**MODE OF ACTION OF AMPHOTERICIN B ON CHICK EMBRYO FIBROBLASTS AND ON MOUSE EHRLICH TUMOUR CELLS: A CYTOLOGICAL AND CYTOCHEMICAL ANALYSIS**

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**SUMMARY**

Chick embryo fibroblasts cultivated *in vitro* and Ehrlich ascites tumour cells (*in vivo* or *in vitro*) have been treated with amphotericin B. Cell multiplication is strongly inhibited. Large clear zones appear in the fibroblast nucleoli (phase-contrast and electron-microscope observations). Many treated fibroblasts and tumour cells have a high DNA content (pre-mitotic or polyploid level; measurements by cytophotometry). However, the RNA content (cytophotometry) and the total protein content (cytophotometry and micro-interferometry) are relatively low in the tumour cells. As shown by autoradiography, DNA synthesis is active but RNA synthesis and, in some cases, protein synthesis are inhibited. Due to this unbalanced growth, the cells cannot divide.

**INTRODUCTION**

Amphotericin B (Am. B; Fungizone Squibb) is an amphoteric polypene fungicide from *Streptomyces nodosus*. It is used for the treatment of mycosis (Dunn, McMillen & Cornbleet, 1963; Montes & Wilborn, 1968; Montes et al. 1971; Bindschadler & Bennet, 1969) but is toxic however, causing renal damage and various other effects (Halde, Newcomer, Wright & Sternberg, 1957; Bindschadler & Bennet, 1969; Douglas & Healy, 1969, and others). The remission of an astrocytoma has been observed by Rubissow (1970) after a treatment with Am. B. In tissue culture, Am. B protects against fungi but is quite toxic for HeLa cells, mouse fibroblasts and Ehrlich tumour cells if the concentration is above 25 μg/ml (Perlman, Giuffre & Brindle, 1961; Perlman & Brindle, 1963; Laskey, 1970). In fungi, Am. B inhibits glycolysis and the incorporation of guanine or uracil (Gale, 1960, 1963; Kobayashi et al. 1972; Medoff et al. 1972). Uridine incorporation is inhibited by Am. B in various mammalian cell lines (Amati & Lago, 1974).

The effects of Am. B on membranes are particularly interesting. It can indeed increase the permeability of artificial membranes by combination with cholesterol (Weissmann & Sessa, 1967; Andreoli, 1974; Andreoli & Monahan, 1968; Andreoli, Dennis & Weigl, 1969; Demel, Crombag, Van Deenen & Kinsky, 1968; Cass, Finkelstein

Only few cytological and cytochemical observations related to the cellular effects of Am. B in higher vertebrates have been published yet. For this reason, the present work was devoted to the cytological and cytochemical analysis of chick embryo fibroblasts and mouse Ehrlich tumour cells treated with Am. B. The normal fibroblasts were cultivated *in vitro*; the tumour cells were grown either *in vitro* (cell cultures) or *in vivo* (as an ascites in the mouse peritoneum). Preliminary observations have been published: F. De Paermentier and R. Bassleer, *C.r. hebdom. Séanc. Acad. Sc., Paris* 273, 2285–2286 (1971) and A. Lepoint, F. De Paermentier, R. Bassleer and Cl. Desaive, *Congrès International d'Histochemie, Tours*, 1973 (in Press).

**MATERIAL AND METHODS**

**Tissue cultures (in vitro)**

Chick fibroblasts directly prepared from embryos were cultivated on Maximow slides in hanging drop (1 drop cock plasma 1/8, 1 drop chick embryo extract 1/3, 1 drop foetal bovine serum 1/4, 1 drop Tyrode-glycosol solution). Fibroblasts to be studied by electron microscopy were cultivated in Leighton tubes, after trypsinization, in a liquid medium (40 % NCTC 109 medium, 10 % chick embryo extract, 10 % foetal bovine serum, 40 % Tyrode-glycosol and 100 U./ml penicillin). Every two or three days, the cells received fresh nutrient medium after rinsing with an isotonic saline solution at 37°C.

Hypertetraploid Ehrlich ascites tumour cells taken from C57 BI mouse peritoneal cavity were washed in undiluted calf serum at 37°C, centrifuged and then immediately cultivated in Leighton tubes (40 % NCTC 109 medium, 40 % Hanks' solution, 20 % foetal bovine serum, 100 U./ml penicillin, 5 % CO₂ and 95 % air). Every day, the nutrient medium was changed. Amphotericin B (10⁻³–10⁻¹ M final concentration) was added to the culture medium 2 days after the beginning of cultivation (duration of treatment: between 6 hours and 4 days).

