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IMPORTANCE OF THE NUCLEOLUS IN THE INITIATION OF DNA SYNTHESIS IN MAMMALIAN CELLS

Studies with an Ultraviolet Microbeam and Low Concentrations of Actinomycin D

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SUMMARY

Two independent techniques have been used to study the effects of nucleolar damage on the entry of cells into the DNA synthetic phase. Experiments have been performed with HEp/2 cells, mouse L cells, freshly cultured embryonic mouse fibroblast cells and freshly cultured monkey kidney cells. All the results confirm previous reports that when cells have proceeded more than 1–2 h into the G1 phase the onset of the next DNA synthetic phase is not delayed by nucleolar damage. However, nucleolar damage at about the time of cell division appears to delay entry into S phase for certain cell types.

There is considerable evidence that RNA and protein must be synthesised during the G1 phase before a cell can initiate DNA synthesis [1, 2, 3]. Since the nucleolus is involved in the synthesis of ribosomal RNA [4], and consequently in the synthesis of protein, it may play an important part in the movement of a cell into the DNA synthetic phase. The purpose of this work was to investigate to what extent, if at all, nucleolar activity controls the initiation of DNA synthesis.

A direct investigation is possible with the ultraviolet (UV) microbeam. Such an instrument, designed to produce a focused spot 2 μm in diameter [5] can be used to damage the nucleolus by selective irradiation. If the nucleoli of a cell of known age within the G1 phase are irradiated, the subsequent entry of this cell into DNA synthesis can be studied whilst the other daughter cell from the same division can be used as a control. If another pair of daughter cells is selected, one of them can be irradiated with an equivalent dose of radiation within the nucleoplasm. In such experiments, the microbeam is being used as a microsurgical instrument and any differences between the effects of nucleolar and nucleoplasmic irradiation may be directly associated with the inhibition of some function specific to the nucleolus.

An independent approach to the problem is possible using the antibiotic, actinomycin

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D (AMD). An interesting finding with this drug has been the identification of a dose of AMD which will inhibit ^{3}H-uridine (^{3}H-UR) uptake into the nucleoli of mammalian cells without appreciable effect on extranucleolar nuclear incorporation [6]. This observation raises the possibility that AMD may be capable of inhibiting nucleolar synthetic activity whilst causing minimal damage to other cellular functions. The finding that comparable doses of AMD in early G1 phase delayed entry of mouse L cells into the DNA synthetic phase in both asynchronous and synchronous cultures suggested that this highly important transition in the cell cycle is dependent upon a functional nucleolus [7, 8]. The present experiments with AMD were designed to test this suggestion by determining whether or not the effect of the drug upon nucleolar synthetic activity could be dissociated from its effect upon the initiation of DNA synthesis [9].

Experiments were conducted both with cells of normal origin in primary culture and with cells from established lines of malignant origin, to try and identify possible differences in the mechanism controlling the initiation of DNA synthesis in different cell types.

MATERIALS AND METHODS

Established cell lines

Both mouse L cells and HEp/2 cells, a human tumour of the nasopharynx maintained in culture for a number of years, were grown in Pyrex feeding bottles in culture medium comprising 90% medium 199 (Glaxo Ltd) and 10% foetal bovine serum for L cells and 90% Eagle medium and 10% foetal bovine serum buffered to pH 7.2 with bicarbonate for HEp/2 cells.

Primary cell lines

Monkey Kidney (MK) cells were supplied as primary cultures by Wellcome Ltd. They were grown in 90% medium 199, 9% foetal calf serum and 1% lactalbumin hydrolysate.

Embryonic mouse fibroblast (EMF) cultures were obtained by standard procedures [10] and were grown in the same medium as MK cells. Both MK and EMF cells were used for experiments in either the second or third passage.

Asynchronous cultures were prepared by trypsinising a monolayer of cells and seeding coverslips 16 mm in diameter with $2–4 \times 10^4$ cells. The cultures were incubated in an atmosphere of 5% CO_2 in air for at least 24 h in the case of EMF cells and MK cells and 36 h in the case of mouse L cells and HEp/2 cells before experimental use.

Synchronous cultures

L Cells, HEp/2 cells and EMF cells. Synchronous cultures were prepared by selectively harvesting mitotic cells from a nearly confluent monolayer growing on a 10 cm Petri dish [11]. A preliminary washing of the parent culture was followed 12 h later by a second washing to provide cells for experimental use. In the case of EMF cells the second washing was preceded by a 1 h incubation in Ca^{2+}-free medium.

