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THE FUNCTION OF THE NUCLEOLUS AS STUDIED WITH THE ULTRAVIOLET MICROBEAM[1]

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By means of a UV microbeam the effects of nucleolar irradiation on some cellular processes are studied. On the basis of the results obtained some conclusions are made concerning the role of nucleoli in mitotic processes and the synthesis of ribonucleic acids and proteins.

In cytological studies designed to elucidate the function of the nucleolus the ultraviolet microbeam has proved to be a very valuable tool. The microbeam serves as a »micro needle« which destroys selectively a small volume within a living cell while leaving the remainder of the cell essentially intact. When directed at a nucleolus it can »enucleolate« a cell with considerably less damage to surrounding parts than conventional micrurgical techniques. It is therefore possible to select a particular attribute of a cell (e. g. ability to divide or the ability to synthesize nucleic acids or proteins) and to establish whether this attribute is in any way connected with nucleolar function.

THE APPARATUS

A UV microbeam was constructed by Tschacotin as early as 1912 (1).[2] An improved apparatus of the type described here was developed by

[1] Text from a lecture delivered at the Institute for Medical Research (incorporating the Institute of Industrial Hygiene), Zagreb, June 1960. The author is indebted to Prof. V. B. Vouk for his kind hospitality.

[2] For a comprehensive account of UV microbeams as well as other types of partial cell irradiation the reader may consult an article by R. E. Zirkle (2).

Uretz and coworkers (3, 4). The basic principle is that a microscope objective lens is used *simultaneously* in two ways: conventionally to view the specimen which is to be irradiated, and in reverse to form a demagnified image of a pinhole which is illuminated by UV light. The optical system, fig. 1, consists of a focussing stage microscope equipped with an incident illumination attachment, reflecting objective, and

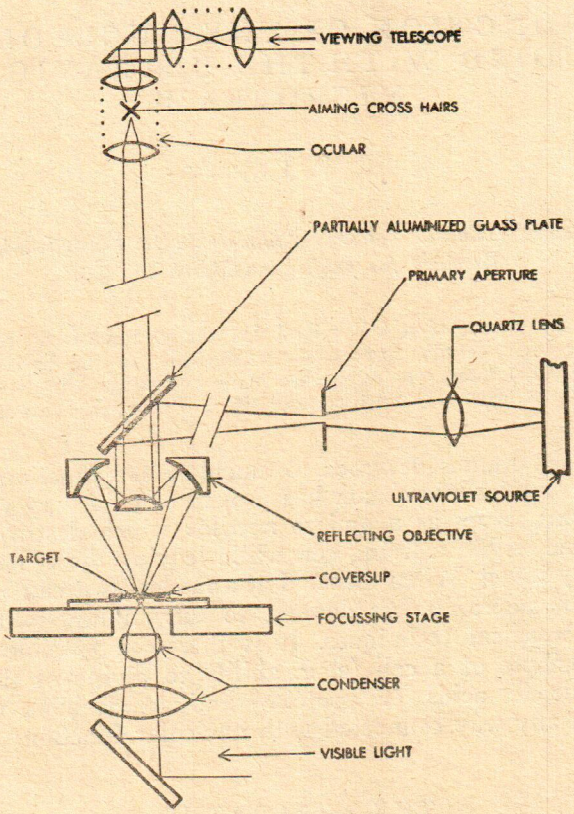

Fig. 1. Principle of the ultraviolet microbeam.

viewing telescope. The pinhole, termed the »primary aperture« is positioned at an optical distance from the objective equivalent to that of a reference cross hairs in the ocular and is illuminated with heterochromatic UV radiation from a high pressure mercury lamp.