**Ehrlich ascites tumour cells propagated in the mouse (in vivo)**

Ten million hypertetraploid Ehrlich ascites tumour cells were injected into the peritoneal cavity of female C57 BI mice. Four days after the inoculation, the mouse received one intraperitoneal injection of Am. B (50, 100, 200, 300, 500 or 1000 μg). The tumour cells were studied a few hours and up to 13 days after the treatment.

**Cytological observations**

Living cells were examined at 37°C with a phase-contrast microscope. Some cells were fixed with osmium tetroxide vapour and ethanol (80 vol. %) or in absolute ethanol-acetone (1/1) at 4°C for 24 h. They were then stained with haematoxylin according to Von Möllendorff or with toluidine blue. Other cells were fixed with saline formol and then stained with Soudan IV for the detection of lipids. Ehrlich ascites tumour cell nucleoli were stained with toluidine blue after digestion with
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neutral deoxyribonuclease (DNase) at 37°C (if this digestion is omitted before staining, nucleoli are hardly distinguishable in this material).

Cells to be studied by electron microscopy were collected by scraping from Leighton tubes and then centrifuged (5 min at 1000 rev/min). Fragments of the pellets were fixed with glutaraldehyde (2.5% in phosphate buffer) and then postfixed with 1% osmium tetroxide. The cells were embedded in Epon 812. Ultrathin sections (Reichert OMU3 microtome) were contrasted with uranyl acetate and lead citrate. The observations were performed with a Siemens Elmiskop 101 or a Philips EM301 at 80 kV.

Mitotic activity, cell number and cell volume

The mitotic activity was estimated by establishing the mitotic index (number of mitotic cells per 1000 cells). In the case of Ehrlich ascites tumour cells cultivated in vitro, they were first resuspended by trypsinization and smeared; the mitotic index was then calculated.

The number and volume of Ehrlich tumour cells and of fibroblasts in suspension were measured with an electronic Coulter counter (model B) equipped with the model J plotter.

Cytochemical methods

Cells were treated by the Feulgen reaction (HCl 3.5 N hydrolysis during 20 min at 37°C). The DNA content was measured in individual cells by absorption cytophotometry in visible light (543 nm) with the GN2 scanning micro-densitometer from Barr and Stroud.

Other cells were stained with naphthol yellow S according to Deitch (1955). The total protein content was measured in individual cells by absorption cytophotometry in visible light (435 nm) with the GN2 scanning micro-densitometer from Barr and Stroud. In order to measure the DNA content and the total protein content in the same cells, the latter were successively stained by the Feulgen reaction and then with naphthol yellow S (Deitch, 1955).

Cells were fixed with Carnoy solution and stained with gallocyanin according to Einarson (1951) after digestion with neutral DNase. The RNA content of these cells was measured by cytophotometry (at 350 nm) with the GN2 micro-densitometer (Sandritter, Kiefer & Rick, 1966).

The total dry mass was measured in the cells by micro-interferometry. Cells which had been fixed with ethanol-acetone were mounted in distilled water and then measured with the M86 interferometer from Vickers (a scanning and integrating automatic device).

The incorporation of tritiated thymidine (2 Ci/min), uridine (2.4 Ci/mM) or leucine (0.3 Ci/mM) was studied by histotautoradiography. The final concentration in the culture medium was 10 μCi/ml in each case. After 30 min (thymidine) or 15 min (uridine, leucine), the cells were rinsed in isotonic saline solution and fixed with osmium tetroxide vapour and 80% ethanol. The stripping film method was applied.

RESULTS

Effects on cell multiplication

When chick embryo fibroblasts are treated in vitro with 10^{-9} or 10^{-8} M amphotericin B (Am. B), a high degree of cell death is observed. At 10^{-6}-10^{-10} M, cell multiplication is inhibited. Cells can be maintained in culture for 8 days if the concentration is 10^{-10} M. The multiplication of Ehrlich tumour cells cultivated in vitro is strongly inhibited by Am. B (10^{-9} M). The growth of the ascites is completely stopped in the mouse peritoneum if 1000 μg are injected (1 dose).

