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Fluorescence Microscope and Electron-Microscope Studies on the Effect of Trenimon on Human Tumour Cells

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Our experience has shown that local chemotherapy is suitable for treatment of effusions of neoplastic origin, on account of its good tolerance and efficacy. Gamma irradiation was often ineffective, diuretic measures were nearly always so (Thom, 1967). A wide spread use of intracavitary radioactive gold is especially complicated by the necessary measures for protection against radiation. Earlier studies showed distinct morphological differences in the effect of local treatment of carcinoses of the serous membranes with various alkylising cytostatics (Thom, 1964). At adequate dosage (determined by the LD50 for rats) Trisaethyleniminobenzochinon = Trenimon was found distinctly superior as regards to the rapidity and intensity of the effect and therefore we used this drug exclusively for local therapy in recent years (Thom, 1965). To obtain data on the optimal dosage and form of application detailed cytological and histological studies were made on cells in the exudate or pleural suction biopsies.

Model experiments on HeLa cell cultures suggested that the cytotoxic effect might be increased by longer exposure to the drug, by means of protracted infusions (Bierling, 1964). In contrast to the favourable conditions in vitro, where the test material is uniform, clinical tests are far more complicated because of the considerable differences in cellular resistance of the various malignant tumours. Our experience with long-term intracavitary infusions in man is still too limited to permit final conclusions. The following findings are limited to large single doses of Trenimon. Usually infusions of 0.015 mg/kg were given intrapleurally or i.p. if the bone marrow was not seriously damaged. We distinguish three different types of administration.

1. The drug was injected into the exudate, which was not aspirated. In this way there was no local irritation or immediate serious disturbance in the patient's subjective condition. In the majority of cases full control of the production of exudate required repeated treatment, e.g. 2-3 times at intervals of a week. In a few cases massive indurations of the pleural cavity developed. This we attribute to the fact that sometimes the entire exudate became jelly-like a few hours after the instillation of Trenimon. If this complication was detected at an early moment injection

of a streptokinase preparation led to liquefaction of the exudate, which could then be aspirated. In this way connective tissue organization could be prevented.

2. If there was distinct depression of the bone marrow, especially in cases of thrombopenia after general cytostatic treatment, we administered smaller doses of 0.2-0.4 mg into the pleural fissure, after aspiration of as much of the exudate as possible. In cases of serious haemorrhagic diathesis we used physiological saline for rinsing after the cytostatic had acted for 30 minutes, to obtain maximal elimination of the drug or its degradation products. This regularly led to primary adhesion of the pleural membrane, sometimes with small encapsulated residual exudates that did not impair the respiratory capacity to any important degree. Sometimes these were demonstrated only at autopsy. This form of treatment was not seen to cause an increase in the haemorrhagic diathesis, which is likely to develop after a single large dose of Trenimon, after 16-18 days, depending on the dose.

3. In most patients good therapeutic results can be obtained with treatment in two phases. If there are relatively large amounts of exudate they are drained until inspiration just results in a negative pressure in the pleural fissure. Then an infusion of 0.015 mg/kg of the cytostatic is given and the residual exudate is aspirated after a few hours. If this aspiration is fractionated information can be obtained on the degree and evolution of the cell damage.

High local concentrations of more than 100 μg% cause marked cytotoxic effects, predominantly in the tumour cells and rarely in the normal cell populations. This selective effect of Trenimon, demonstrated in tissue cultures and on perfusion of isolated tumours with the heart-lung-machine (Rohr and Kersting, 1964; Rohr *et al.*, 1965) apparently also applies to tumour cells suspended in the exudate. However, the results of these animal experiments cannot be applied indiscriminately to man. Intracavitary administration of glycerol aldehyde to patients did not cause damage to the tumour cells or any other therapeutic effect, although Warburg reported that adequate doses caused a 100% recovery in mice with Ehrlich's ascites tumour.

Fractionated aspiration of exudates can be done with a thin ⌀ (2 mm) plastic tube introduced into the body cavity by a puncture cannula. When introduction is sufficiently deep the cannula can be slipped over the tube and removed. We use teflon tubes, stable to temperatures over 400°C, and biologically completely inert. In contrast to PVC and silicone tubes, teflon tubes do not cause changes in the exudate, damage to the cells, and obstructions are avoided. For electron microscopic examination the exudate was directly drained into an isotonic buffered solution of 1.4% glutaraldehyde. For examination with the light microscope we centrifuge the material immediately after collection, before it starts to solidify. Artefacts from fixation cannot be completely eliminated in electron microscopy and we tried to avoid these by supra vital examination of the sediment with combined phase contrast and fluorescence microscope (Haselmann and Wittekind, 1957).

Fluorescencemicroscopical part

Fluorochromation was done with acridine orange purified by chromatography (Zanker, 1952). Like most acridine dyes this has a marked affinity to RNA, DNA and acid mucopolysaccharides (Schümmelfeder *et al.*, 1957; Stockinger, 1964). On account of its concentration dependent metachromasia it causes supra vital staining of the nucleus, nucleolus and various types of cytoplasmic granules and vacuoles in the entire spectrum between green and red. Also, many different types of living cells can segregate and concentrate this supra vital dye with involvement of the RNA (Kedrowski, 1934; Stockinger, 1964; Wittekind, 1959). This leads to the resolution in the light microscope of otherwise non-preformed cytoplasmic structures, first observed by Chlopin (1927) after treatment with neutral red, another alkaline supra vital stain. In analogy to secretory cellular processes these were called the crinome.

It does not seem correct to consider this a granular storage of the dye, as the crinomes may have a fibre-like (Fig. 1)[1] or vacuolar (Fig. 2) structure. Their stability is too low to permit preservation by fixation (Wittekind, 1959). Formation of crinomes is of diagnostic importance as it can be a very sensitive criterium for the

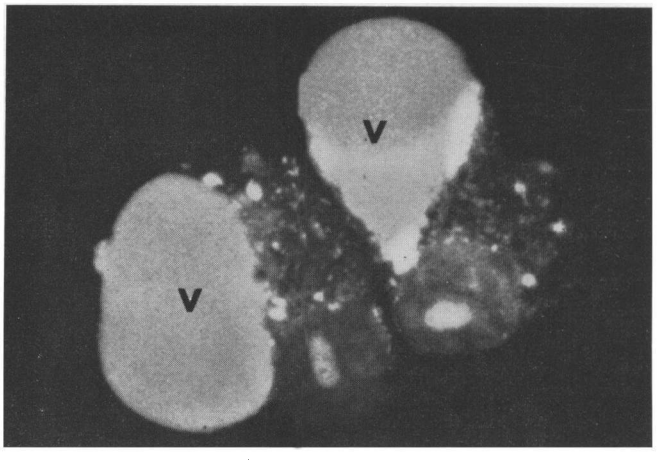

Fig. 1

[1] Explanation of the symbols in the figures:

E Endoplasmatic reticulum
Er Ergastoplasm
Ly Lysosoma
M Mitochondria
N Nucleolus
✱ Nucleolus coarse granular part (RNP granules)
✱ Nucleolus fibrillary part (chromatides)
▽ Nuclear pores
G Golgi apparatus
R Ribosomes (RNP granules)
t Microtubuli

vitality of the cell and often permits easier differentiation of the cells by its characteristic pattern (Braunsteiner *et al.*, 1952; Thom, 1964; Wittekind, 1959; Yasuzumi and Sugittara, 1965).

