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# Effect of cyclophosphamide on meristematic plant cells

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Abstract

The action of cyclophosphamide on meristematic plant cells was checked. A mitostatic influence of this preparation was observed, by way of DNA synthesis inhibition. The disturbance in the course of mitosis is described and the characteristic changes in the structure of the ergastoplasm, analogous to those elicited by respiratory inhibitors.

Cyclophosphamide (Endoxan, Cytoxan) is a drug of the group of chloralkylamines with cytostatic and cytotoxic action. On account of its wide spectrum of action it is one of the most frequently applied preparations in the treatment of neoplasms.

Cyclophosphamide metabolism and the mechanisms of its action have been and continue to be the object of numerous studies (Kondo and Muragishi, 1970; Brock et al., 1971; Alarcon et al., 1972; De Wys, 1972; Hill et al., 1972; Jao et al., 1972; Sheehan and Shklar, 1972). Until recently most authors agreed that cyclophosphamide is inactive *in vitro*, and its activation occurs only *in vivo* in the presence of liver microsomes, molecular oxygen and $NHDPH_2$ (Brock and Hohorst, 1963; Brock et al., 1971; Hart and Adamson, 1969; Connors et al., 1970; Cohen and Jao, 1970). It results, however, from more recent investigations that cyclophosphamide is also active *in vitro*; and this activity is only enhanced by the addition of liver microsomes (Cooper and Goldstein, 1976) and is manifested by inhibition of growth of tumour cells, chromosome aberrations and DNA and RNA synthesis inhibition (Buczko and Popow, 1976; Szewczyk et al., 1975; Stetka and Sheldon, 1976; Carmel and Brown, 1977).

The present experiments were undertaken in order to study the action of cyclophosphamide on meristematic plant cells.

## MATERIAL AND METHODS

The *Allium* test introduced by Levan (1949) for demonstration of the effect of numerous chemical substances, both organic and inorganic, was applied to reveal the mitostatic properties of cyclophosphamide. The mitotic activity of the meristem was expressed as the number of mitoses and the rate of synthesis of nucleic acids and proteins.

Adventitious roots of onion were cultured hydroponically in glass vessels of 250 ml capacity at 20°C in tap water changed daily. After 3—4 days of culture, when the roots reached length of about 3 cm they were transferred with the bulbs for period of 6, 12 and 24 h to a cyclophosphamide solution (Germed, East Germany) of 0.1, 0.2 and 0.4 per cent concentration, prepared freshly before use from tap water. The remaining part of the material growing further in water served as control. After 6, 12 and 24 h of exposure to the action of cyclophosphamide, part of the material was fixed, and the rest was postincubated in water to check the reversibility of the changes. The concentration of the incubation solutions used was established on the basis of literature data concerning the effectiveness of doses in tumour therapy and of preliminary trials. Most active towards *Allium* roots proved the 0.4 per cent solution.

The experimental material was fixed in acetoalcohol (A : A = 1 : 3) for 2 h and stored in 80 per cent ethanol. From this material squashes were prepared in acetoorcein. Mitotic activity of the meristem was determined by counting all the mitoses in a 2-mm segment of the root apex. The number of the particular phases was recorded. The rate of NA and protein synthesis was determined by the autoradiographic method with the use of $^{3}$H-thymidine, -uridine and -leucine. The incorporation of labelled precursors into the roots treated with 0.4 per cent cyclophosphamide for 24 h, was analysed, the precursor being added in 10 μCi/1 ml concentration for the last hour of incubation. For checking the reversibility of the occurring changes, the incorporation of the labelled precursor into the root tips treated with 0.4 per cent cyclophosphamide for 24 h and then transferred for 72-h postincubation to water was determined. The labelled precursor was administered in the last hour of postincubation. The material was then fixed in AA, squashes were prepared in acetoorcein and covered with AR Kodak emulsion for 2 weeks.