The beam is in the form of a hollow cone converging to a spot, the size of which is determined by the pinhole diameter and the magnification of the objective. The angle of convergence is determined by the numerical aperture of the objetive and the index of refraction of the specimen. The geometrical situation illustrated in fig. 2 represents a beam from a Beck reflecting objctive (Na = 0.65) converging to a 2.2 micron diameter spot in a nucleolus of refractive index 1.42. The intensity of biologically effective radiation at the focal plane can be as high as 10^5 ergs(mm^2)sec. The intensities in out-of-focus planes diminish as shown in fig. 2. It is estimated that in a nucleolar irradiation of a

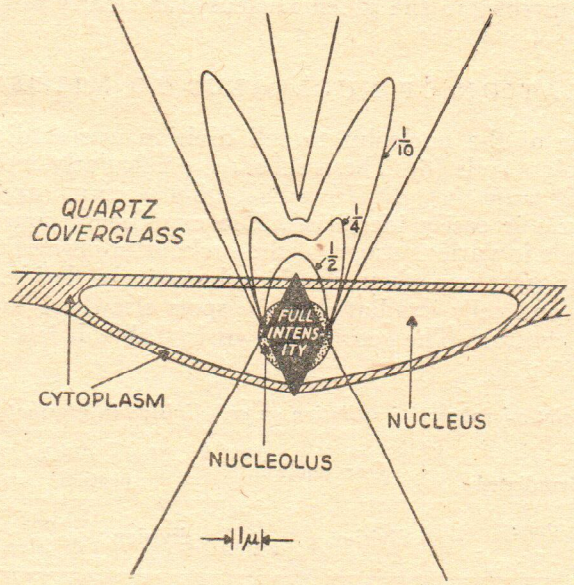

Fig. 2. Idealized profile of a cell being bombarded by an ultraviolet microbeam which is focussed on its nucleolus. The cell geometry is estimated from measurements on fixed material. Above and below the bicone of full intensity are shown isointensity contours of diminishing intensity.

tissue culture cell about 0.7 of the intercepted radioation is confined to the nucleolar volume, the other 0.3 being absorbed by the cytoplasm and other portions of the nucleus. It is obviously desirable to use thin flat cells, such as tissue culture cells, for these studies.

The effect of localized irradiation on nucleic acids is exemplified by the damage to chromosomal DNA shown in figure 3. When a chromosome is irradiated in a 4 micron diameter area there is a decrease at the irradiated site in the index of refraction and in the 260 mμ absorption (5). From the absorbance drop the diminution in DNA concentration is calculated to be more than 60%. The effect on nucleolar RNA has not been measured in this way. However it has been shown *(vide infra)* that irradiation will completely inhibit nucleolar RNA synthesis. Another example of the degree of localization is illustrated in fig. 4. In this case the pinhole primary aperture was replaced by a ring aperture so that the resultant microbeam had a central obstruction. After irradiation the cell was stained with acridine orange and a colorless ring (arrow) in the green nucleus was clearly observable.

RELATION OF NUCLEOLUS TO MITOSIS

The relation of the nucleolus to cell division was studied in grasshopper neuroblast cells (6). These cells are particularly well suited for such a study because they divide rapidly (4 to 8 hours) and their stage in the cell cycle is easily identified. The experiment consisted simply in choosing cells in various stages of the cycle, irradiating their nucleoli, and observing whether they would subsequently divide. Both unirradiated cells and cells irradiated in 3μ spots elsewhere in the nucleus were used as controls. The results, shown in Table I, indicate that the

Table I

Effect of ultraviolet microbeam irradiation on cell division in grasshopper neuroblasts*

Cell part irradiated / Mitotic stage		One nucleolus		Non-nucleolus portion		Total No. of cells
		division	no division	division	no division	
Pre-middle prophase		0	4	2	1	7
Middle prophase	(A	1	5	5	1	12
	(B	3	3	5	3	14
	(C	3	4	4	1	12
Late prophase	(A	5	1	6	0	12
	(B	5	0	7	0	12
Very late prophase		1	0	11	0	12
				Total cells irradiated		18

Diameter of microspot: slightly less than 3μ.

presence of a functioning nucleolus during the early stages of the cell cycle is essential for the mitotic process to occur, but that the nucleolus ceases to be important with respect to mitosis after middle prophase, even though it is present as a morphological entity until prometaphase.

THE NUCLEOLUS AND NUCLEIC ACID METABOLISM

Having established that the nucleolus is vital for mitotic activity, we then asked the question, »What metabolic processes in the cell are dependent on the nucleolus?« Since the RNA in the nucleolus is at a higher concentration than in any other cytologically distinguishable part of the cell, we first focussed our attention on RNA metabolism (7,8). The metabolic activity was assayed as the incorporation of tritium labelled nucleosides and measured by autoradiography. Under normal conditions, when cells are introduced to labelled nucleosides the label appears first in the nucleus and nucleoli and afterwards in the cytoplasm.