For microbeam work the cells were allowed to settle on to a quartz coverslip in the bottom of a specially designed tube. This tube was made from Perspex and the cap covering its base could be removed (Tolmach. Personal communication). After 1–2 h the mitotic cells had attached themselves to the coverslip and the latter could be removed from the tube and inverted on to the culture chamber. These chambers were used routinely for microbeam work and were similar in design to those described in previous communications [12, 13]. Because the exact time of division was not known for individual cells, and because the cells spread out rather slowly after washing off, they were not irradiated during the G1 phase following harvesting. However, this procedure did provide, 16–20 h later, a large number of mitotic cells which could be followed from the precise moment of anaphase at the second division until they had spread out sufficiently for irradiation in the next G1 phase.

L cells exposed to AMD medium were either treated for 2 h starting 3 h before washing off mitotic cells ($m-3$ to $m-1$ h), or mitotic cells were washed off in AMD medium and allowed to grow for 2 h before being returned to AMD free medium (m to $m+2$ h), or cells were treated with AMD for 2 h starting 3 h after washing off ($m+3$ to $m+5$ h). EMF cells exposed to AMD medium were either treated for 45 min starting 1.5 h before washing off ($m-1.5$ to $m-0.75$ h), or were washed off in AMD medium and allowed to grow for 45 min before being returned to AMD free medium (m to $m+0.75$ h). Fortyfive-minute exposures to AMD were chosen for EMF cells because the G1 and G2 phases in these cells are much shorter than in L cells (see table 1 later).

MK cells are extremely difficult to synchronise. For microbeam irradiation of single cells it was considered more satisfactory to follow by eye a particular pair of daughter cells in an asynchronous culture from the time of division to the time of irradiation.

Microbeam apparatus. The apparatus is similar to that first described by Uretz & Perry [5]. However the reflecting objective has been replaced by a Zeiss (32×) 'Ultrafluar' objective which allows the cells to be viewed with phase contrast optics during irradiation [14]. A series of experiments to check the optical properties of the objective and to measure the diameter of the focused UV beam spot can be summarised as follows: (i) Using a 50 μm diameter primary aperture, the Ultrafluar objective produced a focused UV spot which, when recorded on thin autoradiographic emulsion, had an effective diameter of 2.0 μm. The amount of UV radiation scattered outside the beam was measured using different exposure times on the same emulsion and found to be very small—0.5 μm from the edge of the beam the intensity had fallen to 10 %, and 1 μm from the edge, the intensity was only 1 %. (ii) The focal plane of the objective shifted by 0.9 μm when the wavelength was changed from 550 to 275 μm. However, the diameter of the beam had increased by less than 10 % of its minimum value when measurements were made in planes 2 μm above and below the focal plane, and, therefore, the focal shift was considered not to be significant.

The two wavelengths chosen for irradiation were 260 nm where nucleic acid bases absorb strongly and 280 nm where certain of the amino acids in proteins show strong absorption.

Microbeam dosimetry

The flux of UV radiation in the microbeam was measured by reflecting the beam with a periscope arrangement into a photomultiplier. The geometry was carefully arranged so that the UV light passed to a calibrated photomultiplier along a ray path similar to the one it normally traversed during irradiation experiments. The spectrum of UV radiation delivered by the mercury arc lamp was measured and the spectrum of UV passed by the monochromator when it was set at 260 and 280 nm was then calculated [14].

The flux used for cellular irradiation was estimated to be approx. 4×10^{-4} erg/sec at 280 nm. Although the photomultiplier method could not provide this absolute figure with an accuracy of better than 30 %, it was possible using this arrangement to monitor at the beginning of each experiment and arrange for the total UV exposure to be reproducible within an error of 5 %.

The absorption of UV radiation by the nucleolus, nucleoplasm and cytoplasm of living HEp/2 cells has been measured using a specially designed microspectrophotometer [15]. It was thus possible to calculate the absorbed dose of radiation for each part of the cell when it was irradiated with the microbeam.

Irradiation procedure

The time of division was recorded for individual cells and the position of the two daughter cells was checked by redrawing the field of view at hourly intervals. All of the nucleoli in one of the two daughter cells were irradiated at a known time after mitosis. A nearby pair of cells, which had divided at approximately the same time, was chosen and one of the daughters was irradiated at different places in the nucleoplasm—the number of places irradiated being equal to the number of nucleoli irradiated in the first cell. All four cells were then followed until the control cells could be presumed to be in S phase (from table 1).