The second part of the experiment aimed at establishing the consequences of cyclophosphamide treatment for mitosis and chromosome morphology. The experimental material consisted of adventitious onion roots cultured and treated with cyclophosphamide analogously as in the first part of the experiment. The effect of the drug was additionally checked on the morphology and chromosome structure in plants with large chromosomes convenient for observation such as: *Tradescantia* and

*Haemanthus*. For this purpose 3—4 cm cut adventitious roots of *Tradescantia* cultured in water were treated with cyclophosphamide of 0.4 per cent concentration for 3 and 6 h. Analogously were treated several centimetre long *Haemanthus* roots growing previously in a pot with soil. The observations were made on squashes in acetoorcein and microtome slices. The growth apexes of roots for the paraffin method were fixed in chrome-aceto-formalin (CrAF) in a percentual proportion of 0.5 – 1 - 20, and stained with Heidenhain haematoxylin.

The last part of the experiment concerned the effect of cyclophosphamide on the protoplast morphology in meristematic cells. The object of observations were the growth apexes of *Allium*, *Tradescantia* and *Haemanthus* roots treated with the drug in a 0.4 per cent concentration for 3 and 12 h, and additionally treated with a 1 per cent solution for 3 h.

For observation in a light microscope the material was fixed in CrAF in a percentual proportion 0.5 – 1 - 20 and preparations were made by the paraffin method and stained with iron haematoxylin. For observation in the electron microscope the material was fixed in two ways: in 2 per cent $KMnO_4$ and in glutaraldehyde after Karnowsky (1965). The preparations were embedded in Epon and contrasted according to Reynolds (1963). A BS 500 Tesla electron microscope was used.

## RESULTS

It was found that under the given experimental conditions cyclophosphamide exhibits distinct mitodepressor properties manifested in a decrease of the number of mitoses in the experimental material, inversely proportional to the concentration and time of exposure of the drug (Diargram 1). The decrease of the number of mitoses is strictly dependent on the concentration of cyclophosphamide. At lower concentrations (0.1, 0.2%) the mitotic activity of the roots is only slightly depressed as compared with the control. Only a concentration as high as 0.4 per cent caused a rapid fall of the number of mitoses (Fig. 1) and blocked the metaphases. The mitostatic effects of cyclophosphamide are largely reversible. In the course of 24 h postincubation a marked increase, and even a certain explosion of mitoses were observed. The course of the curve illustrating the increase of the number of divisions in the postincubated material is also dependent on the concentration of the drug (Fig. 1). Since it was suspected that the observed depression of mitosis under the action of cyclophosphamide significantly dependent on the concentration of the solution is conditioned by inhibition of NA and protein synthesis, in the next step of the experiment the rate of synthesis of these substances was determined by autoradiography.

The results obtained prove that cyclophosphamide has a specific action on DNA synthesis. Its rate is decreasing in the experimental ma-

terial markedly (Fig. 30) as compared with the control (Fig. 29). The depression of RNA and protein synthesis is less drastic (Figs 26, 27, 32, 33).