The protocol for the experiments is illustrated in fig. 5. HeLa tissue culture cells were grown in micro-cuvettes on a complex medium, ϕ_{10}. A group of 40 or so were selected and were either: irradiated in one or more nucleoli, irradiated in the nucleoplasm adjacent to a nucleolus, or left unirradiated. The cells were then transferred to ϕ_{10} supplemented with tritiated cytidine and incubated for a given duration. They were then fixed and coated with autoradiographic emulsion.

In figure 6 are illustrated a group of cells in phase contrast before irradiation (A) and the subsequent autoradiograph of the group (B). Note (i) that there is at least a 10 fold diminution in the grain count over irradiated nucleoli compared to nucleoli in unirradiated cells, (ii) irradiation of one nucleolus in a cell which has two nucleoli does not affect the incorporation of the unirradiated nucleolus, and (iii) irradiation of a 2μ diameter spot in the nucleoplasm does not affect either the incorporation of the nucleoli or the overall incorporation of the nucleus. These three facts prove that it is possible to greatly reduce the RNA activity of a nucleolus without producing indirect radiation side effects on other parts of the cell. Hence a change in the RNA activity of any other part of the cell subsequent to nucleolar irradiation can be considered solely a response to the loss of nucleolar function.

In the cells which had their nucleoli inactivated one can see a fairly high incorporation into the nucleoplasmic RNA, which on the average is only about $^1/_3$ lower than in the control nuclei. This points to a system of RNA incorporation in the nucleus which is independent of the nucleolus. Since the cells of fig. 6 were incubated in cytidine for only two hours there is no significant labelling in the cytoplasm, even in the con-

trols. However when the cells are incubated for longer periods (4 to 8 hours) so that the control cytoplasms have an appreciable amount of label one finds that nucleolar inactivation causes a $^2/_3$ diminution in cytoplasmic incorporation. This emphasizes a cytoplasmic RNA system which is strongly dependent on the nucleoli.

The relationship between the nucleolus and DNA synthesis was studied by performing companion experiments to those described above in which thymidine replaced cytidine as the source of label (9). At the

Fig 5

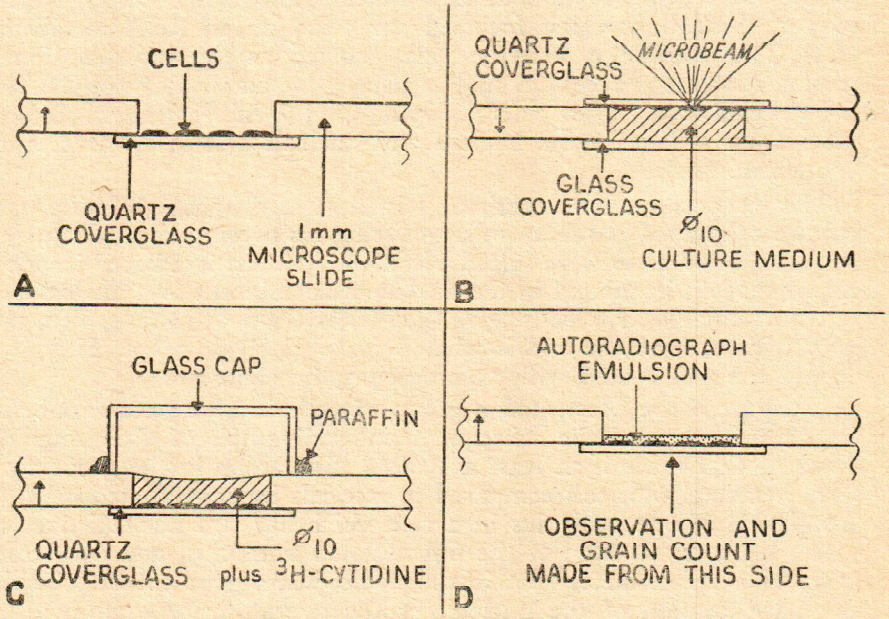

Fig. 5. Sequence of the treatment of cells in a microbeam experiment. A. Growth, B. Irradiation, C. Incubation, D. Autoradiography.

doses used in the foregoing experiments the irradiation of a spot in the nucleoplasm was as effective as a nucleolar irradiation in producing a 50–60% attenuation in thymidine incorporation. For this reason it was not possible to ascertain whether the nucleolus has a role in DNA synthesis.