Opinion on the morphological substrate of these concentrations of the dye, also called coacervates or acridine orange particle (AOP) (Rolbins and Marcus, 1963), vary

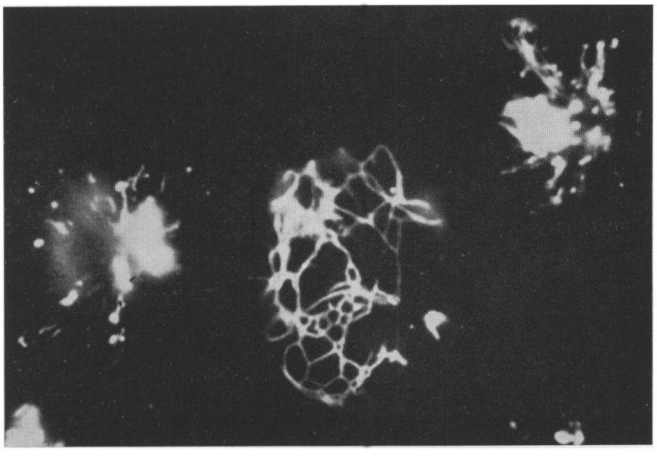

Fig. 2

and is somewhat contradictory. Critical studies showed that AOP and mitochondria are not identical (Rolbins and Marcus, 1963; Wittekind, 1959), although this has been repeatedly maintained (Austin and Bischof, 1959). Some authors think the AOP originates from the Golgi apparatus (Robbins and Marcus, 1963), others reject this (Stockinger, 1964). As well as separation of vacuoles from the membrane system of the Golgi apparatus or unfolding of entire membrane sacs, alveolar dilatation and separation of the endoplasmic reticulum or vesiculation of the plasmolemma are possible (Schmidt, 1962). In some cell types development of storage vacuoles from mitochondria and dilatation of the nuclear membrane have been described (Schmidt, 1963).

A clear differentiation between " paraplasmic granules " secondarily stained with cridin orange and newly formed, non-preformed AOP is of decisive importance for the assessment of pharmacodynamically induced cellular changes. Reliable differentiation is only possible by continuous optical checking during fluorochromation. For this purpose the combined phase-contrast fluorescence microscope (Carl-Zeiss, Oberkochen) mentioned above was found especially valuable.

Fluorescence microscopy can also be combined with dark field, or Nomarski's interference contrast. This causes a considerable reduction in resolving power, brightness of the image and continuous superposed visualization of fluorescent and non-fluorescent structure is not possible.

Well-differentiated carcinoma cells often contain numerous preformed granules

that stain with acridin orange, which makes differentiation from the newly developed crinome granules difficult. The following studies are, therefore, restricted to a case especially suitable for demonstration, where metaplasia of the pleura had resulted from a retothelial sarcoma. The pleural exudate almost exclusively contained sarcoma cells without granules that stained panoptically and with discinct differences in ultrastructure in comparison with the local cells.

Fig. 3 shows the untreated supra vital preparation in a survey photograph. Immediately after collection, acridin orange was added (1/50000). Sarcoma cells are by far the most abundant. They are characterized by the large, numerous and often bizarrely shaped, bright green nucleoli. In many cells the nuclei are difficult to see in the reproduced picture. In cells with a very high concentration of cytoplasmic the nucleoplasm RNA, which cause a strong diffuse green fluorescence, the nucleoplasm is clearly seen as a dark hole. (4) In the right upper part of the picture a cell in anaphase is seen. (3) Like all mitotic cells this shows only very slight granular storage of dyes. The interphase cells show numerous crinome granules, mostly in the nuclear hilus region. (5) In the right upper part of the picture there is one polymorphonuclear granulocyte, and two of these are seen in the right lower corner. (1) The preformed (paraplasmic) granules are so abundant that they give the cytoplasm a red fluorescence, whereas it usually shows a very weak dark-green fluorescence. In histiocytes the nuclei can often not be seen, due to the density of the granules. (2) Picture 4 shows a single cell from the same preparation under a higher power. Due to the small depth of field of objective with great resolution and the three-dimensional overlapping, confluence of the crinome granules is simulated. (1) The cytoplasm is extremely rich in RNA and shows a distinctly stronger nuclear membrane fluorescence than the karyoplasm, so that the nucleus appears darker. Only the nuclear membrane is seen as a bright zone in the tangential aspect. The nucleolar apparatus is enormously increased in size and shows bright green fluorescence. (2) In contrast to the necrobiotic cells in Figs. 5 and 7, and in agreement with the ultrastructure, the nucleoli do not show membrane-like boundaries with the karyoplasm.

Fig. 6 was made 3 hours after intracavitary administration of 1 mg of trenimon. A very early and sensitive sign of the reduced cellular vitality is the almost complete inhibition of development of crinome granules in all sarcoma cells. The cytoplasmic RNA, seen in bright field as basophilia and in the electron microscope as free ribosomes is not appreciably reduced. In contrast to the malignant cells the local cells do not show morphological changes. One histiocyte, (1) one polymorphonuclear granulocyte (2) and two monocytes (3) are seen. It must be pointed out, however, that these cells do not show a reduction of crinoma granules as readily, due to the abundance of preformed granules that stain passively with acridin orange. Characteristic changes preceding necrobiosis can be seen in the nuclei of the tumour cells. Besides a distinct hyperchromasia of the nuclear wall there are characteristic changes especially in the nucleoli, consisting of retiform extrusions and a vague outline. The complete absence of mitoses is also seen in a greater number of cells than shown in the illustration.

Some 6 hours after intracavitary infusion of trenimon all cases so far studied

showed massive infiltration of polymorphonuclear granulocytes and histiocytic elements. The absence of subjective symptoms and the interval between instillation and the development of leucocytosis are arguments against a chemically induced excitation of the serous membranes. The active onset of the phagocytosis rather suggests a chemotactic stimulus from the tumour cells.

In Fig. 7, which was taken 12 hours after the instillation, all tumour cells show distinct signs of cell death. Metachromasia of the nucleoli and outlining by a pseudomembrane (1) homogenization of the nuclear contents (2) inflation necrosis (3) karyorrhexis (4) are seen. The cell in the lower right corner probably corresponds to polymorphonuclear granulocyte with tumefaction necrosis. In contrast to the necrotic tumour cells the great majority of the granulocytes did not show impaired vitality, even in phase-contrast. After 24 hours there are a very few giant tumour cells in addition to the local cell population. In contrast to the early inflation necrosis after trenimon instillation these showed cell death from Strugger's concentration effect (Strugger, 1949; Fig. 5), (the colour of the nucleoli ↙ and the cytoplasm changes to red) (Schümmelfeder, 1950, 1957, 1958).

To summarize: with this method toxic effects can be seen in tumour cells after both local and humoral administration of cytostatics. The particular aspects of the cytoplasmic dye concentrations related to cellular vitality facilitate differentiation between the normal and foreign cell population and also permit control of the chemotherapy of malignant exudates.

Electron-microscopical part

Aethyleneimines not only inhibit the synthesis of nucleic acids by the production of alkyl radicals with positive charge, but they also react with the normal DNA already produced. Although it is known that alkylizing of the N7 atom in the guanine molecules in DNA occurs, and the double strands begin to form a network this primary cell has not been seen thus far in electron-microscopic examination of thin sections. Current methods of preparation do not permit visualization of the macromolecular structural elements of the higher chromosomes in interphase nuclei in such a manner that good assessment of their arragement, especially the teritiary structure, is possible (Gisbrecht, 1965). Thus the electron microscope survey (Fig. 8) shows a nucleus (N) with relatively little structure, in which the sections of three nucleoli * dominate. The cytoplasm (Z) shows considerably greater structural variation with deep invaginations. In metabolically active cells these enlarge the surface of the boundary between the nucleus and the cytoplasm, which favours exchange of substances, and they apparently also have a special relationship to the nucleoli. The ergastoplasm is scarce (Er), as is the case in many malignant tumour cells with marked proliferation. The ribosomes, the morphological substrate of the basophilia of the cytoplasm, are not close to the tubules of the endoplasmic reticulum but lie freely and apparently without order in the cytoplasm. The internal structure of the mitochondria is also characteristic for anaplastic cells; branching of the lamellae system