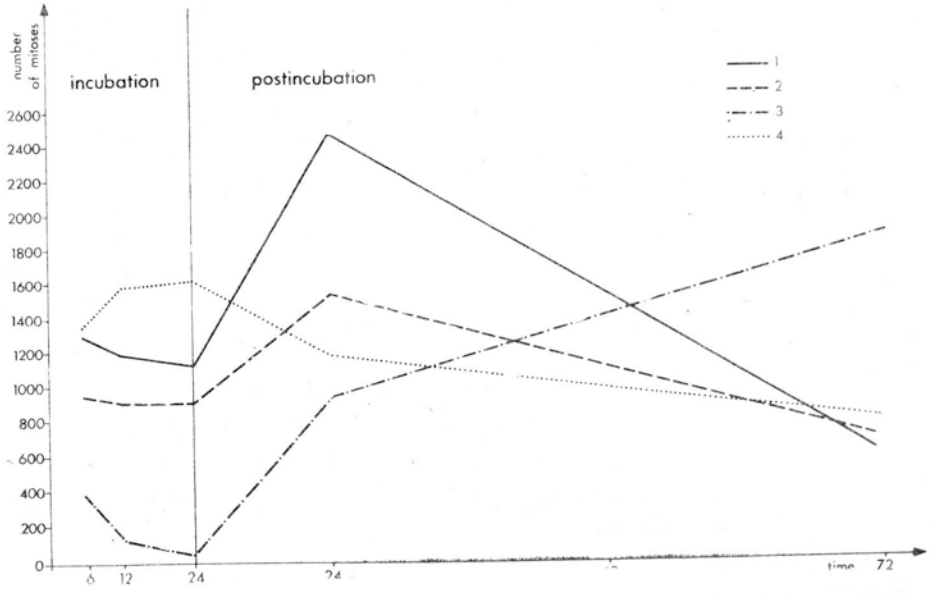
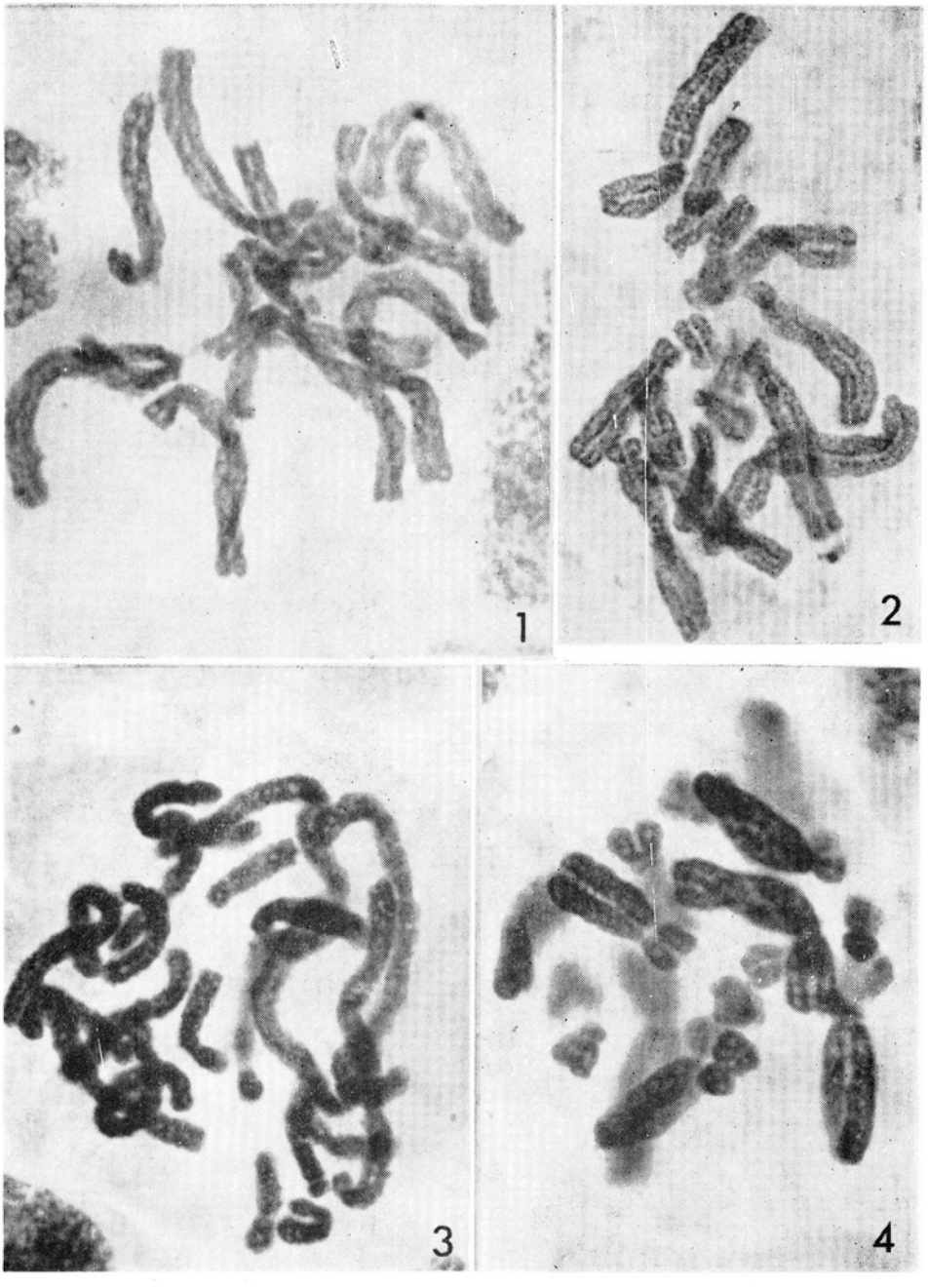


