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NUCLEOLAR "PALING" FOLLOWING ULTRAVIOLET MICROBEAM IRRADIATION OF MAMMALIAN CELLS OF NORMAL AND TUMOUR ORIGIN

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ÖZET:

Işınlandırılan canlı sistemlerde, ışınlandırmayı takip eden kısa süreler içinde radyasyon etkisi ile morfolojik bir değişikliğin görülmesi olağan değildir. U.V. microbeam sistemi ile yapılan ışınlandırmayı müteakip, DNA bakımından zengin kromozomlar üzerinde görülen "Paling" veya "DNA steresis" adı verilen olay, bu çalışmada aynı tip bir sistemle ışınlandırılan 3 farklı tip hücrenin daha ziyade RNA içeren nukleoluslarında da farklı değerlerde tesbit edilmiştir.

SUMMARY:

A microbeam apparatus has been used to irradiate with heterochromatic ultraviolet light areas 3.5 μm in diameter in the nucleoli of mammalian cells in culture. For all three cell types some nucleoli showed paling but the percentage of cells showing strong paling was much higher for the short-term cultured rat tumour cells than for freshly cultured embryonic rat fibroblasts or for mouse L-strain cells that have been in culture for many years.

Further experiments are suggested to elucidate the mechanism of this effect which has not been reported previously.

INTRODUCTION

There have been several reports of changes in refractive index at sites irradiated with an ultraviolet (UV) microbeam in cells growing in monalayer culture.

Naruse et.al (1967) used a U.V. microbeam 2 μm in diameter to irradiate a region in the nucleoplasm of interphase cells for a variety of species. Grasshopper spermatocytes and newt peritoneal leukocytes lost material from the irradiated region but all the mammalian cells showed darkened areas under positive phase contratst and this change was identified cytochemically with an accumulation of DNA in the irradiated region.

Conversely loss of absorbing material from chromosomes irradiated with a U.V. microbeam ("paling" or DNA stresis) has been reported frequently (Uretz et al 1954; Boom and Leider 1962; Zirkle and Uretz 1963;

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Bloom and Ozarslan 1963). Bloom and Leider (1962) studied this phenomenon in detail for mitotic cells of *Triturus viridescens* and concluded that there was a loss of DNA or possibly deoxyribonucleoprotein form the irradiated area.

The effect has also been studued in the bands of the giant chromsome of *Chironomus* which is clearly visible under the phase contrast microscope even during interphase. In these experiments it was noted that paling could be induced readily in the bands which contained densely accumulated DNA but not in those where the DNA was decondensed (Algüneş et al 1987).

Mammalian chromosomes have not been studied because they are too small. Homever, during interphase the nucleolus contains nucleic acid in a densely compacted form. Although previous reports on paling have all been concerned with changes of DNA in the irradiated area, it was thought that the nucleic acid, which is mostly RNA, in nucleoli might behave similarly.

These experiments were therefore designed to look for a paling effect in the nucleoli of mammalian cells growing in tissue culture.

METHODS

Three types of cells have been used in these experiments. Normal embryonic rat fibroblasts were prepared from embryos taken from pregnant albino rats (Rattus norvegicus) by standard methods. They were maintained in medium 199 supplemented with 10% calf serum and were used for experiments in the second passage. Rat tumour cells were obtained from tumours induced by treatment of albino rats with croton oil and 7,12 – dimethylbenzanthracene (Uçer 1972). They were maintained in the same medium as the rat fibroblast cells and were used for these experiments between the 20th and 44th passegas. Mouse L cells were obtained from the Department of Radiotherapeutics, University of Cambridge, England, by the courtesy of Dr.P. Dendy. They have been in culture for many years and for these experiments were cultured in the same medium as the other two cell types.

Cultures for experimental use were obtained by seeding 3×10^4 cells onto a quartz coverslip. 48 hrs later, chambers suitable for microbeam work (Algüneş, 1974) were prepared at pH 7.0–7.2 and used immediately. Cells were chosen for irradiation in two ways. In most experiments a cells which contained only one prominent nucleolus was selected and a nearby cell which contained only one nucleoulus was used as a control. In some experi-

ments cells which contained two similar nucleoli were chosen; one was selected for irradiation and the other was used as a control. All cells were photographed under positive phase contrast at this stage.