Drug

A commercial preparation of AMD was used (Merck, Sharp & Dohme Ltd) and an intermediate dilution of 4 μg/ml stored in the dark in medium 199 at −20°C showed no appreciable deterioration over a period of 1 month, after which time a fresh dilution was made [16].

Pulse labelling and autoradiography

Cells were treated for 15 min with medium containing ^{3}H-thymidine (^{3}H-TdR) at 5 μCi/ml, 2 Ci/mM, or ^{3}H-uridine (^{3}H-UR) at 20 μCi/ml, 3 Ci/mM, rinsed twice in Ringer, fixed in ethanol/acetic acid (3:1) for 15 min and then rinsed in 70 % ethanol for 5 min and distilled water for 1 min. In the mitotic labelling experiments the culture was rinsed thoroughly in Ringer after the 15 min pulse of ^{3}H-TdR and the label was flooded by adding 10^{-5} M cold thymidine to the medium for 1 h. Cultures were fixed at intervals thereafter. ^{3}H-UR labelled cultures were digested with DNAase (Worthington Biochemical Corp.) at 15 mg/100 ml in buffered solution for 30 min after fixation.

Labelled coverslips were mounted on slides with "DePeX". Slides were dipped into diluted 1:1 Ilford K-2 emulsion, exposed for 3 days and developed in ID 19.

In microbeam experiments, counts were made over all the irradiated nuclei, usually about 20 in each culture, and over all their paired controls. The count over each irradiated nucleus was expressed as a percentage of the count over the unirradiated sister nucleus and the results for pairs of cells which had been treated similarly (usually about 20 in all) were averaged. For AMD work, average values for the percentages of cells labelled with ^{3}H-TdR were determined by counting 2 000 cells/point for asynchronous cultures and at least 200 telophase pairs for synchronous cultures. Average grain counts in ^{3}H-UR experiments were obtained from counts on 50 cells.

Microspectrophotometry

The total DNA content of the nuclei of synchronous HEp/2 cells was measured on Feulgen stained preparations using a Barr & Stroud Integrating Microdensitometer.

Table 1. *Cell cycle parameters for four different cell types*

Cell type	Intermitotic time (h)	G1 (h)	S (h)	G2 (h)	M (h)
EMF	11.4	2.3	7.2	1.6	0.3
MK	17.0	5.2	8.0	3.5	0.3
L	22.5	7.6	9.7	4.8	0.4
HEp/2	19.0	7.5	7.0	4.0	0.5

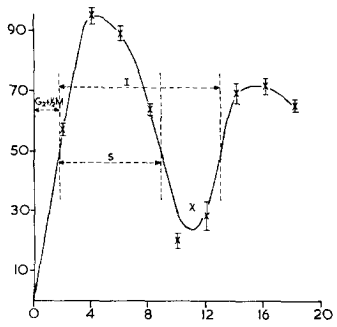

Fig. 1. Abscissa: time (hours) after exposure to ^{3}H-TdR; *ordinate:* % labelled mitoses.
The percentage of labelled mitoses in a culture of EMF cells at various times after a pulse exposure to ^{3}H-TdR is shown on the graph from which the durations of the G2 phase + ½ mitosis (G2 + ½*M*), the DNA synthetic phase (*S*) and the intermitotic time (*I*) can be deduced.

RESULTS

Cell cycle analysis

The percentage of labelled mitotic cells on preparations of asynchronous cultures fixed at various times after a short pulse of ^{3}H-TdR was measured for EMF cells (fig. 1), MK cells and HEp/2 cells. The duration of mitosis (*M*) was estimated by visual observation under the microscope. From this data the intermitotic time and the length of each of the phases of the cell cycle have been calculated. The data for L cells are taken from Rickinson [8].

For the microbeam experiments and some AMD experiments a more accurate value of G1 was desirable. This was obtained for L cells and HEp/2 cells by harvesting mitotic cells into medium containing ^{3}H-TdR and fixing at intervals thereafter (e.g. the control curve on fig. 5).

All the relevant information on cell cycle parameters is summarised in table 1.

Effects of microbeam irradiation during early and mid G1 phase

^{3}H-UR uptake into HEp/2 cells and MK cells: Fig. 2 shows results for HEp/2 cells irradiated at 280 nm between 3 and 5½ h after mitosis ($m+3$ h to $m+5\frac{1}{2}$ h), pulse-labelled with ^{3}H-UR between 2 and 3 h after irradiation and fixed immediately. Similar results were obtained when the cells were irradiated at 260 nm; when they were irradiated with either wavelength between $m+1\frac{1}{2}$ and $m+3$ h, and when the experiments were repeated with MK cells (see the broken lines in fig. 3).