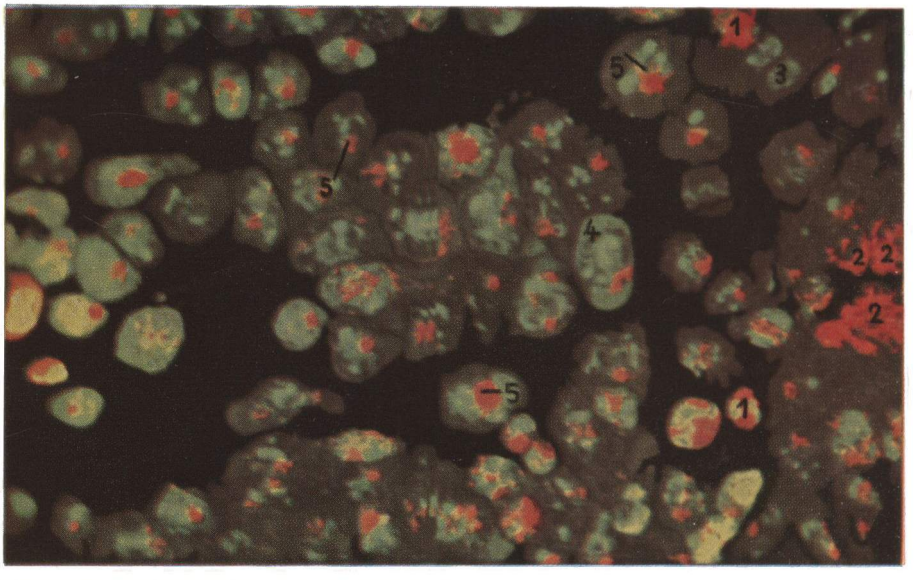

Fig. 3

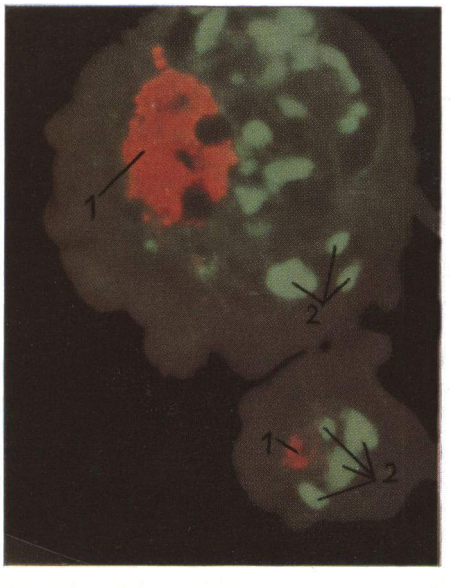

Fig. 4

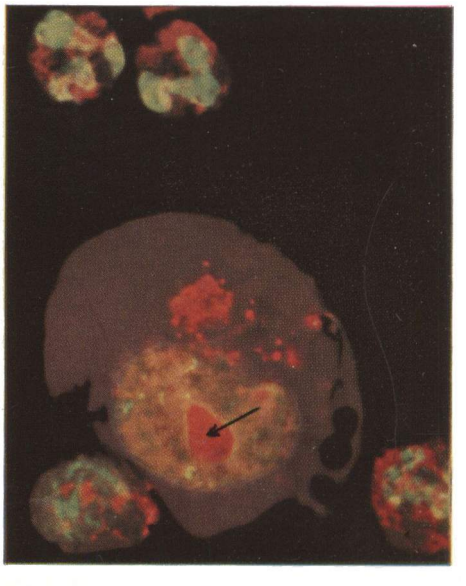

Fig. 5

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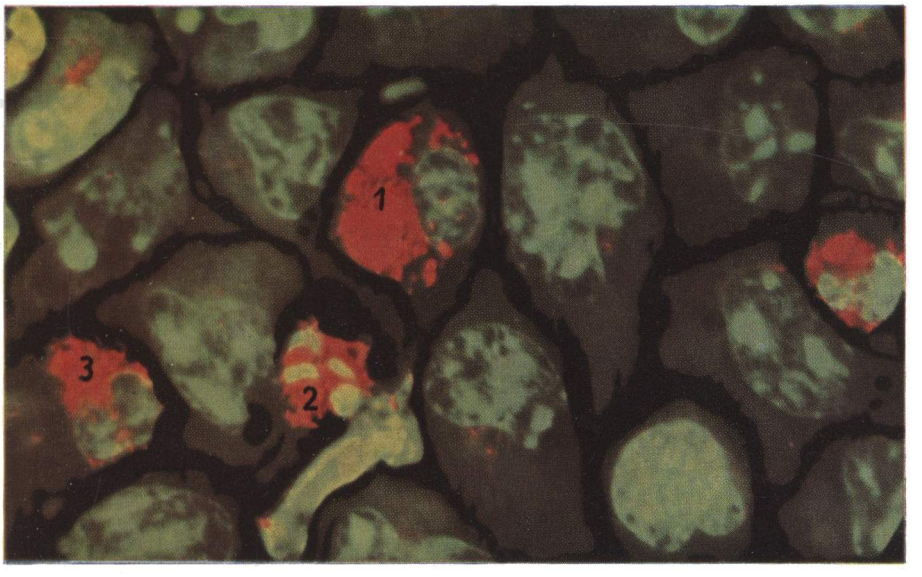