UV microbeam used in this work was bassed on the design first suggested by Uretz and Perry (1957).

A 74X reflecting objective demagnified the light emerging from a primary aperture 200 μm in diameter to a theoretical spot size of about 2.7 μm. Owing to the finite thickness of the nucleoli, which are frequently most spherical and about 3–4 μm across, the effective minimum diameter of the spot was about 3.5 μm (Hatfield 1970). Heterochromatic ultraviolet light with a small component of visible green light, obtained by filtering the lamp output through 5 cm $NiSO^4$ / $CoSO^4$ solution (Kasha 1948) was used throughout. All cells were irardiated for 45 sec. Monitoring equipment is not available but careful comparison with a similar piece of equipment in Cambridge indicated a dose rate of about 2×10^{-4} erg / μm^2 / sec.

The temperature in the microbeam room was not strictly controlled but varied between 30 and 36°C.

The UV microbeam equipment is not fitted with phase contrast, so immediately after irradiation the culture chambers were transferred back to the phase contrast microscope for observation and photography. In some experiments cells were fixed with 25% acetic acid in ethanol followed by 70% ethanol 15 min after irradiation. These cells were stained with Geimsa at pH 7.2.

RESULTS

Nucleolar paling was observed in all cell types. Under optimal conditions it could be detected immediately on returning to the phase contrast microscope but did not reach a maximum until about 5 min after irradiation. An example of strong paling is demonstrated for rat tumour cells in Fig. 1. One nucleolus in the cell on the left and one nucleolus in the cell on the right (shown arrowed) were irradiated. Densitometer traces through photographic negatives of the cell on the left, before and after irradiation, show the paled region clearly (Fig. 2). The trace obtained after irradiation has been displaced slightly to the right for clarity. Note that the traces obtained for the unirradiated nucleolus before and after microbeam exposure are very well matched, suggesting good photographic reproducibility and no paling due to scattered UV light. Not all nucleoli paled strongly as shown in Table 1.