All these experiments indicate that there is a range of absorbed doses in the region of $(10–30)\times10^{-4}$ ergs/cell nucleus within which nucleolar ^{3}H-UR incorporation is reduced to a much greater extent by nucleolar irradiation than by nucleoplasmic irradiation. Within the same dose range, however, nucleoplasmic ^{3}H-UR incorporation is affected equally and only slightly by irradiation at either site.

^{3}H-TdR uptake into HEp/2 cells and MK cells: These cultures were allowed to grow after irradiation until the control cells were judged to have entered S phase on the basis of information in table 1, (e.g. until about $m+6$ h to $m+7$ h in the case of MK cells), then pulse-labelled and fixed immediately. The results for MK cells irradiated at 280 nm between $m+1$ and $m+2$ h are shown in fig. 3. The effects of similar treatment on nucleolar ^{3}H-UR incorporation

have been included for comparison. Once again very similar results were obtained at 260 nm and when the experiments were repeated with HEp/2 cells.

No significant difference between nucleolar and nucleoplasmic irradiation could be detected in any of these experiments on ^{3}H-TdR uptake.

Increase in total DNA content of HEp/2 cells: The total DNA content at a time corresponding to late S phase was measured for cells irradiated with 280 nm UV between $m+1\frac{1}{2}$ and $m+3$ h, again using the other daughter cell as an unirradiated control. The exact age at fixation could not be standardized because it was necessary to work on the same coverslip with a number of cells which had divided at different times. However, the time of fixation was chosen so that each cell had proceeded at least 4 h into S phase. Results are shown in fig. 4 and fully confirm the results of the ^{3}H-TdR experiments.

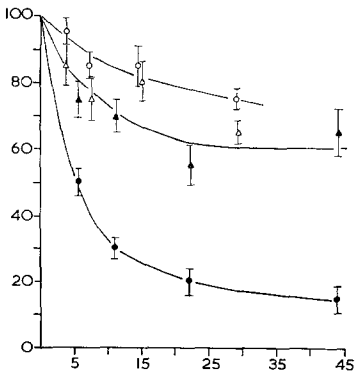

Fig. 2. Abscissa: absorbed dose in ergs ($\times 10^{-4}$); *ordinate:* ^{3}H-UR uptake following irradiation (as % of controls).

RNA synthesis in synchronous HEp/2 cells irradiated at 280 nm between 3 and 5 h after mitosis. ^{3}H-UR uptake into nucleolus, following nucleolar (●) and nucleoplasmic (○) irradiation. ^{3}H-UR uptake into nucleoplasm following nucleolar (▲) and nucleoplasmic (△) irradiation.

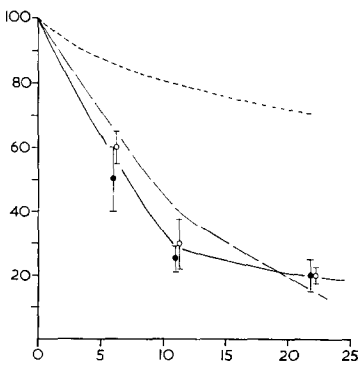

Fig. 3. Abscissa: absorbed dose in ergs ($\times 10^{-4}$); *ordinate:* ^{3}H-TdR uptake following irradiation (as % of controls).

DNA synthesis during early S phase in MK cells irradiated at 280 nm between 1 and 2 h after mitosis. ^{3}H-TdR uptake following nucleolar (●) and nucleoplasmic (○) irradiation. ^{3}H-UR uptake into nucleolus following nucleolar (– –) and nucleoplasmic (----) irradiation.

Effects of microbeam irradiation during late G1 and early S phase on ^{3}H-TdR uptake into HEp/2 cells

The results for cells irradiated at 280 nm between $m+5$ and $m+7\frac{1}{2}$ h (late G1 phase) and for cells irradiated more than $7\frac{1}{2}$ h after mitosis (early S phase) are shown in table 2.

These cells were pulse labelled 2–3 h after irradiation and there was no significant difference between the effects of nucleolar and nucleoplasmic irradiation at either time. Moreover, there was no significant difference between cells irradiated in late G1 phase and cells irradiated in early S phase.

Irradiation in the cytoplasm had virtually no effect on subsequent uptake of ^{3}H-TdR into the nucleus.

