERAGE TIME, WITH STANDARD ERROR, TO REACH MID-MITOSIS (24° ± 2° C.) Unirradiated	Irradiated: Nucleolus	Irradiated: Non-nucleolus
Middle prophase	3.1 ± 0.6 hr. (16)	18.0 ± 1.4 hr. (4)	16.2 ± 0.7 hr.† (11)
Late prophase	34 ± 3 min. (13)	38 ± 6 min. (7)	50 ± 5 min. (6)

* Numbers of cells in parentheses. Data taken only from cells observed to reach mid-mitosis or from those for which this time could be fairly accurately estimated.
† Two cells not included in this calculation reached mid-mitosis 30 and 48 hours after irradiation.

the cells (MPb), however, it is definitely known that the microbeam was focused in such a way that the edge of the microspot was about 3 μ from one nucleolus. This observation suggests the diffusion of a radiation-produced substance from the irradiated region to the nucleolus, or the presence of a nucleolar sensitive region that extends beyond the visible boundaries of the nucleolus itself, or scattering of radiation from the microbeam. Further experiments are needed to resolve this point.

Temporary Mitotic Inhibition.—Cells that were nucleolar-irradiated during their transition period of sensitivity (MPb and MPc) and that divided were greatly retarded in reaching mid-mitosis (nuclear membrane breakdown), as were MP cells irradiated in non-nucleolar regions (Table 2). Late prophase cells nucleolar-irra-

diated showed no mitotic delay, but those irradiated in non-nucleolar regions did exhibit some delay that statistical analysis[22] suggests is a real retardation. Cells nucleolar- or non-nucleolar-irradiated at very late prophase for as long as 30–60 seconds show little or no mitotic delay. These data indicate that the cell becomes less sensitive to ultraviolet microbeam irradiation as it approaches mid-mitosis and that this loss of sensitivity is more pronounced in nucleolar- than in non-nucleolar-irradiated cells.

Morphological Changes.—The only morphological change observed in the nucleolus after irradiation was an occasional slight decrease in size. Irradiated nucleoli disappear in the usual manner if the cells reach very late prophase.[23] The fragmentation and spheration of nucleoli observed by Carlson and McMaster[24] after whole-cell exposure of neuroblasts to monochromatic ultraviolet radiation were not observed after microbeam irradiation. The reason for this lack of spheration, even in a few cells irradiated with a 30-μ microspot, remains to be determined. It seems plausible, however, to consider the nucleolus as having been "inactivated" structurally and functionally by the microspot of ultraviolet. The dose of polychromatic radiation in the microspot focused on the nucleolus in a 3-second exposure is 3×10^6 ergs cm^{-2}, about 1,500 times the mean effective dose of 2650 A found by Carlson and McMaster to be the most effective in producing spheration. This tremendous quantity of radiation most probably alters the nucleolar material so drastically, perhaps by denaturing it, that spheration is not possible.

Mitotic retardation after non-nucleolar irradiation of MP and LP cells is associated with a change in the chromatin best described as "reversion"—the chromatin assumes an appearance resembling that of chromatin of earlier mitotic stages. This phenomenon also occurs occasionally in nucleolar-irradiated cells, but it is slower and considerably less extensive than that observed in non-nucleolar-irradiated cells. Reversion has been observed in neuroblasts of embryos exposed to X-radiation[25, 26] or to ultraviolet radiation.[27] The present study reveals that reversion of chromatin occurs eventually throughout the entire nucleus (15–20 μ in diameter) although irradiation is confined to an area less than 3μ in diameter.

Immediately after non-nucleolar irradiation, the chromatin at the focal point of the microbeam "pales," as described by Uretz *et al.*[10] and Perry[28] to give a clear region, the size of the microspot, in the nucleus. The remainder of the chromatin continues prophase development for about 15–20 minutes, after which the chromatin immediately around the paled spot begins to show reversion. Within the next 5 minutes, all of the chromatin in the nucleus has undergone reversion, the greatest extent of which is to the early prophase condition. If a microspot of chromatin is paled at one side of a nucleus, a longer time is required for reversion of all the chromatin, i.e., about 10–15 minutes. The chromatin reappears in the microspot about 1 hour after irradiation; several hours later, the cell begins to recover and progresses through prophase again.

DISCUSSION

Although the present study does not indicate the specific role of the nucleolus, it does demonstrate that this structure is directly involved in the mitotic process. This makes more plausible the speculation of Norman[29] to the effect that the nucleolus, or possibly the nucleolar organizer, is the cell structure inactivated to give the

non-genetic effects of ultraviolet radiation observed in *Neurospora* conidia. The effect of the ultraviolet microspot on the neuroblast nucleolus is assumed to result from direct damage to the nucleolus; there is no evidence that implicates the nucleolar organizer.

Since it has been shown[30, 31] that deoxyribonucleic acid (DNA) synthesis does not occur after very early prophase in the neuroblast, the cessation of mitosis that results from irradiation of a nucleolus at early and middle prophase cannot be related to disturbances in DNA synthesis. Removal of nutrient yolk from cultures of grasshopper embryos has been shown to affect the ability of neuroblasts in stages through MP and LP to complete mitosis but to have no effect on the mitotic progress of VLP cells.[32] Evidently, the neuroblast requires extracellular materials for completion of the major part of the prophase processes. That the nucleolus may be involved in the utilization by the cell of such materials is suggested by autoradiographs of neuroblasts cultured with tritiated cytidine.[33] These indicate that the nucleolus can incorporate cytidine (into its RNA, presumably) at mitotic stages from late telophase through MPb.[34] The nucleolus appears, therefore, to be metabolically active in those mitotic stages in which it is susceptible to ultraviolet radiation.

Vincent[35] in his comprehensive review has discussed the many functions that have been ascribed to the nucleolus. The most attractive is that proposed by Caspersson[36, 37]: the nucleolus is connected with protein synthesis and the production of cytoplasmic RNA. Evidence to substantiate this view is equivocal, but data obtained with a number of techniques on a variety of cells are accumulating[1, 3, 35, 38–40] that point to the relation of the nucleus to formation of at least part of the cytoplasmic RNA and protein. That the synthetic capabilities of the nucleolus are requisites for the mitotic process can be inferred from the present work. Further studies utilizing a monochromatic ultraviolet microbeam and labeled RNA precursors may reveal the importance of nucleolar RNA to mitosis.

SUMMARY

Exposure of one nucleolus in a grasshopper neuroblast to a microspot of high-intensity ultraviolet radiation for 3 seconds permanently stops mitosis of cells irradiated in stages from late telophase to the middle of middle prophase. During the latter half of middle prophase, the cell becomes mitotically less sensitive to nucleolar irradiation and by late prophase is completely insensitive to it. Microspot irradiation of a non-nucleolar region of the nucleus causes mitotic delay but does not usually prevent cell division. These observations support the hypothesis that the nucleolus has an intimate functional relation to the mitotic process.

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[20] A number of cells in tissue fragments have been seen in division in the cultures for as long as 2 or 3 weeks. Differentiation has been observed to begin 3 days after preparation of the cultures.

[21] Neuroblasts are considered "dead" when all Brownian movement of mitochondria in the cytoplasm ceases or when the entire protoplast contracts into a highly refractile, amorphous mass.

[22] The authors are indebted to Dr. A. W. Kimball, of the ORNL Mathematics Panel, for the statistical analysis.

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