Fig. 6

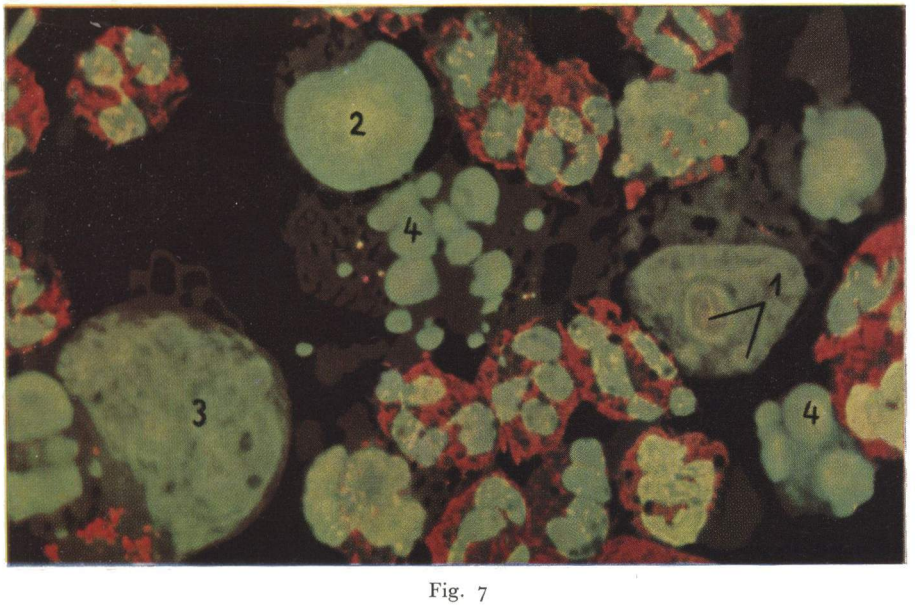

Fig. 7

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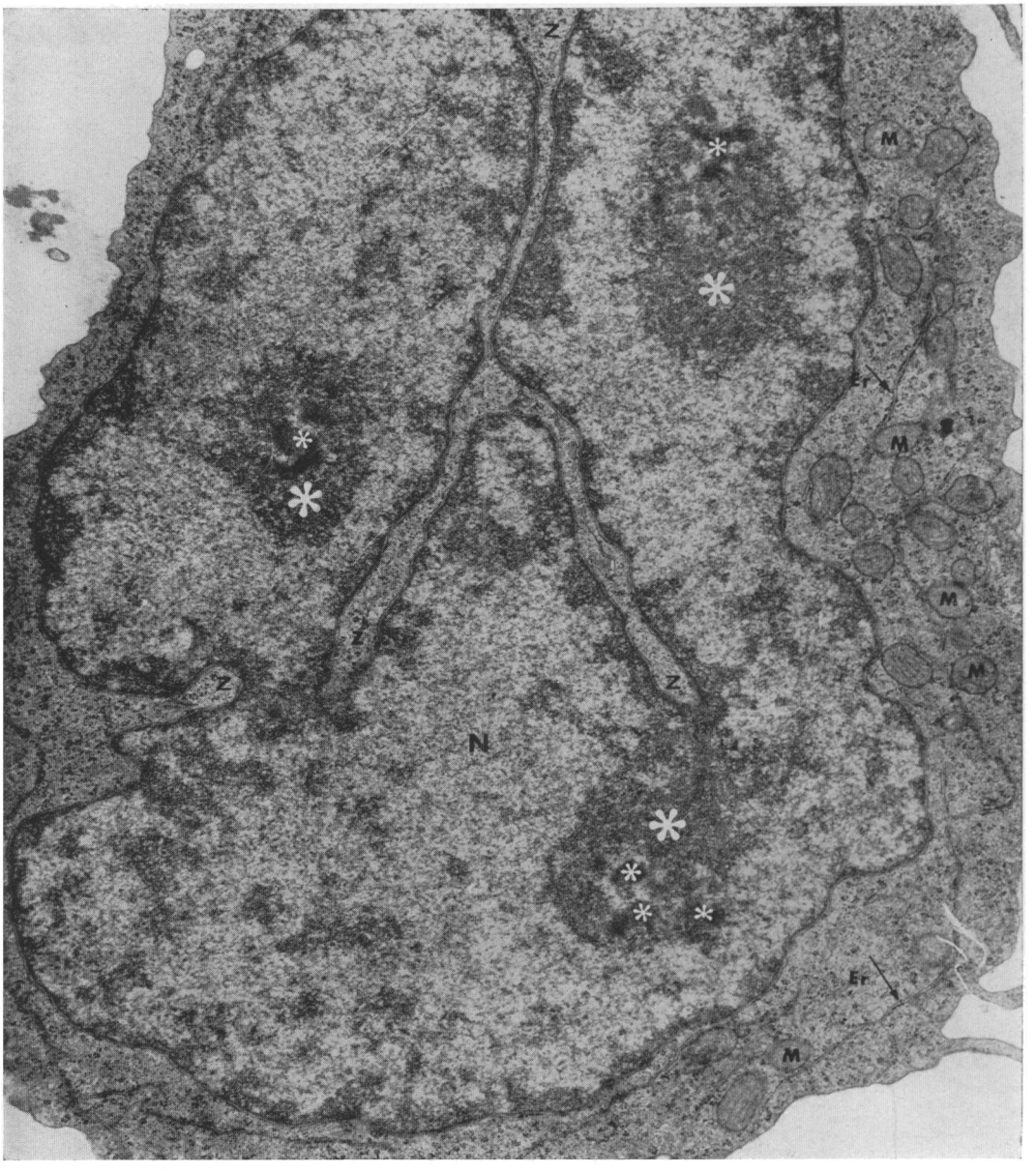

Fig. 8

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is slight in comparison to the matrix. Fig. 9 shows a higher power view of a sarcoma cell prior to chemotherapy. In the nucleolus two parts can now be clearly distinguished. In a coarse granular marginal zone ✱ with electron-dense particles probably identical with ribosomes structures are seen that consist of fine fibrils * lying close together. In transverse section their aspect is one of fine granules. This is the nucleonema (Estable and Sotelo, 1951) which, according to recent studies, is a part of the chromatids (Altmann *et al.*, 1963; Weissenfels, 1966). In the cytoplasmic invagination the double membrane of the nuclear wall shows typical pore complexes (Watson, 1959) with hyalinization of the neighbouring karyoplasm ⍋.

In the first few hours after instillation of trenimon the ultrastructure of the tumour cells does not show evidence of regressive changes (Fig. 10). All organelles and the microtubules (t) are intact, the structure of the Golgi zone (G) and the extrusion of small separated parts of the karyoplasm into the endoplasmic reticulum (↙) rather suggest an increased cell activity. Remarkable changes are seen in increasing numbers at the nucleus-cytoplasm boundary, however. In the terminal parts of the deep cytoplasmic invaginations (Figs. 11 and 12) vacuolar structures with strikingly thickened membranes (E) are seen to form. The round electron-dense particles with a diameter of some 600 Å (↙) have not been described thus far, to our knowledge, and probably they correspond to tangential sections of these membrane swellings. Longitudinal sections of such invaginations show that there is swelling and thickening of the outer nuclear membrane (Fig. 13) and of the endoplasmic reticulum (Fig. 14). At the periphery of the nucleus saccular protuberances of the external nuclear membrane are seen in increasing number (Fig. 15) and pouch-shaped invagination of the internal nuclear membrane is also seen. This may correspond to the double membranes in pre-prophase nuclei (G-2 phase) of Ehrlich's ascites tumour cells described by Yasusumi (1965) and later by Merker (1966) (Fig. 16). The characteristic clear areas in the cytoplasm near the nuclear pore complexes are no longer so distinct at this stage, whereas the fusion of the inner and outer nuclear membranes forming the outine of the nuclear pores persists at first.

In contrast to other authors (Debiasi and Pajadia, 1965) we did not observe ultrastructural changes in the nucleoli in this early stage that permitted the conclusion that the regressive process has occurred. Depending on the interval between instillation of trenimon and collection of the exudate there was an increasing percentage of nucleoli that were in contact over a great part of their surface with the lateral terminal parts of the cytoplasmic invaginations (Fig. 17).

Unlike the relatively uniform structure of the pore complexes the double membrane of the nucleus showed irregular loosening in this area. It also showed large interruptions not attributable to tangential sectioning, as shown by serial sections.

Insofar as fixed preparations permit conclusions on cellular kinetic processes it may be concluded that in this area swarms of ribosomes (R) are released into the cytoplasm (Fig. 18, a high-power view). These observations are in agreement with the findings of studies with the light microscope and with radiobiological data, which show that in the early phase of radiation damage transfer of nucleolar substance into

Fig. 9

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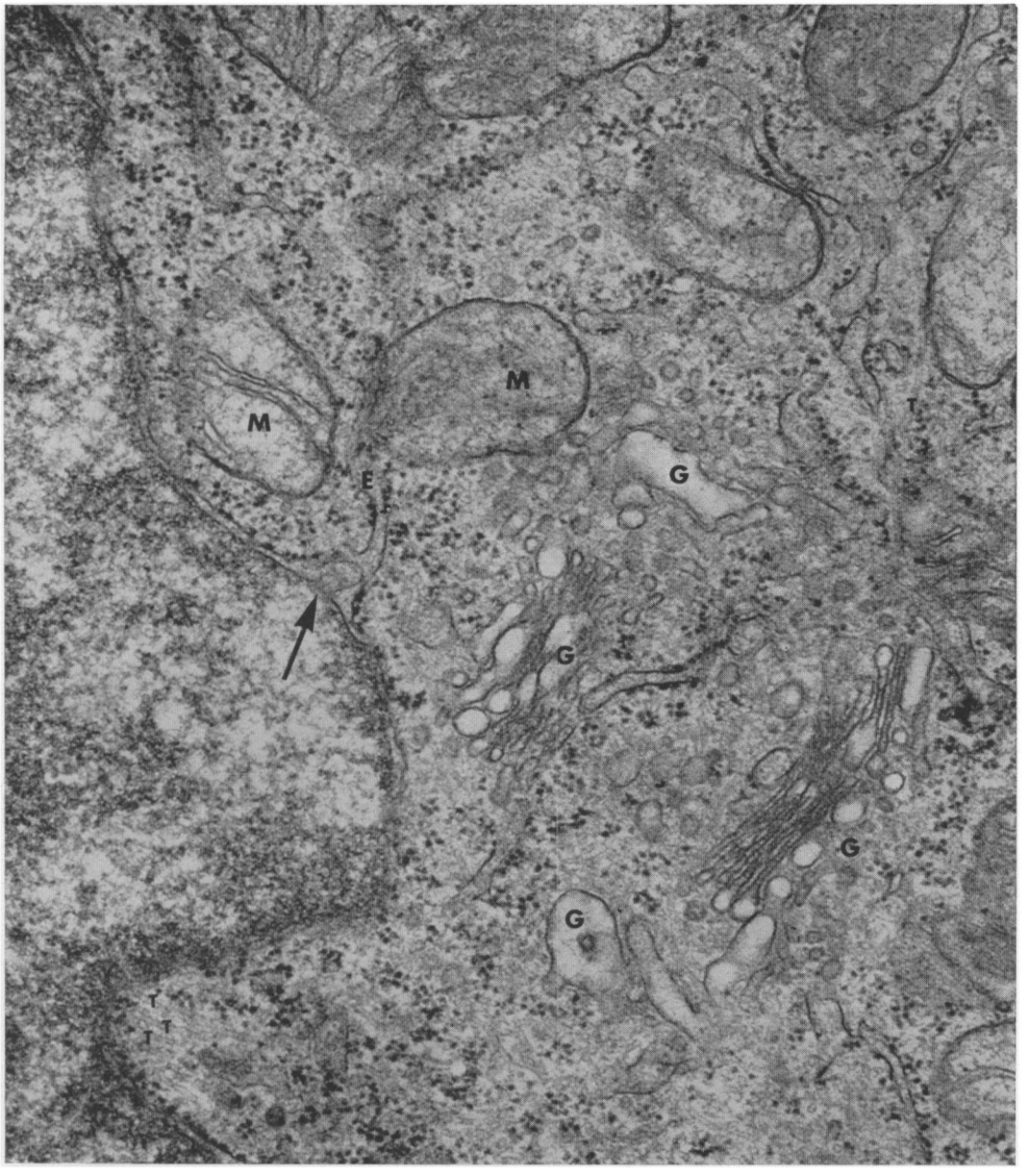