THE NUCLEOLUS AND AMINO ACID INCORPORATION

Similar experiments were performed using the amino acids lysine, leucine, or phenylalanine instead of the nucleic acid precursors. In contrast to the strong nucleolar dependence of cytoplasmic nucleoside incorporation it was found that both nuclear and cytoplasmic amino acid incorporation are practically normal after nucleolar irradiation, even when incubations were extended for about one half generation following irradiation (10). Thus in the nucleolar irradiated cell we have an artificially constructed system in which there is essentially no RNA metabolism associated with the nucleolus, less than 1/3 of the normal RNA activity in the cytoplasm, and yet almost normal amino acid incorporation.

CONCLUSIONS

Mitosis. The failure of cells to divide after nucleolar irradiation indicates that certain metabolic processes associated with the nucleolus are necessary prerequisites to mitotic division. Furthermore the loss of nucleolar sensitivity during prophase suggests that a cessation of function precedes, by a measurable interval, the morphological disappearance of the nucleolus. Although at the present time it is still not certain which metabolic processes are involved in the mitotic inhibition, the above studies make it reasonable to suppose that RNA synthesis is a primary factor. It is possible that DNA synthesis is also involved although not uniquely so. This latter conclusion is derived from the fact that nucleolar irradiation specifically inhibits mitosis in the neuroblast when delivered at cell stages subsequent to the completion of DNA synthesis. Since overall inhibition of protein synthesis is not observed after nucleolar inactivation it is concluded that if the system is at all connected to protein synthesis then it must be limited to a small specific class of proteins.

RNA and Protein Synthesis. The data presented here are consistent with the hypothesis that a large portion of cytoplasmic RNA is synthesized at the nucleolus. It is not yet clear from these studies whether the RNA moves as an intact molecule from nucleolus to cytoplasm or whether it is altered in some way before being incorporated into the cytoplasm. Our experiments have also definitely shown the existence of a system of RNA synthesis in the nucleoplasm which is independent of the nucleolus. A model in which cytoplasmic RNA is derived from these two nuclear sources was tested with quantitative data from measurements of nucleoside incorporation kinetics and found to be tenable (11).

Our inability to observe any appreciable effect on amino acid incorporation in cells where cytoplasmic RNA incorporation was greatly reduced is in agreement with the concept that RNA synthesis does not have to occur simultaneously with protein synthesis. If the experiments could be extended beyond a generation time then perhaps an effect due to gradual depletion of protein synthetic centers would be noticed.

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Sadržaj

ISPITIVANJE FUNKCIJE NUKLEOLUSA POMOĆU ZRAČENJA MIKRO-SNOPOM ULTRAVIOLETNIH ZRAKA

Opisan je uređaj za zračenje tzv. »mikro-snopom« (micro-beam) ultravioletnih zraka.

Pomoću ovog uređaja ozračeni su selektivno nukleolusi ili područja odgovarajućeg promjera (oko 3 mikrona) u nukleoplazmi živih stanica.

Ozračivanje nukleolusa neuroblasta skakavaca u raznim stadijima staničnog ciklusa pokazalo je da je funkcija nukleolusa bitna za odvijanje mitotskih procesa, ali samo do sredine profaze.

Studij inkorporacije nukleozida, markiranih tricijem, u RNK (ribonukleinsku kiselinu) i DNK (deoksiribonukleinsku kiselinu) He La stanica u kulturi, iza ozračivanja nukleolusa »mikro snopom« ultravioleta, pokazao je slijedeće.

Razaranje nukleolusa ultravioletom reducira inkorporaciju prekurzora u nukleoplazmatsku RNK svega za $^1/_3$, dok je markiranje citoplazmatske RNK smanjeno za $^2/_3$. Inkorporacija prekurzora u DNK jednako je reducirana iza ozračenja nukleolusa ili odgovarajućeg područja u nukleoplazmi.