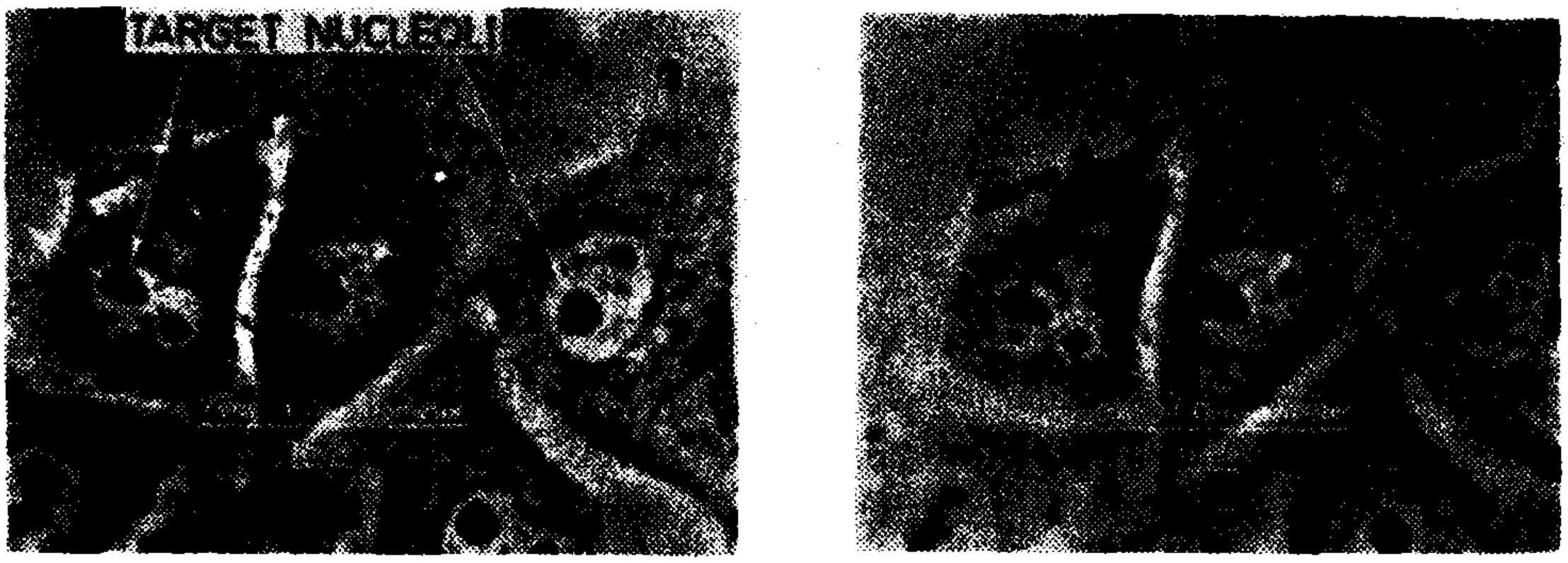

Fig. 1. Rat tumour cells in monolayer culture.
(left) before the large nucleolus in the left hand cell and the large nucleolus in the right hand cell had been irradiated (shown arrowed).
(right) After microbeam irradiation.
(Magnification of final photographs approximately 800 X.)