Mitotic activity. The mitotic index is progressively lowered when chick fibroblasts are treated in vitro (Fig. 1). Mitoses disappear after 72 or 120 h if the concentration of Am. B is 10^{-9} or 10^{-10} M. After 24 h, a complete block in metaphase is usually observed. In the controls, the mitotic index remains near 20%. When Ehrlich tumour
cells are treated *in vitro* with Am. B ($10^{-8}$ M), the mitotic index is about 6% after 24 and nearly 0% after 48 h against 30% in the controls. The mitotic index in the control ascites tumour cells (*in vivo*) is also around 30%. After one injection into the mouse peritoneum ($50-1000 \mu g$), the mitotic index is about 15% after 24 h and near zero % after 4 or 5 days. No block in metaphase has been detected in these tumour cells.

![Graph](image)

**Fig. 1.** Effect of amphotericin B ($10^{-8}$ M) on mitotic activity and percentage of tetraploid cells (with regard to DNA content) in a population of chick embryo fibroblasts cultivated *in vitro*. A, the mitotic index (number of mitoses per 1000 cells) is indicated on the ordinate; abscissa, duration of treatment. B, percentage of tetraploid cells (post-synthetic or $G_2$ cells) detected by cytophotometry in the same population (ordinate); abscissa, duration of treatment. ●, controls; ○, treated cells.

**Cytological analysis**

*Phase-contrast observations.* Living chick fibroblasts and Ehrlich tumour cells cultivated *in vitro* have been studied at 37 °C (Am. B concentration $10^{-6}$ M). Twelve hours after the beginning of the treatment, several small spherical zones appear in the fibroblast nucleoli (Fig. 5). The latter are usually spherical and denser than in controls. The mitochondria do not seem to be altered. After 48 h, large lipid droplets accumulate in the cytoplasm and compress the nucleus. Some cells die. Similar nucleolar alterations have not been observed in Ehrlich tumour cells (Fig. 7). Lipid droplets also accumulate in these cells before they die.

*Observations after fixation and staining.* The nucleolar clear zones can usually be observed in the treated fibroblasts after toluidine blue staining. Caryorrhexis occurs during cell degeneration. Lipid droplets are detected in the cytoplasm after Soudan IV staining but the nucleolar vacuoles give a negative reaction. The number of nucleoli per nucleus is not altered in either the fibroblasts or the tumour cells.
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Electron-microscope observations. In fibroblasts (Fig. 8) and in Ehrlich tumour cells, the nucleoli contain granules, fibrils and clear circular zones ('fibrillar centres'). The latter are particularly large in control Ehrlich tumour cell nucleoli but are not always visible in untreated fibroblast nucleoli. However, fibrillar centres are much larger after treatment of the fibroblasts with Am.B (Fig. 9). But this nucleolar modification is less clearly observable in treated Ehrlich tumour cells. No other specific alterations have been detected in the treated cells.

Quantitative cytological and cytochemical analysis

*DNA measurements by cytophotometry.* These measurements have been performed in order to study the effects of Am. B on the cell cycle. Under our culture conditions, 20–30% of the fibroblasts are in post-synthesis as regards DNA \((G_2, \text{ Fig. } 1)\). These cells have doubled their DNA content (4 DNA) and are thus ready to divide. The mitotic index in these control cultures is about 20%. As also shown in Fig. 1, 12–72 h

![Graph showing DNA, total protein, and cell volume measurements](image)

Fig. 2. The contents of DNA and total protein, and the volume of Ehrlich tumour cells cultivated *in vitro* and treated with Am. B \((10^{-6} \text{ M, } 48 \text{ h})\) compared with control values. A, B, the DNA contents in arbitrary units (cytophotometry after Feulgen reaction) of control and treated cells, respectively. C, D, the total cell protein contents in arbitrary units (cytophotometry after naphthol yellow S staining) of control and treated cells, respectively. E, F, cell volumes (electronic counter) of control and treated cells, respectively. DNA, protein and cell volume measurements were performed on the same cell populations. Ordinates show the percentages of cells in each class. In A, B, the full lines situated at the top indicate the mean DNA diploid amount measured in leucocytes and the dotted lines indicate the mean post- or pre-mitotic values (hypertetraploid line); 50 cells in interphase were used in each case. In C, D, the dotted lines indicate the mean values found for post-mitotic or mitotic cells; 50 other cells in interphase were measured in each case. In E, F, the full lines indicate the modal values; 25 \(\times\) 10⁶ cells were used in each case.
after the beginning of a treatment with Am. B (10⁻⁴ M), 40–50% of the cells are in G₂. During the same period, the mitotic activity decreases progressively (Fig. 1).