Fig. 10

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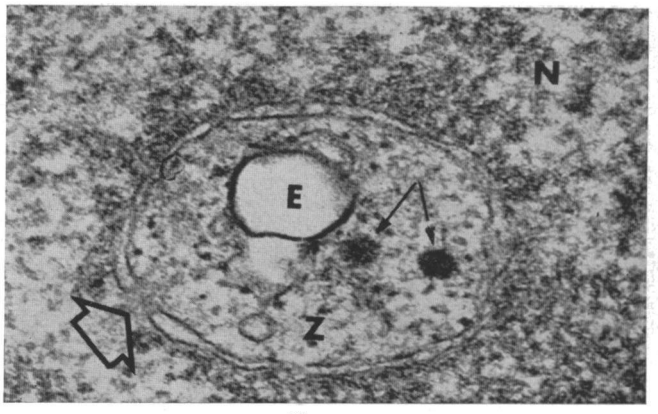


Fig. 11

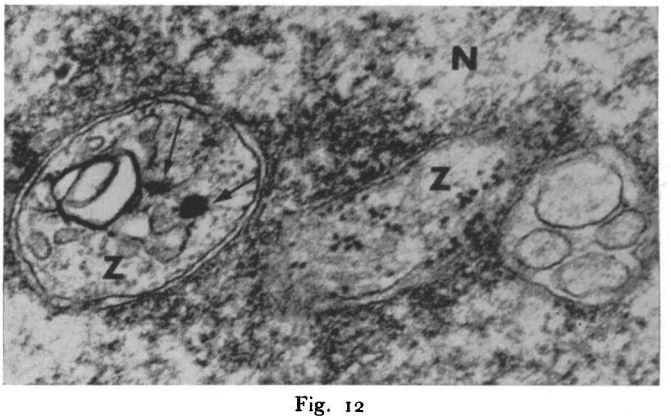
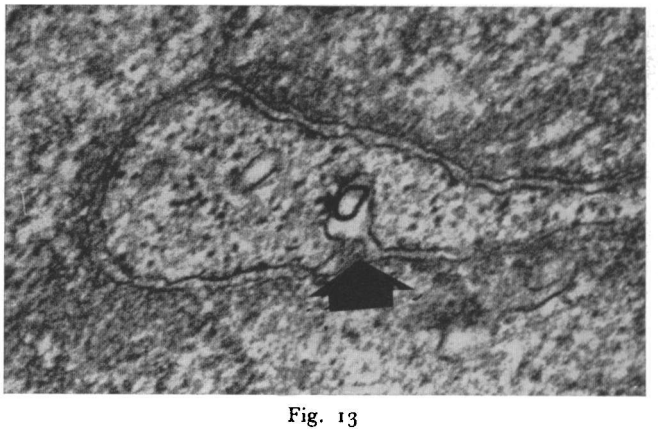


Fig. 12



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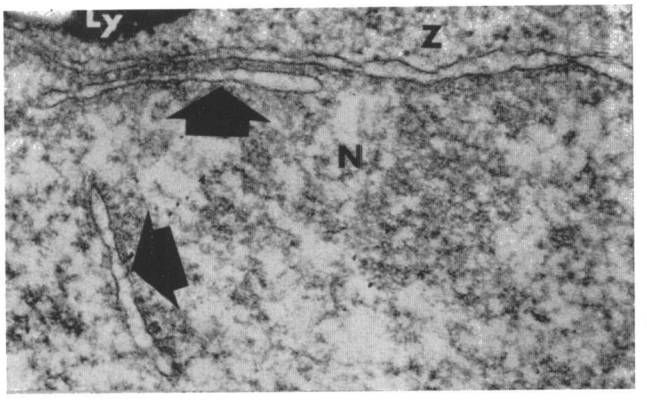


Fig. 14

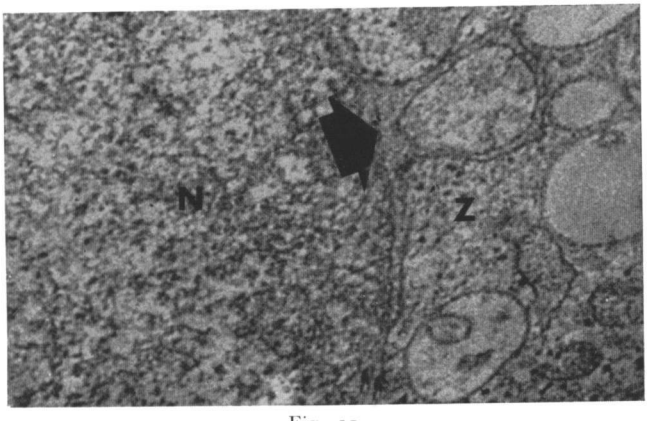
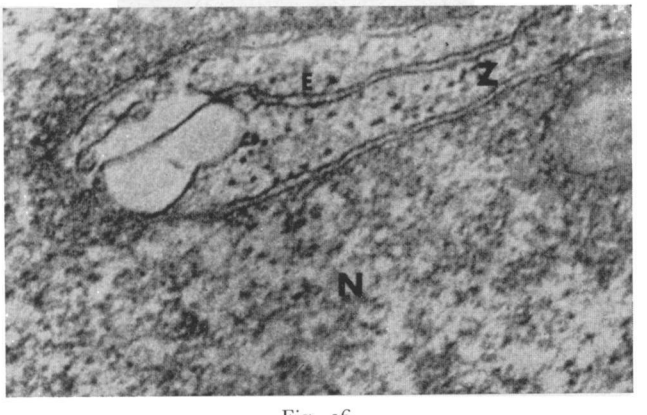