Effects of AMD in early G1 phase on mouse L cells

Entry into DNA synthesis: Synchronous cultures of mouse L cells were exposed from m to $m+2$ h to doses of AMD which ranged from 0.005 to 0.04 μg/ml. They were pulse-labelled with ^{3}H-TdR at different times

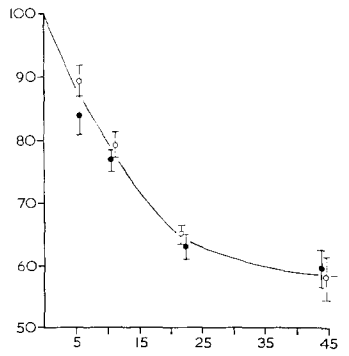

Fig. 4. Abscissa: absorbed dose in ergs ($\times 10^{-4}$); *ordinate:* Feulgen content after irradiation (as % of controls).

Total DNA content in synchronous HEp/2 cells irradiated at 280 nm in early G1 phase (between 1½ and 3 h after mitosis) and fixed at about $m+14$ h. Intensity of Feulgen stain after nucleolar (●) and nucleoplasmic (○) irradiation.

thereafter and fixed immediately. The results, averaged over three experiments, are shown in fig. 5. It is clear that the onset of the next DNA synthetic phase is delayed at doses as low as 0.005 μg/ml AMD.

^{3}H-UR uptake: Mouse L cells which had been exposed to varying concentrations of AMD from m h to $m+2$ h were pulse-labelled with ^{3}H-UR at $m+3$ h and fixed immediately. The results in fig. 6 show that nucleoplasmic incorporation of ^{3}H-UR is unaffected by doses of AMD from 0.005–0.04 μg/ml. However, inhibition of incorporation of ^{3}H-UR into the nucleoli of G1 cells in this dose range follows quite closely the pattern of delay in the onset of DNA synthesis which has been redrawn from the results of fig. 5.

Effects of AMD during mid G1 phase on mouse L cells

Mouse L cells were exposed to two different concentrations of AMD (0.0075 μg/ml and 0.04 μg/ml) either from m h to $m+2$ h or from $m+3$ h to $m+5$ h. The percentage of cells which had entered DNA synthesis by $m+12$ h was measured (table 3). These results suggest that delayed entry into S phase is not affected by the timing of the lower dose of AMD. Exposure to the higher concentration from m to $m+2$ h has a much greater delaying effect than exposure from $m+3$ to $m+5$ h.

Effects of AMD during late G2 phase in mouse L cells

Fig. 7 shows the effect on the onset of DNA synthesis in L cells when AMD at 0.04 μg/ml was given from $m-3$ h to $m-1$ h compared with the standard treatment from m to $m+2$ h. Both treatments seriously delayed the onset of DNA synthesis, but cells eventually entered S phase after exposure to drug during early G1 phase.

Table 2. *^{3}H-TdR uptake into HEp/2 cells after irradiation at 280 nm either between $m+5$ h and $m+7\frac{1}{2}$ h or later than $m+7\frac{1}{2}$ h expressed as a percentage of uptake in matched control cells*

Absorbed dose $\times 10^{-3}$ (erg)	Nucleolar irradiation		Nucleoplasmic irradiation		Cytoplasmic irradiation	
	Between $m+5$ and $m+7\frac{1}{2}$ h	After $m+7\frac{1}{2}$ h	Between $m+5$ and $m+7\frac{1}{2}$ h	After $m+7\frac{1}{2}$ h	Between $m+5$ and $m+7\frac{1}{2}$ h	After $m+7\frac{1}{2}$ h
0.55	30±5	30±7		45±5	100±1	95±2
1.1	25±3	25±3	30±4	30±5	95±3	95±4
2.2	15±5	25±10	20±10	35±10	100	95±9

Comparative experiments with synchronous EMF cells

The results of similar experiments with EMF cells are also presented in fig. 7. Again the onset of DNA synthesis was seriously delayed by both schedules of drug exposure and for early G1 phase cells the block appears to be more effective than for L cells.

Continuous incubation of asynchronous cultures of L cells, EMF cells and HEp/2 cells in 0.04 μg/ml AMD. In preliminary experiments for this section asynchronous cultures of each cell type were exposed to 0.04 μg/ml AMD for 2 h, then pulse labelled with ^{3}H-UdR and fixed immediately.

Results in table 4 show that for all three cell types, this concentration of AMD will cause a highly selective inhibition of nucleolar ^{3}H-UdR incorporation at the end of the 2 h exposure period.