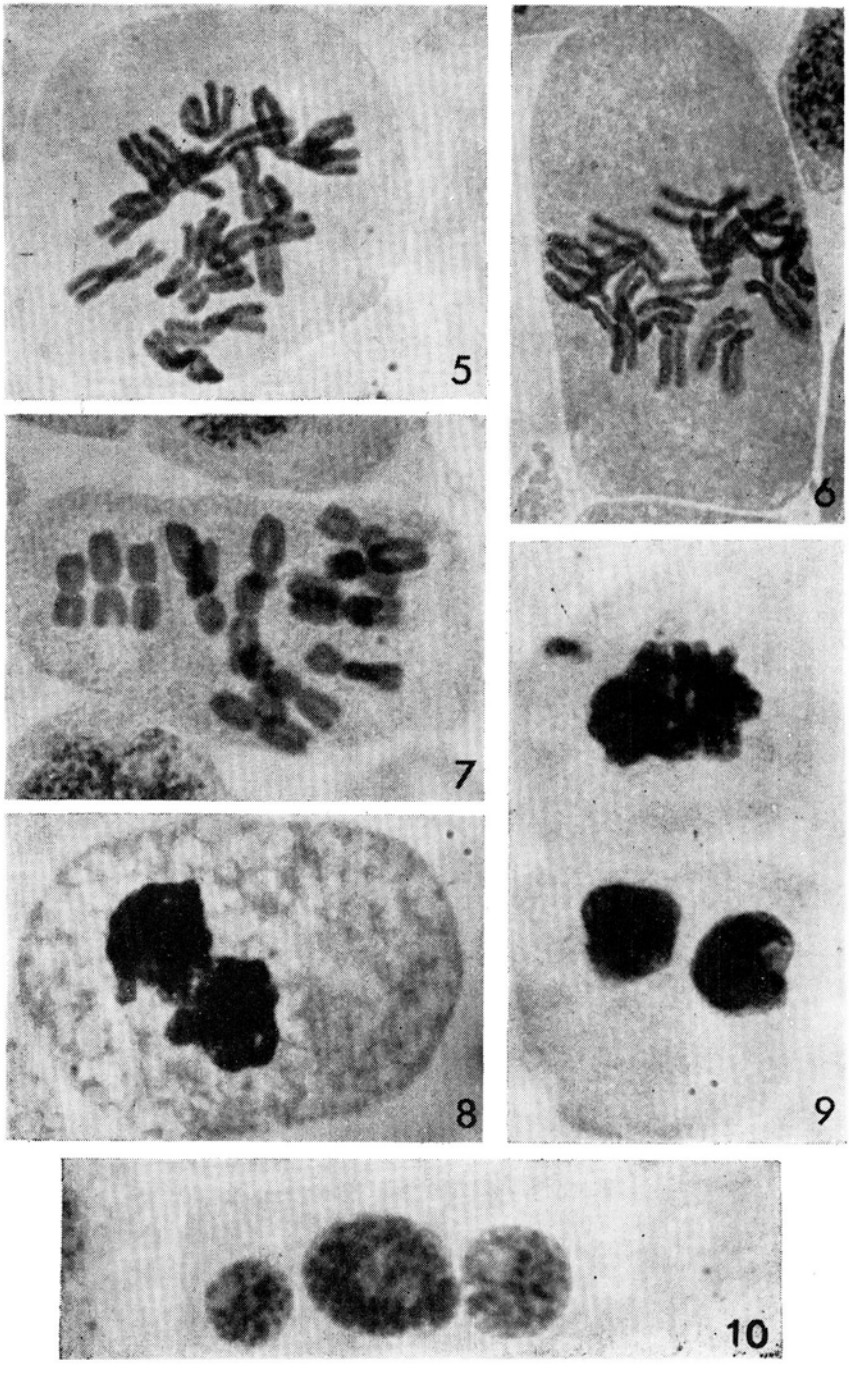

Diagram 1. Number of mitoses in meristem of apical roots of *Allium cepa* treated with cyclophosphamide for 24 h and postincubated in water for 24 and 72 h; 1 — 0.1% cyclophosphamide, 2 — 0.2% cyclophosphamide, 3 — 0.4% cyclophosphamide, 4 — control

Cyclophosphamide exerted also a characteristic influence on the course of mitosis. This conclusion was reached on the basis of observations of microtome preparations from root apexes of *Allium, Tradescantia* and *Haemanthus,* stained with iron haematoxylin or squashed in acetoorcein.

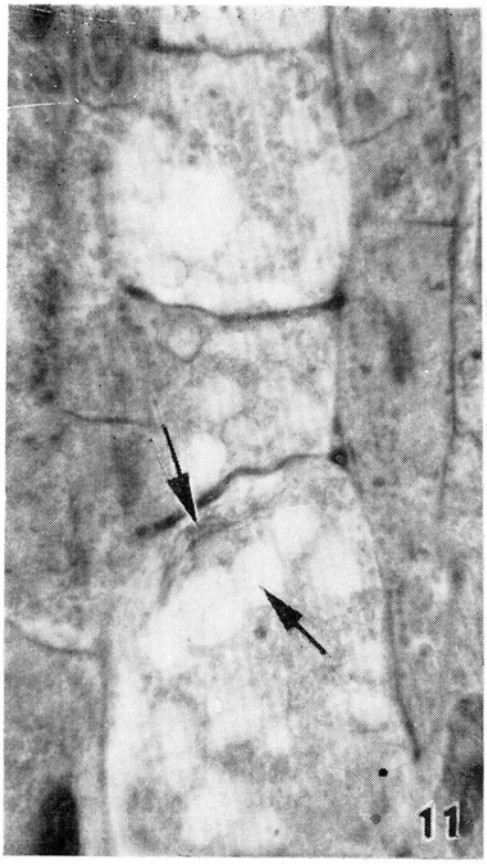
Independently of the drug concentration and the time of its action, numerous prophases were observed with a loose arrangement of prophase chromosomes (Fig. 3), probably this was the consequence of a slowed down spiralisation of chromonemata. The looser structure of subchromatids visible in the preparations (Fig. 3) seems to confirm this supposition. In metaphase the arrangement and morphology of chromosomes depend on the concentration and time of the drug action. Metaphasal plates were noted with only slightly thickened and shortened chromosomes (Fig. 2) as compared with the control (Fig. 1). There were observed plates with chromosomes dispersed, owing to a partially injured spindle (Figs 4, 5), also figures analogous to c-metaphases (Fig. 7). Retarded centromere division typical for c-metaphases was observed (Figs 4, 5, 7). The chromosomes were shortened, but their internal structure