Inkorporacija markiranih aminokiselina u proteine nukleusa i citoplazme ostala je (do polovice prve generacije) praktički normalna iza ozračenja nukleolusa »mikro snopom« ultravioletnih zraka.

Inkorporacija markiranih prekurzora u nukleinske kiseline i proteine praćeno je autoradiografskom tehnikom.

Na osnovu navedenih rezultata, autor iznosi neke zaključke o ulozi nukleolusa u mitotskim procesima, te u sintezi nukleinskih kiselina i proteina.

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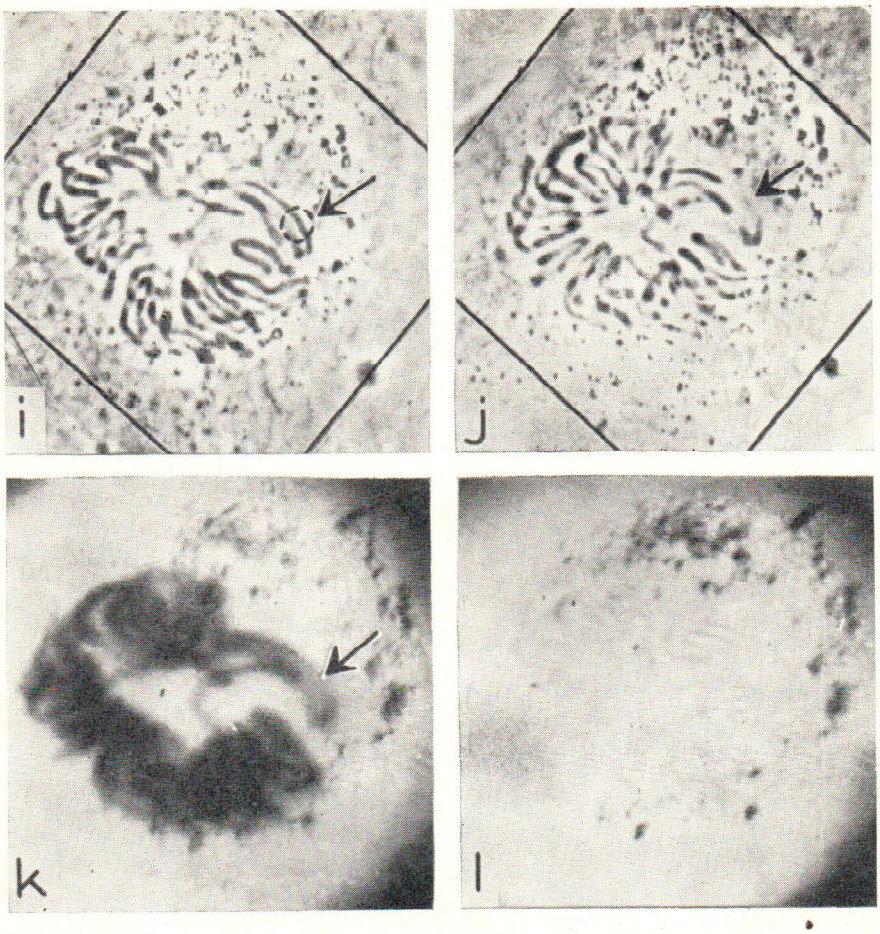

Fig. 3. (i) Phase contrast. Metaphase before irradiation. Dotted circle (arrow) shows size of 4μ diameter microspot. (j) Phase contrast. Same cell as (i) after irradiation with 4μ diameter microspot. »Paled« chromosome indicated by arrow. (k) 260 mμ. Same cell as (i) after irradiation. (l) 310 mμ. Same cell as (i) after irradiation showing that (k) represents true absorption and not refraction effects.