Asynchronous cultures of each cell type were then incubated continuously in the nucleolar-specific AMD concentration of 0.04 μg/ml and the percentage of cells in the DNA synthetic phase at various times was recorded. The results (fig. 8) showed marked variations in the responses of the different cell types.

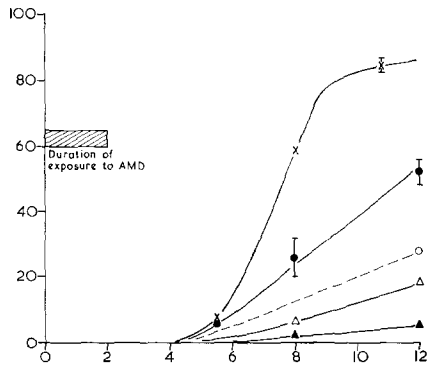

Fig. 5. Abscissa: time (hours) after washing off mitotic cells; *ordinate:* % cells labelled with ^{3}H-TdR. ×—×, control; ●—●, 0.005; ○—○, 0.0075; △—△, 0.01; ▲—▲, 0.04 μg/ml AMD

Measurements on the entry of synchronous L cells into the DNA synthetic phase following different doses of AMD from *m* to *m*+2 h.

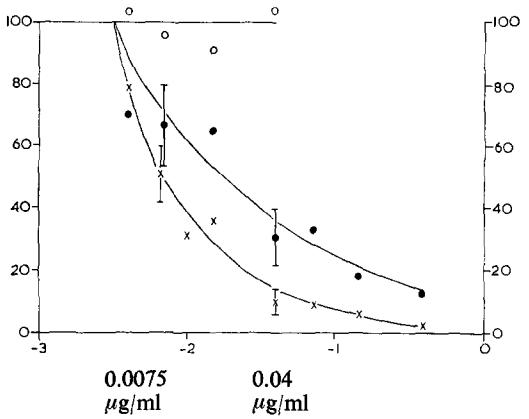

Fig. 6. Abscissa: $\log_{10}$ (AMD conc. in μg/ml); *ordinate: (left)* % ^{3}H-UR uptake relative to controls; *(right)* cells in S phase by 12 h as % of controls.

The figures (0.0075 and 0.04 μg/ml) concern ranges of doses of particular interest.

Effects of exposure of synchronous L cells to different doses of AMD from *m* to *m*+2 h on incorporation of ^{3}H-UR into nucleolus (●) and nucleoplasm (○) at *m*+3 h. ×, % ^{3}H-TdR-labelled cells at *m*+12 h, expressed as % labelled control cells at the same time. Each point has been averaged from the results of 6 expts in the case of low doses of AMD and 2 expts for doses above 0.04 μg/ml.

DISCUSSION

The discussion can most easily be developed by dividing the G1 phase into two parts, considering first those cells which had proceeded more than 1–2 h beyond mitosis at the time of treatment, and secondly those cells which had not reached this stage when treated.

Results of the microbeam experiments relate to cells in the first group. Fig. 2 confirms the observation of Perry et al. [17] that it is possible to choose a dose of UV radiation which, when delivered to the nucleoli, will severely interfere with nucleolar ^{3}H-UR incorporation but will have very little effect on that process when delivered to nucleoplasmic regions. On the other hand the same absorbed dose of radiation delivered either to nucleoli or nucleoplasm has only

Table 3. *Effects of doses of AMD which cause little nucleolar inactivation (0.0075 μg/ml) and substantial nucleolar inactivation (0.04 μg/ml) on the entry of synchronised L cells into S phase*

Results are averaged over 12 expts

	% cells labelled with ^{3}H-TdR at $m+12$ h	
	Drug from m to $m+2$ h	Drug from $m+3$ to $m+5$ h
Control	81±4	83±4
0.0075 μg/ml AMD	35±8	41±4
0.04 μg/ml AMD	10±2	33±3