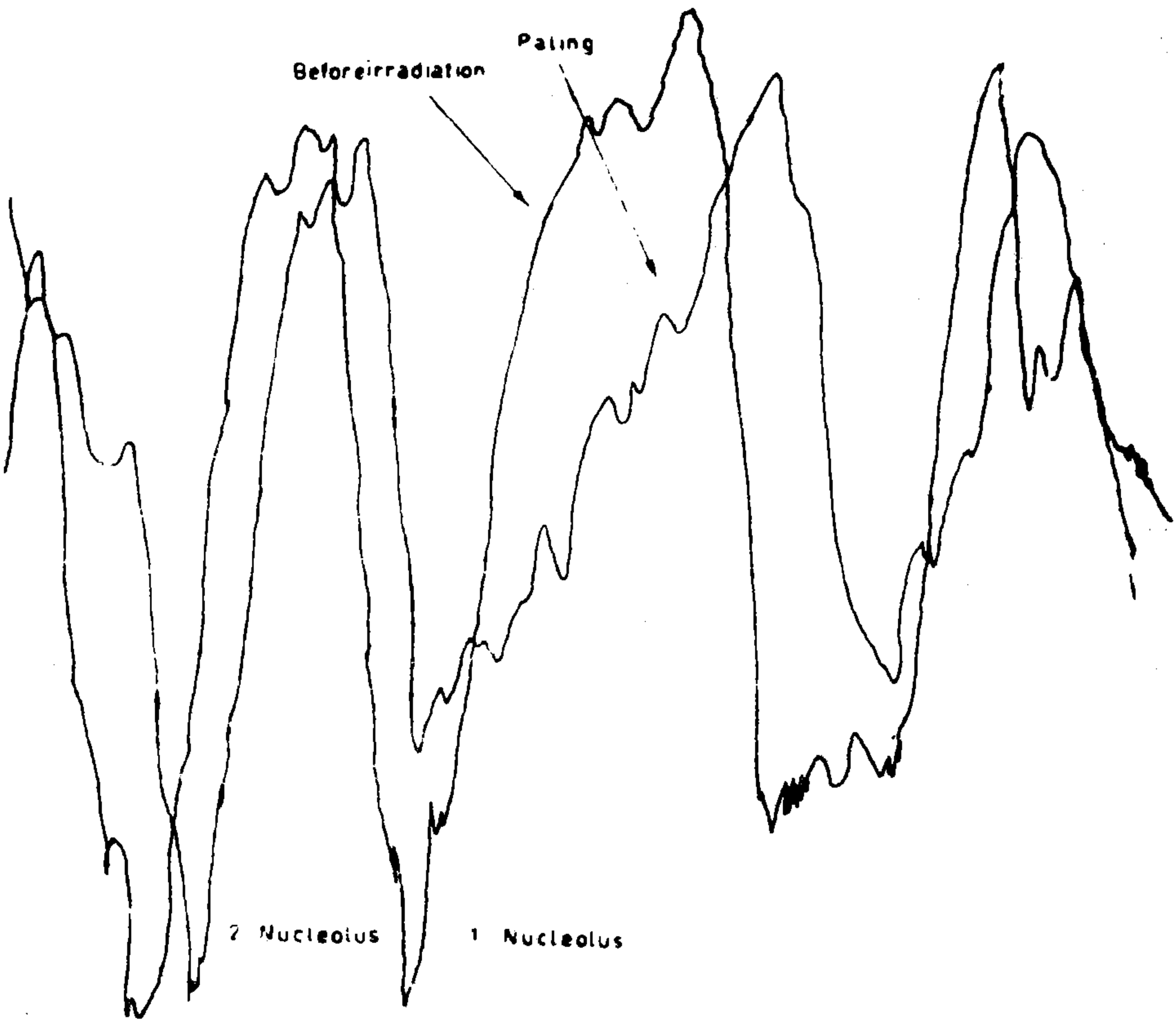

Fig 2. The negatives from which Fig. 1 was prepared were used for densitometry. Traces are shown along the line indicated. The profilo obtained from the negative after irradiation has been displaced slightly to the right for clarity. (Magnification of negatives approximately 350 X).

Table 1.

Cell Tyoe	Total number of cells irradiated	% Paling	Percentage of cells showing: Strong Paling	Some Paling	No Paling
Embryonic rat Fibroblasts	104	% 71 (74 / 104)	41 % (43)	30 % (31)	29% (30)
Rat tumor cells	120	% 89 (107 / 120)	68 % (82)	21 % (25)	11 % (13)
L strain cells	34	% 70 (24 / 34)	47 % (16)	23 % (8)	30 % (10)

The percentage of cells showing paled nucleoli after UV microbeam irradiation for the three cell types studies.

Many cells were followed visually for 2 hr after irradiation. During this time the degree of paling remained unaltered but the diameter of the paled area increased. No difference in behaviour was detected after irradiation of mononucleolar cells and irradiation of a single nucleolus in binucleolate cells.

The paled area was also clearly visible in cells which had been fixed and stained with Giemsa but no attempt has been made to quantify this effect because it depends on the density of staining of each culture.

DISCUSSION

Paling has been observed for both normal and tumour cells and for both rat mouse cells. However the proportion of cells which showed paling was different, being much higher for rat tumour than for the other two types. Failure to pale a small percentage of cells might be due to errors of focussing since the microbeam is not equipped with phase contrast. This could account for 10% of rat tumour cells showing no paling and 20% showing poor paling. But the proportion of cells showing little or no paling was much higher for the other two cells types suggesting that some other factor is present. Under the phase microscope, the nucleoli of freshly cultured embryonic rat fibroblast cells look slightly paler that rat tumour nucleoli, so the amount of nucleic acid, which is mostly RNA in the nucleoli, may be different for the three cell types. From the evidence with *Chironomus*, paling might be weaker in nucleoli where the density of nucleic acid is lower.

Alternatively, the molecular structure of the nucleic acids in the nucleolus and in particular the degree of binding to form nucleoprotein might be different for the three cell types. Weak paling would be associated with strong binding.

Using a similar microbeam, Dendy (Personal communication)* has failed to observe nucleolar paling over the course of many years. A comparison of the physical dimensions of the $CoSO_4$ / $NiSO_4$ light filters which are used to remove most of the visible light from the beam but also remove some of the ultraviolet light shows that the dose rate used in this work was about 10X lower than that used by Dendy. This figure is confirmed by a comparison for the two microbeams of the exposure times required to reduce the rats of uptake of ^3H thymidine to 40% immediately after nucleoplasmic irradiation in the DNA synthetic phase of the cell cycle. The earliest report of Uretz et al (1954) showed that chromosome paling depended on microbeam spot size and no paling was observed if the irradiated area was too large. This paper suggests that diffusion of materials away from the irradiated area may also depend critically on dose rate.

Several further detailed investigations are suggested by this study and will form the basis of future work. The first is the development of a quantitative assay method for paling, based on densitometric measurements of photographic negatives. Secondly studies are required into the physical factors affecting the paling process in nucleoli including dose rate, spot size, temperature and the spectral quality of the radiation. Identification of the material leaving the paled area is required and finally the quantitative difference between freshly cultured normal cells and tumour cells in short term culture must be confirmed.

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