In these treated cultures, 4–14% of the cells are octoploid with regard to DNA (8 DNA) after 48 or 72 h, versus 0% in the controls. According to these results, Am. B can block the cells in G₂ but some of them become polyploid in DNA content. This explains why the mitotic activity diminishes and shows that no inhibition of DNA synthesis occurs. If the concentration of Am. B is lower (10⁻¹⁰ M), the effects are identical but take place later on.

In a population of Ehrlich tumour cells growing in vivo, about 40% are in G₂ and 2% are polyploid (in the latter, the DNA content is twice as high as in G₂ cells). One, 2, 3 or 5 days after an injection of Am. B into the mouse peritoneum (50–1000 μg), 55–70% of the cells are in G₂ and 10–20% become polyploid. The mitotic activity is strongly inhibited, as shown before. When grown in vitro (Fig. 2), the Ehrlich tumour cells actively divide and about 35% of them are in G₂; generally, about 7% are polyploid. After addition of Am. B to the culture medium (10⁻⁶ M, 48 h), 50% of the cells are in G₂ and 27% become polyploid (Fig. 2); the mitotic index is very low (6% against 30% in the controls). The effects of Am. B on the cell cycle are thus the same for the Ehrlich tumour cells as for the fibroblasts.

Cell volume measurements. The measurements of the cell volume were performed with an electronic counter. The modal cell volume (1440 μm³ for the Ehrlich tumour cells, Fig. 2; 600 μm³ for the fibroblasts) is not modified after treatment with Am. B.

Total protein content and total dry mass of the cells. Measurements of the total cell protein content by cytophotometry (after naphthol yellow S staining) and of the total cell dry mass by scanning micro-interferometry have been performed in fibroblasts and in Ehrlich tumour cells.

It is well known that the cell dry mass is usually considered as essentially representing the total cell protein content. We have shown recently that the results obtained in the same material by cytophotometry after naphthol yellow S staining and by interferometry agree very well (Lepoint, De Paermentier, Bassleer & Desaive, 1973). In protein histograms (Fig. 2) as well as in dry mass histograms related to actively multiplying cells, the cell content varies between minimum values (post-mitotic cells) and doubled values (pre-mitotic cells). This fact is due to the duplication of the amount of total proteins occurring in cells preparing for division.

In Ehrlich tumour cell populations treated in vitro with Am. B (10⁻⁴ M, 48 h; Fig. 2), the total cell protein content is nearly the same as in controls but the percentages of cells with a relatively low content are a little higher. The cell dry mass histograms are also nearly identical with the controls. DNA measurements performed on other cells from the same treated populations however showed an increase of the percentages of cells in G₂ (as to DNA) and of polyploid cells. Cell protein and dry mass measurements performed in fibroblasts treated with Am. B (10⁻⁶ M, 24 h) gave the same conclusions as for the tumour cells.

Ehrlich tumour cells (treated in vivo 3 days before with 1 mg Am. B) were successively stained by Feulgen reaction and by naphthol yellow S. The total protein content and the DNA content were successively measured in the same cells. In the
controls (for details, see Lepoint, Bassleer & De Paermentier, 1974; Lepoint et al. 1973), there is a strong correlation between these 2 cellular parameters. In other words, the total protein content and the DNA content quite simultaneously double during the cell cycle in these Ehrlich tumour cells; the ratio total protein/DNA remains constant or nearly so during the preparation for mitosis. After treatment with Am. B, this ratio becomes much lower in many cells: some cells with a post-mitotic total protein content have a doubled or even quadrupled DNA content; others have a pre-mitotic (doubled) protein content and a pre-mitotic (doubled) or polyploid (quadrupled) DNA content. Only those with a pre-mitotic DNA and protein content divide; indeed, only cells with such a protein and DNA content have been found in mitosis.

Fig. 3. Relation between content of DNA and of total protein in fibroblasts treated with Am. B (10^-6 M, 24 h) and in corresponding controls. Abscissa, total protein content in arbitrary units (cytophotometry after naphthol yellow S staining); ordinate, DNA content in arbitrary units (cytophotometry after Feulgen reaction). Each point corresponds to the values obtained for the same cell by the 2 methods. ○, control cells in interphase; ●, treated cells in interphase. Fifty cells were used in each case.