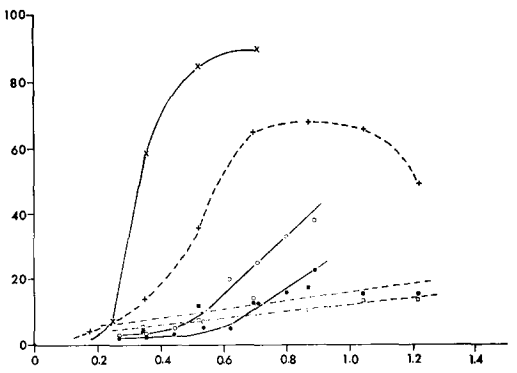

Fig. 7. Abscissa: time after mitosis (in fractions of intermitotic time); *ordinate:* % ^{3}H-TdR-labelled cells. Effects on entry into the next DNA synthetic phase of exposure to 0.04 μg/ml AMD during very late G2 phase compared with exposure during very early G1 phase. Synchronous L cells ×, Control; ○, AMD from m to $m+2$ h, ●, AMD from $m-3$ to $m-1$ h; synchronous EMF cells + Control; □, AMD from m to $m+\frac{3}{4}$ h; ■, AMD from $m-1\frac{1}{2}$ to $m-\frac{3}{4}$ h.

a small effect on nucleoplasmic ^{3}H-UR incorporation. It is, therefore, reasonable to conclude that as far as ^{3}H-UR metabolism is concerned the UV microbeam is able to cause appreciable nucleolar inactivation with a fairly high degree of specificity within the dose range $(10–30)\times10^{-4}$ ergs/nucleus.

When these doses of radiation were delivered to either the nucleoli or nucleoplasm of HEp/2 cells in late G1 phase or early in S phase and the effects on DNA synthesis were measured, results confirmed those of Dendy & Smith [12]. For irradiation at about this time in the cell cycle it is not possible to find a dose of UV which will have a greater effect on ^{3}H-TdR incorporation when delivered to the nucleoli than when delivered to the nucleoplasm.

Irradiations were performed even earlier in the cell cycle to study specifically the onset of S phase; thus HEp/2 cells were irradiated $1\frac{1}{2}$–3 h, and MK cells as early as 1–2 h after mitosis with wavelengths which would be preferentially absorbed either in nucleic acid bases (260 nm) or in certain amino acids in proteins (280 nm). In none of these situations was it possible to find a selective effect of nucleolar irradiation on ^{3}H-TdR incorporation, nor was it possible to find a selective effect on the total DNA content in HEp/2 cells several hours after irradiation at 280 nm between $1\frac{1}{2}$ and 3 h after mitosis.

Because cells require some time to spread out on the quartz surface after cell division, it is not possible for them to be irradiated earlier than $m+1\frac{1}{2}$ h in the case of HEp/2 cells or $m+1$ h in the case of MK cells. Whether or not one can exclude nucleolar control over the onset of DNA synthesis at very early stages of the cell cycle has therefore been investigated using AMD. Earlier work by Rickinson [8] has shown that there was no significant effect on nucleolar ^{3}H-UR uptake when asynchronous cultures of L cells were exposed to AMD at concentrations of 0.005–0.0075 μg/ml. The results shown in fig. 5 where it was possible to delay appreciably the onset of DNA synthesis if these very low drug concentrations were administered from m to $m+2$ h, appeared to have dissociated this effect of the drug from its action upon nucleolar RNA synthesis. However,

Table 4. *Effects of a 2 h incubation with 0.04 μg/ml AMD on the incorporation of ^{3}H-UR into asynchronous cultures of various cell types*

Cell type	Control (grains)		Treated (grains)	
	Nucleolus	Nucleoplasm	Nucleolus	Nucleoplasm
L cells	7.3	14.5	2.2	14.7
EMF cells	5.6	23.7	1.6	25.6
HEp/2 cells	7.6	19.6	1.7	20.1

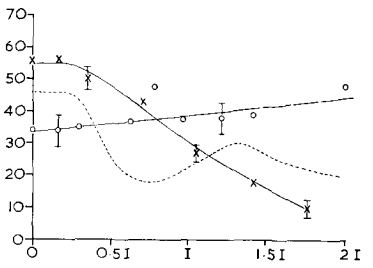

Fig. 8. Abscissa: time in AMD (in fractions of intermitotic time); *ordinate:* % ^{3}H-TdR-labelled cells. Effect of continuous incubation in 0.04 μg/ml AMD on the percentage of cells in an asynchronous culture labelled during a brief exposure to ^{3}H-TdR. ×, EMF; ○, HEp/2 cells; ····, L cells redrawn from Rickinson [8].

measurements of nucleolar ^{3}H-UR uptake by synchronous cells shortly after a 2 h drug exposure very early in G1 phase (fig. 6), suggest that these cells are more sensitive than cells in other parts of the cycle. When a strict comparison is made for cells of the same age, the inhibition of nucleolar ^{3}H-UdR labelling in early G1 phase cells and the inhibition of their subsequent entry into DNA synthesis show a similar dependence upon the AMD concentration from m to $m+2$ h and thus the two effects could be related.