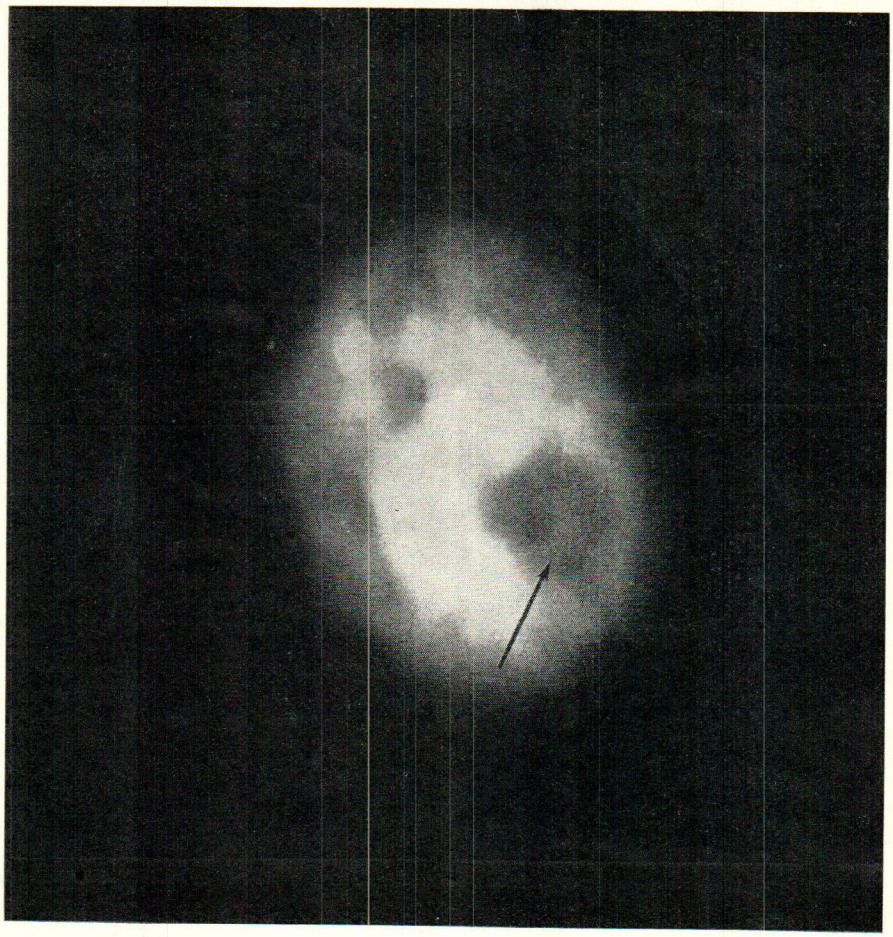

Fig. 4. Nucleus from a cell stained with acridine orange. Arrow shows spot irradiated with a »ring« microbeam of 3μ inner diameter, 6μ outer diameter.

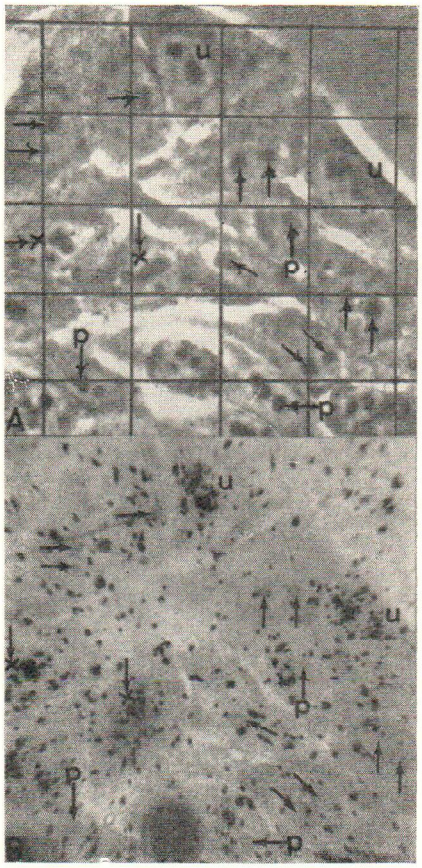

Fig. 6. A. Phase contrast. Field of living HeLa cells before microirradiation and incubation for 2 hours in 3H-cytidine. B. Same field after fixation, exposure of autoradiograph, and staining with methyl green pyronine. Arrows indicate irradiated nucleoli or irradiated spots (x) in nucleoplasm. Arrows marked »p« indicate the irradiated nucleolus of a cell which contains at least one other unirradiated nucleolus. The cells, u, were not irradiated.