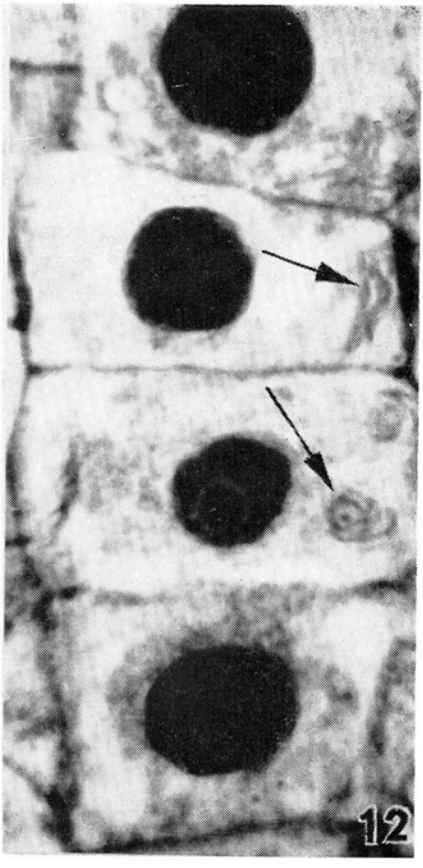


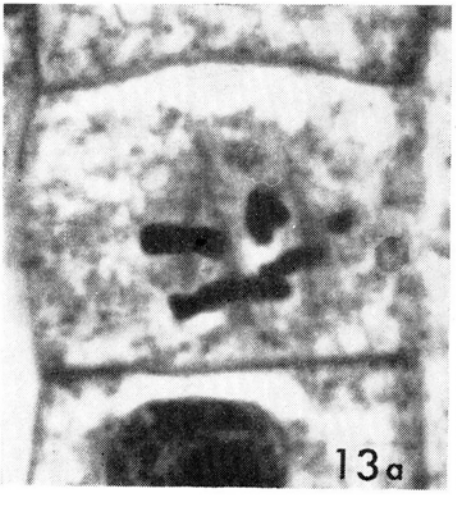

Figs 1—4. Meristematic cells from growth apex of *Haemanthus albiflos* root; acetoorcein. Fig. 1 — control; Figs 2—4 — treated for 3 h with 0.4% cyclophosphamide, Figs 2, 4 — structure of metaphase chromosomes, Fig. 3 — prophase. × ca. 1400

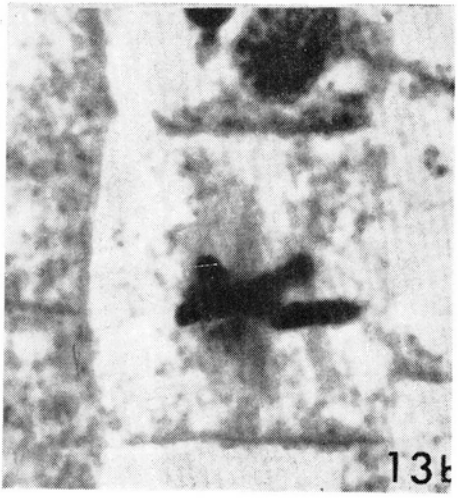



Figs 5—10. Meristematic cells from growth apex of *Allium cepa* root treated with 0.4% cyclophosphamide for 6 h. Figs 5, 7 — metaphase, retarded centromere division, Fig. 6 — anaphase; Figs 8, 9 — coalescing telophase chromosomes; Figs 9, 10 — 2- and 3-nucleate cells. Figs 5—10 × ca 1400