From fig. 6 the effects of a 2 h exposure to 0.0075 μg/ml AMD on nucleolar incorporation of ^{3}H-UR are measurable but small, whereas a 2 h exposure at 0.04 μg/ml causes substantial nucleolar inactivation. These drug concentrations were therefore chosen for the experiments summarised in table 3 and show that the percentage of cells in S phase by 12 h is not affected by the timing of drug treatment when using the lower dose which causes little inactivation of nucleolar ^{3}H-UR uptake. Treatment with a dose which strongly inactivates nucleolar uptake however reduces substantially the percentage of cells in S phase by 12 h if given for the first two hours after mitosis. The small delay in the onset of DNA synthesis following a 2 h treatment with 0.0075 μg/ml AMD at either time or with 0.04 μg/ml AMD from $m+3$ h to $m+5$ h appears to reflect an action of the drug which must be distinguished from the effects when very early G1 phase cells are exposed to the higher drug concentration.

Experiments to compare the effects of exposures to AMD at 0.04 μg/ml either in early G1 phase or late in the previous G2 phase are shown in fig. 7. A marked disturbance of entry into DNA synthesis was observed in each case for both cell types, the effect of exposure in the late G2 phase being at least as great as that in early G1 phase for EMF cells and even greater in the case of L cells. Interpretation of these results is difficult because a 'pulse exposure' in the true sense, is not possible with AMD which binds strongly to DNA and cannot be removed by simple washing procedures [18]. Our own observations upon autoradiographs of mouse L cells labelled for 2 h with 0.04 μg/ml ^{3}H AMD and then returned to normal medium suggest that the level of ^{3}H label falls to 50% after about 5 h. Since the binding of this label to DNA is known to persist during cell division [19], the progeny of cells exposed to the drug in G2 phase probably still contain a considerable amount of AMD during the next interphase. For this

reason the similarity in response to the two drug schedules shown in fig. 7 could again relate to a specific action of the drug in early G1 phase. On the other hand it could mean that AMD-sensitive events necessary for undisturbed entry into S phase also occur at the end of the previous G2 phase.

If nucleolar activity plays an important role in the progression of cells through the cell cycle and into the DNA synthetic phase in particular, then continuous incubation of asynchronous cultures in a nucleolar specific concentration of AMD (deduced from table 4) should cause marked changes in the distribution of cells in the various phases of the cell cycle. Moreover if cellular growth is equally dependent upon nucleolar activity in a number of different cell types then the effects of such incubation should be similar in each case.

The effects of continuous incubation of asynchronous cultures in 0.04 μg/ml AMD on the percentage of cells in the DNA synthetic phase are shown in fig. 8 and are strikingly different for the three cell types studied. The curves for EMF cells and L cells show a similar response in the early stages with the first detectable reduction in the percentage of cells in S phase at the end of a time interval which corresponds approximately to the length of the G1 phase. Rickinson has shown for L cells [8] that at this time cells are leaving S phase for G2 at the normal rate but entry into S phase from G1 has been blocked. The rise some hours later in the L cell curve reflects a substantial recovery of entry into DNA synthesis which does not appear to be shown by EMF cells. In contrast, the percentage of HEp/2 cells in S phase does not seem unduly affected by continuous exposure to this level of AMD for at least one generation time.

This increased disturbance of the cell cycle from HEp/2 cells to L cells to EMF cells, is similar to observations of Studzinski & Ellem [20] who noted that heteroploid cells showed a greater growth capacity than diploid cells when ribosomal RNA synthesis was preferentially depressed by puromycin aminonucleoside. In both sets of experiments the observed differences may be a consequence of the degree of constraint which nucleolar inhibition places upon different types of cell, particularly in the case of diploid EMF cells whose natural rate of ribosomal RNA turnover may be higher than that of heteroploid cells [21].

All the experiments reported here confirm by two independent approaches the earlier suggestion that, when a cell has proceeded more than 1–2 h into the G1 phase, any interruption of normal nucleolar function will not prevent DNA synthesis starting on time and proceeding at the normal rate. However it has not been possible to dissociate completely the disturbances of nucleolar function at about the time of cell division and delays in the onset of the next DNA synthetic phase. The results must therefore lend further support to the suggestion that at least for EMF and L cells subribosomal particles produced in very early interphase and possibly those made in the previous G2 phase and inherited from the parent cell are important for the initiation of DNA synthesis. The extent to which their depletion can disturb the cell cycle may be different for the two cell types.

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