Fibroblasts which had been treated with Am. B (10^-6 M during 24 h) were studied by the same method (Fig. 3). In the majority of the treated cells, the ratio total protein/DNA is much lower than in controls: many cells have a minimal (post-mitotic) protein content but a doubled or even higher DNA content. This effect (unbalanced growth) is thus the same as in the tumour cells.

Total cellular RNA content. These measurements were performed by cytophotometry in Ehrlich tumour cells and in fibroblasts cultivated in vitro. As shown in
Table 1, the variations found in the 2 cell types for the cellular RNA contents are quite large because, as is known, this cellular parameter doubles during preparation for mitosis. After treatment with Am. B, cells with a relatively low RNA content are more frequent than in control populations. Some inhibition of RNA synthesis thus occurs in these treated fibroblasts and tumour cells.

Analysis of the incorporation of nucleic acid and protein precursors by autoradiography. This analysis was performed in cultured cells (fibroblasts and Ehrlich tumour cells) treated with $10^{-6}$ M Am. B for 24 h.

Table 1. Mean cellular RNA content in controls and cells treated with Am. B

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control, a.u. ± S.E.</th>
<th>Treated with Am. B, a.u. ± S.E.</th>
<th>Test of Kolmogorov-Smirnov</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlich tumour</td>
<td>50.63 ± 23.72</td>
<td>39.82 ± 15.21</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>17.67 ± 4.83</td>
<td>11.30 ± 3.50</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

Ehrlich tumour cells ($10^{-6}$ M Am. B, 48 h) and fibroblasts ($10^{-6}$ M Am. B, 48 h) were subjected to cytophotometry after staining with gallocyanin; the results are expressed in arbitrary units. The observed differences between control and treated cells are statistically significant.

After the treatment with Am. B, the percentages of fibroblasts or Ehrlich tumour cells which have incorporated $[^3H]$thymidine into their nuclei are the same as in the corresponding controls (about 70%); the intensity of labelling of the nuclei is also the same.

After a short time of contact with $[^3H]$uridine (15 min), 42% of the control fibroblasts have incorporated the precursor into their nucleoli; only 2% are radioactive after treatment with Am. B. In the case of Ehrlich tumour cells, only 37% have incorporated $[^3H]$uridine into their nucleoli against 94% in the controls.

Very little inhibition of $[^3H]$leucine incorporation has been detected in treated fibroblasts (57% of the cells are radioactive compared with 63% in the controls). The inhibition is stronger in treated Ehrlich tumour cells: 40% have incorporated $[^3H]$leucine against 76% in the controls.

DISCUSSION

Amphotericin B can be toxic to animal cells; it is shown here that the multiplication of normal or cancer cells is strongly inhibited by this drug under our experimental conditions. Some degree of cell killing has also been observed.

Morphological nucleolar modifications appear in the fibroblasts under the action of Am. B; they consist of condensation of the nucleolus and the appearance of small spherical zones inside the latter. These zones are in fact 'fibrillar centres', as described by Recher, Whitescarver & Briggs (1969) in non-treated cells deriving from a human adenocarcinoma and by Goessens (1973) in non-treated Ehrlich tumour cells. These fibrillar centres could be related to the nucleolus organizer in Ehrlich tumour cells and in fibroblasts according to Goessens & Lepoint (1974) and Goessens (1974, unpublished). These fibrillar centres are much larger and seem to be more numerous.
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in the fibroblast nucleoli after Am. B treatment. It is interesting to note that incorporation of uridine into the nucleolus is strongly inhibited in these treated cells. Large fibrillar centres also exist in treated Ehrlich tumour cell nucleoli, but they can also be observed in controls.

Under the action of Am. B, many cells (either fibroblasts or Ehrlich tumour cells) are blocked in post-synthesis with regard to DNA content \((G_2\) phase of the cell cycle), others become polyploid in relation to DNA but their volume does not increase. However, the total cell protein content is relatively low in many treated cells. Am. B is thus responsible for unbalanced growth of these cells in which DNA content increases but protein synthesis seems to be inhibited. Their RNA content is abnormally low and incorporation of uridine and leucine is to some extent inhibited; thymidine incorporation is, however, unaffected. The ratio \(\text{total protein}/\text{DNA}\) remains constant or nearly so during the major part of the preparation for mitosis in untreated fibroblasts and Ehrlich tumour cells. Under the action of Am. B, the ratio sharply diminishes, and we think this is the reason why many of these cells fail to divide. Only rare treated cells, with a pre-mitotic DNA and protein content, have been observed in mitosis. However, cells with a \(\text{total protein}/\text{DNA}\) ratio larger than normal can divide, as we have shown before in Ehrlich tumour cells treated with daunomycin (Bassleer et al. 1973).