Fig. 15



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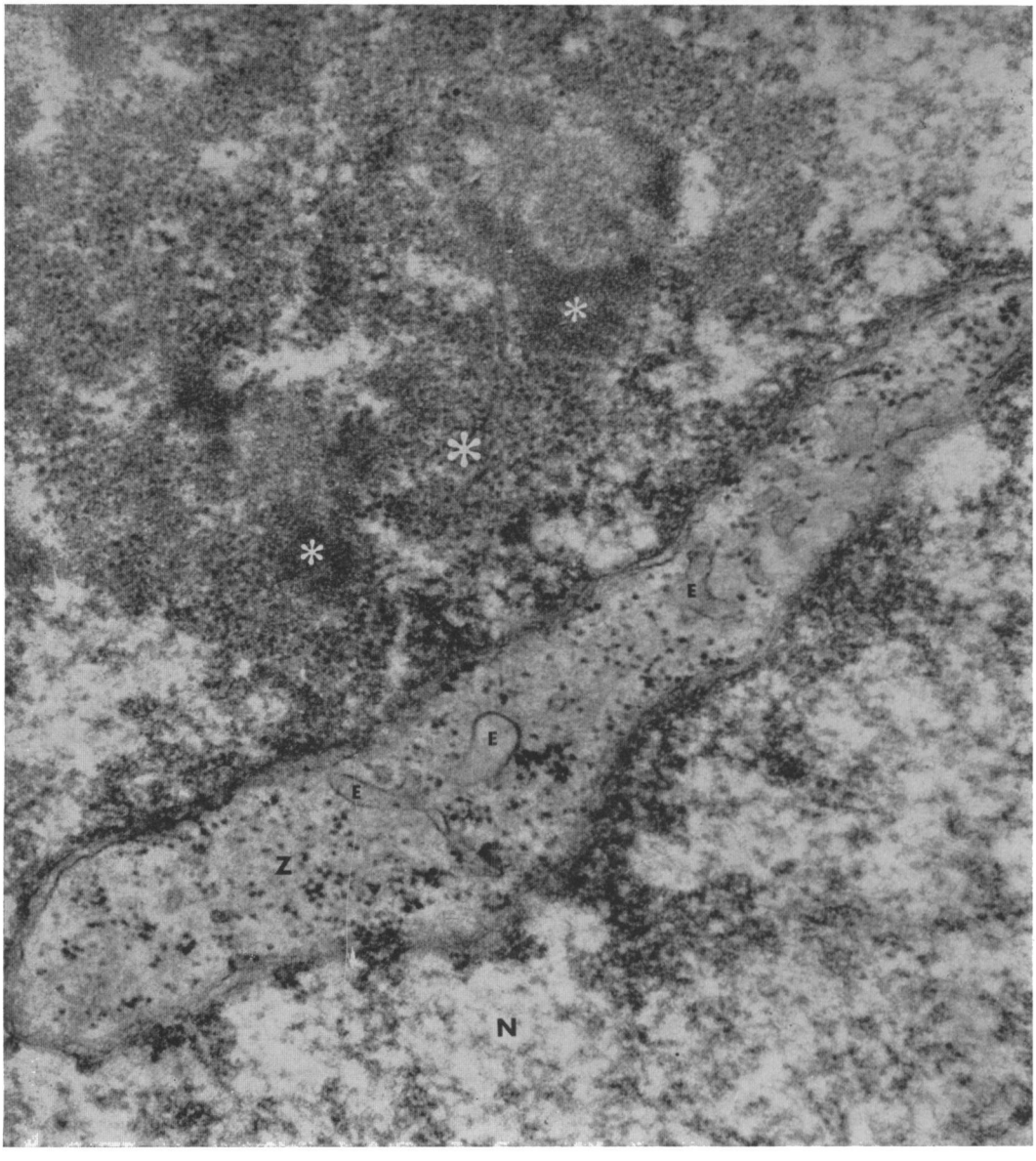

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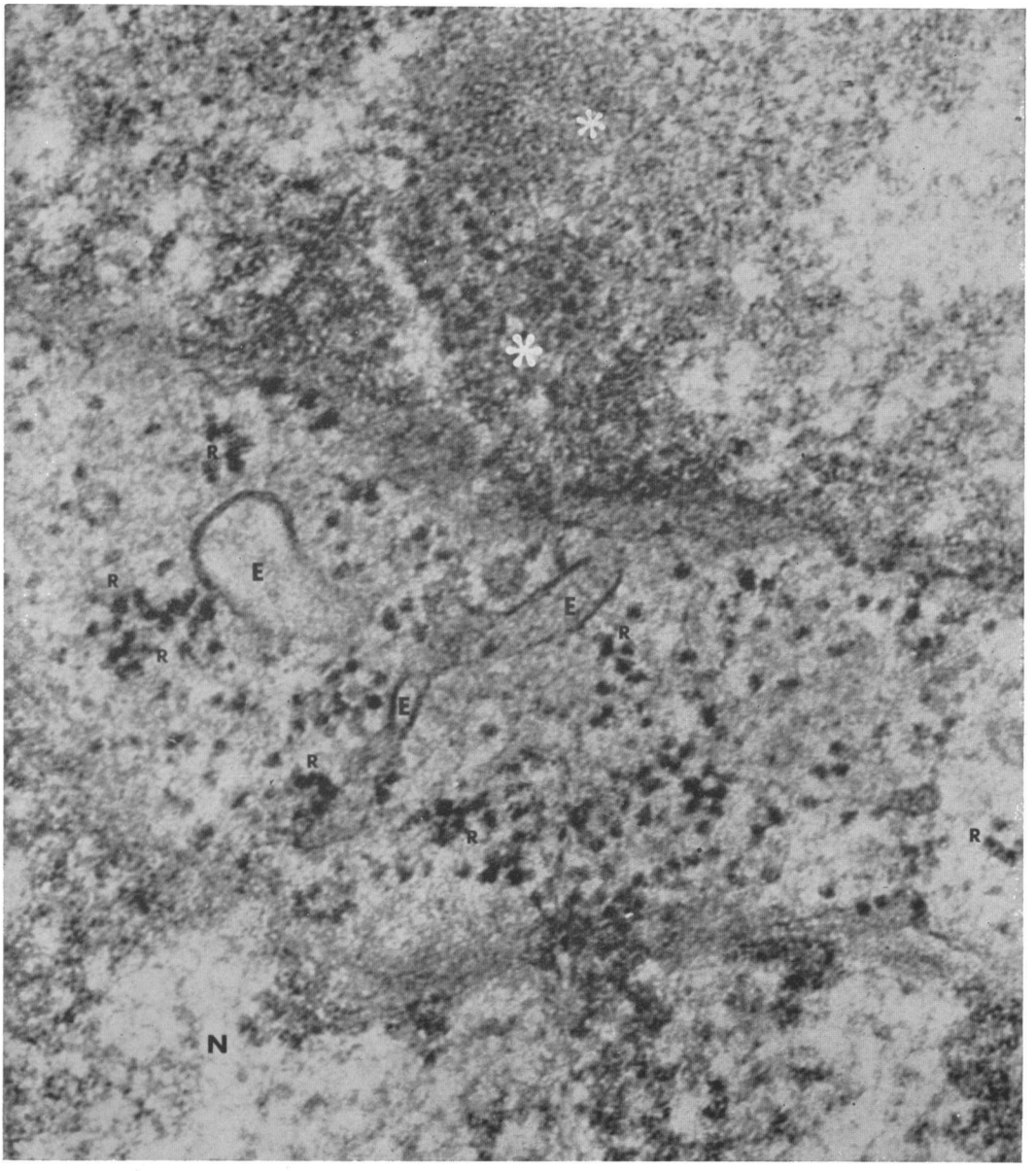

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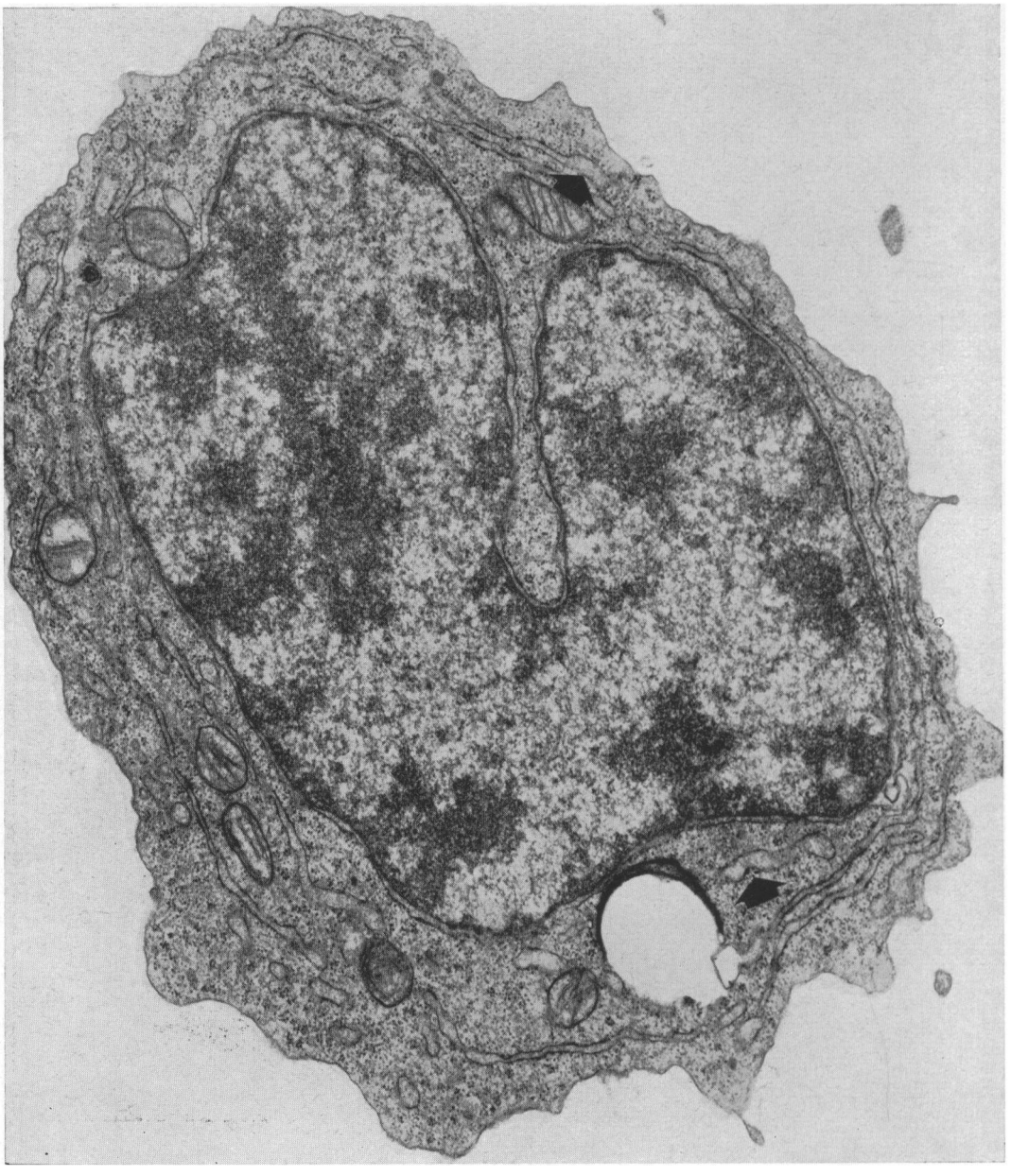

Fig. 19

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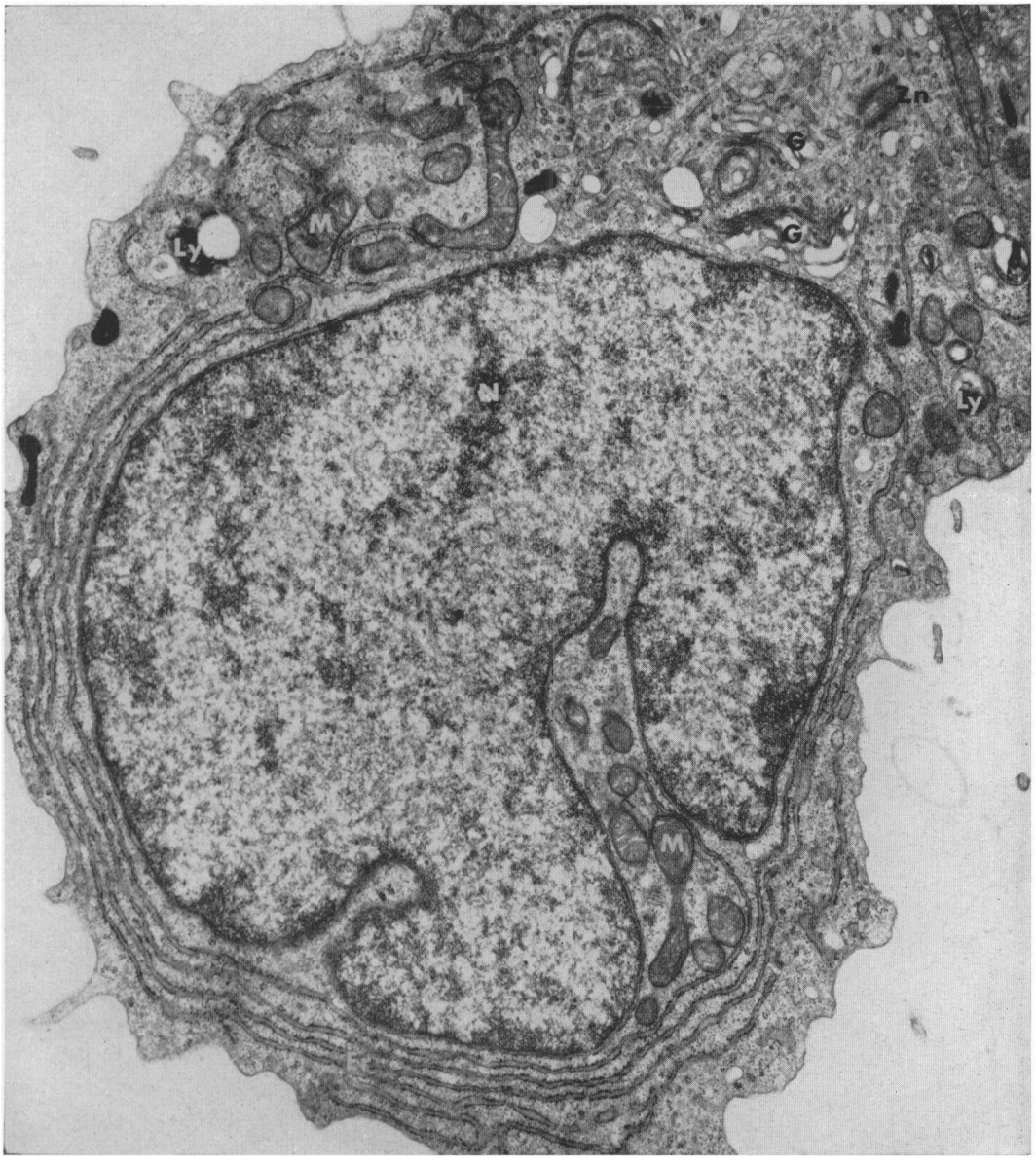