According to the results in Fig. 3, some protein synthesis would occur in the control fibroblasts before DNA synthesis is initiated (no DNA synthesis occurs in cells with a protein content situated between 30 and 40 arbitrary units).

Similar results have been obtained by Killander & Zetterberg (1965), for mouse fibroblasts (L-929 line) analysed by cytochemical methods. In fibroblasts treated with Am. B, active DNA synthesis occurs in cells with a post-mitotic protein content. This would suggest that, under these experimental conditions, initiation of DNA synthesis is not dependent on a ‘critical’ cell protein content. In actively growing Ehrlich tumour cells, DNA synthesis is initiated a very short time after mitosis. This conclusion has been drawn from results obtained by cytochemical measurements (Lepoint et al. 1974). It agrees with observations made by autoradiography in the same cell type (Hornsey & Howard, 1956; Baserga & Lisco, 1963; Lepoint, 1974, unpublished). It is worthwhile noting that we have observed here a very good correlation between results obtained for the same cellular material by micro-interferometry (cell dry mass) and by cytophotometry after naphthol yellow S staining (cell protein content; see also Lepoint et al. 1974).

The mode of action of Am. B could be as follows. Membrane alterations (may be associated with some loss of ions) would be responsible for the unbalanced cell growth. RNA synthesis (especially in the nucleoli) and protein synthesis are inhibited but DNA synthesis takes place. These cells, relatively small but with a high DNA content, cannot divide and often die, probably because of a lack of important proteins. They are blocked in post-synthesis \((G_2)\) or become polyploid with regard to DNA content. An alternative explanation for the dissociation between cell volume and DNA content could be that the drug is responsible for enhanced protein breakdown. This fact could not be checked with the available cytochemical methods but inhibition
of protein synthesis seems to be more likely since strong inhibition of RNA synthesis has been demonstrated by 2 different cytochemical methods. Under the action of daunomycin or alkylating agents (Bassleer, 1972), cells behave in the same way as far as DNA is concerned but they become very large, with a high protein content and giant nucleoli. In cells treated with Am. B, on the other hand, the volume of the cell and of its nucleoli are not increased; the nucleolar modifications detected in the fibroblasts could be in some way related to the high DNA content of the cells and/or due to the inhibition of nucleolar RNA synthesis. Indeed, we have recently observed similar nucleolar alterations in fibroblasts treated with cordycepine or tubercidine, two inhibitors of RNA synthesis.

The results we have obtained here by different cytological or cytochemical methods agree very well all together: high DNA content (cytophotometry) and unaltered thymidine incorporation into DNA; low cellular RNA content (cytophotometry) and inhibited uridine incorporation into RNA; absence of increase in cell volume (electronic counter) and in cellular total protein content (cytophotometry, interferometry) and some inhibition of leucine incorporation (autoradiography).

No fundamental differences have been detected between normal fibroblasts and tumour cells as far as their sensitivity to Am. B and the major alterations produced are concerned.

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REFERENCES


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(Received 21 January 1975)
Figs. 4–7. Phase-contrast observation of the effects of Am. B on chick fibroblasts or Ehrlich tumour cells cultivated in vitro. × 2180.

Fig. 4. Living chick fibroblasts, non-treated. One of the cells is binucleated.

Fig. 5. Living chick fibroblasts treated with Am. B (10^{-4} M, 48 h). The nucleoli are dense, fairly spherical and contain clear spherical zones.

Fig. 6. Living Ehrlich tumour cells, non-treated. Note the presence of large spherical clear zones inside the nucleoli. This feature is perfectly normal in this material and is observed in actively growing cells.

Fig. 7. Living Ehrlich tumour cells treated with Am. B (10^{-4} M, 48 h). No important alterations are visible.
Fig. 8. Nucleolus of untreated fibroblast. Note the presence of granules and of dense fibrillar zones in the nucleolus. At the bottom, a portion of the nuclear membrane and a thin layer of cytoplasm are visible. $\times 44,000$.

Fig. 9. Nucleolus of fibroblast treated with Am. B ($10^{-4} \text{M}, 24 \text{ h}$). A large circular zone ('fibrillar centre'), surrounded by dense fibrils and granules, is visible inside it $\times 38,000$. 