Fig. 20

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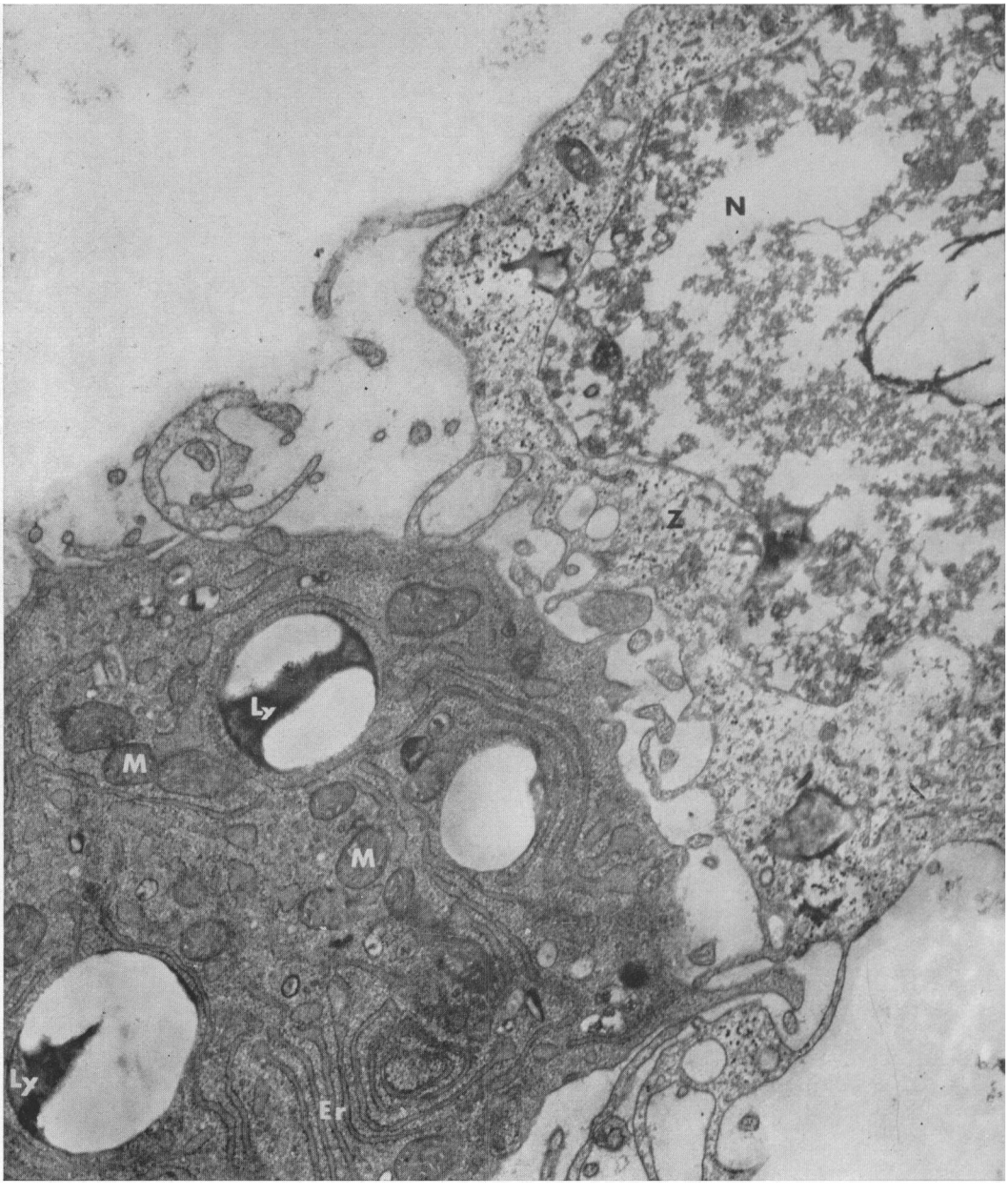

Fig. 21

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the cytoplasm occurs regularly, and is dependent on the endoplasmic reticulum (E) (Figs. 11, 12, 14, 17, 18), that is especially seen in the area of ribosome transfer, is of regressive origin, as similar changes, although less marked, are always observed in a small percentage of untreated cells.

After 3 hours degenerative changes develop in a great number, and very rapidly as shown by vital staining. These did not show specific particularities. The necrobiotic cell (Fig. 19) shows similar changes and these have been described after treatment with cytostatics ((Themann and Schmidt, 1960), autolysis in vivo and after death (Caesar, 1961).

The figure shows the incipient separation of the karyoplasm components; the chromatin agglomerates and the periphery (hyperchromasia of the nuclear wall) and in the inner parts of the nucleus. All cytoplasmic membrane systems show more or less marked swelling, at first of the ergastoplasmic tubules (⇧). Then they remain stationary for a relatively long period. Vacuolar transformation of the mitochrondria develops later on and is considerably more rapid. At the same time the deep cytoplasmic invaginations become less marked. Only when vacuolar degeneration has occurred does the number of cytoplasmic free ribosomes show a distinct decrease. In contrast to the sarcoma cells the histiocytes normally present maintained their characteristic cytoplasmic organelle structure with numerous ergastoplasmic tubules (Er), lysosomes (Ly) and an internal structure of the mitochondria (M) distinctly different from that of the sarcoma cells (Fig. 20).

Fig. 21 shows the onset of a process of phagocytosis developing after approximately 6 hours (as mostly occurs, after local chemotherapy with trenimon) and in which histiocytes and especially granulocytes are involved.

The early changes in tumour cells after Trenimon treatment described above can be interpreted in different ways. Our studies did not show whether the nucleoli are stimulated to accelerated release of RNA or whether the percentage increase in the nucleoli connected with the cytoplasmic invaginations is caused by a blocking in the late S-phase. At least the studies by Weissenfels (1966) on the nucleolar function in chicken heart myoblasts also permit this interpretation.

Summary

A report is given on various forms of administration of Trenimon for local treatment of neoplasms of the serous membranes. For supervision and adjustment of the chemotherapeutic effect supravital staining with acridin orange and phase-contrast microscopy of the untreated effusion sediment are especially suitable. This facilitates distinction between newly formed and preformed granules, which is of importance for the diagnosis. Inhibition of dye-induced production of granules is a very sensitive criterium for assessment of cell vitality. The development of regressive changes in the tumour cells caused by chemotherapy is discussed with reference to micrographs of a metastatic pleural sarcomatosis. Electron-microscope studies on the exu-

date suggest that prior to necrobiosis an increased transfer of the granular nucleolar component into the cytoplasm takes place. This may be caused by a true increase in the transfer of nucleolar RNA towards the cytoplasm or by an inhibition in the late S-phase.

Acknowledgment

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RIASSUNTO

Vengono discusse le varie forme di somministrazione di Trenimon nel trattamento locale dei neoplasmi della membrana sierosa. Per la regolazione ed il controllo dell'effetto chemioterapico è particolarmente efficace l'uso combinato di Acridinorange-Vitalfluorocromation del sedimento di un versamento non trattato e di microscopia in contrasto di fase. Ciò facilita l'importante distinzione diagnostica fra granuli preesistenti e granuli di recente formazione. L'inibizione della produzione di granuli indotta con coloranti è un criterio molto sensibile per accertare la vitalità cellulare. Lo sviluppo nelle cellule tumorali di cambiamenti regressivi causati dalla chemioterapia, viene discusso in base a microfotografie di una sarcomatosi pleurica metastasica. Studi elettromicroscopici dell'essudato indicano che un aumentato trasferimento citoplasmatico della componente granulare nucleare ha avuto luogo prima della necrobiosi. Ciò può essere dovuto ad un reale aumento nel trasferimento di RNA o ad una inibizione nella tarda fase-S.

RÉSUMÉ

Il a été traité des différentes applications du Trenimon lors des thérapies locales des néoplasmes dans les muqueuses séreuses. L'Acridinorange-Vitalfluorochromation du sédiment d'un épanchement natif combinée avec le processus de phases contrastées convient particulièrement pour la régulation et le contrôle des effets chimiothérapeutiques; elle simplifie la distinction, importante pour le diagnostic, entre les grains de nouvelle ou ancienne formation. Le freinage de la formation granuleuse colorée artificiellement, offre un critère sensible pour juger de l'activité des cellules. Le processus de transformation regressive en cellules tumorales provoquée par la chimiothérapie, est expliqué par des microphotographies d'une sarcomatose pleurale métastatique. L'examen electromicroscopique indique que, avant l'entrée en necrobiose, un passage plus important des composants du noyau a lieu dans le cytoplasme. Ceci peut être dû soit à une réelle augmentation dans le transfert de RNA, soit à une inhibition dans la phase-S.

ZUSAMMENFASSUNG

Es wird über verschiedene Applikationsformen von Trenimon zur lokalen Therapie bei Neoplasien der serösen Häute berichtet. Zur Steuerung und Kontrolle des chemotherapeutischen Effektes eignet sich besonders die mit dem Phasenkontrastverfahren kombinierte Acridinorange-Vitalfluorochromierung des nativen Erguss-Sedimentes. Sie erleichtert die diagnostisch bedeutungsvolle Unterscheidung zwischen neugebildeten und präformierten Granula. Die Hemmung der farbstoffinduzierten Granulabildung bietet ein empfindliches Kriterium zur Beurteilung der Zellvitalität.

Der Ablauf chemotherapiebedingter regressiver Veränderung in Tumorzellen wird anhand von Mikrofotographien einer metastatisch bedingten Pleurasarkomatose erläutert. Die elektronenoptische Untersuchung liess vor Eintritt der Nekrobiose einen vermehrten Uebertritt granulärer Nukleolus-Komponenten in das Cytoplasma erkennen.