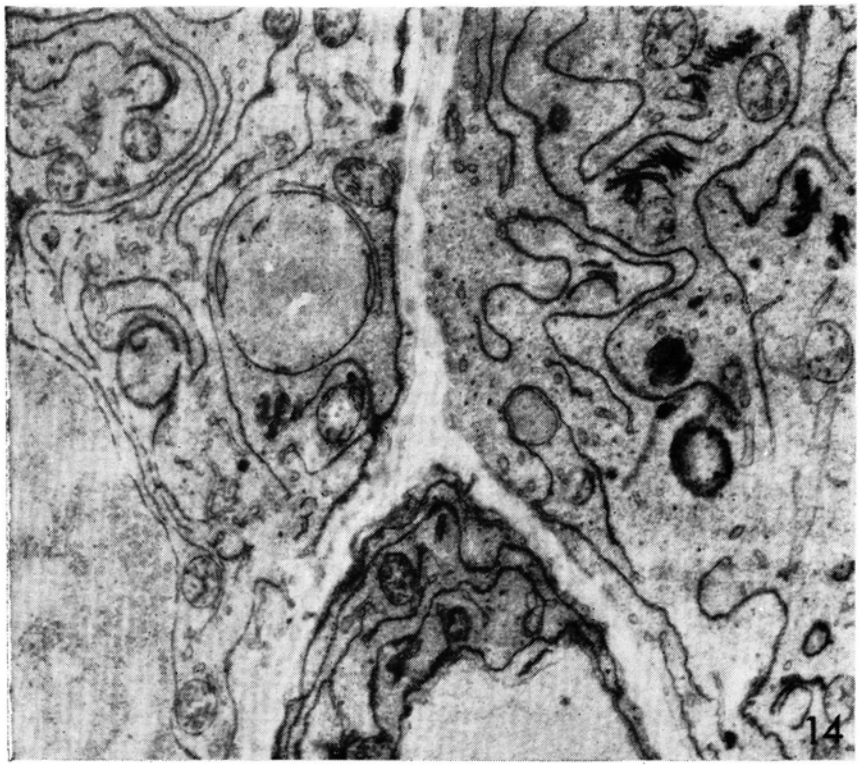


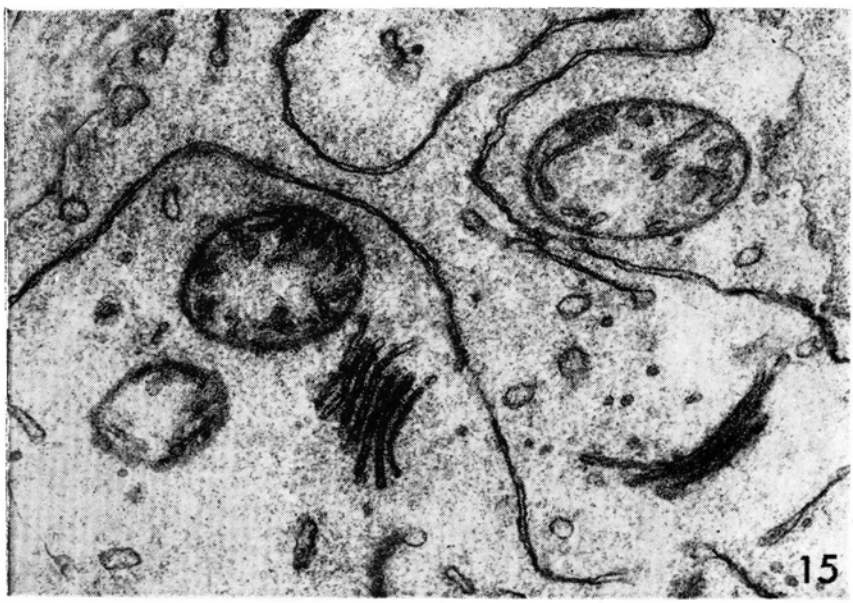



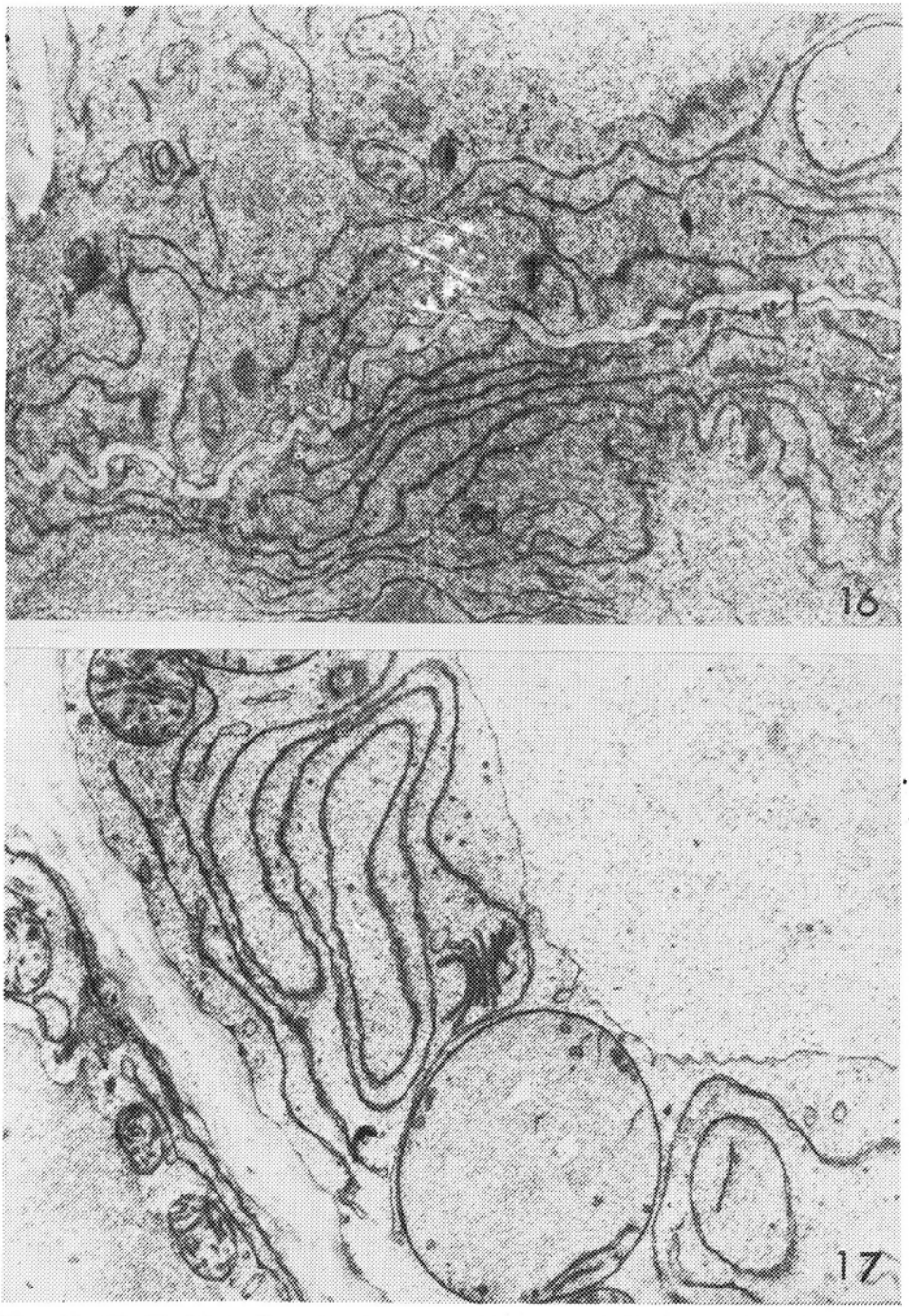




Figs 11—13. Meristematic cells from growth apex of *Tradescentia virginica* root treated with 0.4% cyclophosphamide for 12 h, fixed in CrAF 0.5—1—20; iron haematoxylin; Figs 11, 12 — membraneous structures parallel and spherical within cytoplasm; Fig 13, b — chromosome fibres in parallel arrangement. Figs 11, 12 × ca. 1400, Fig. 13 × ca. 2000

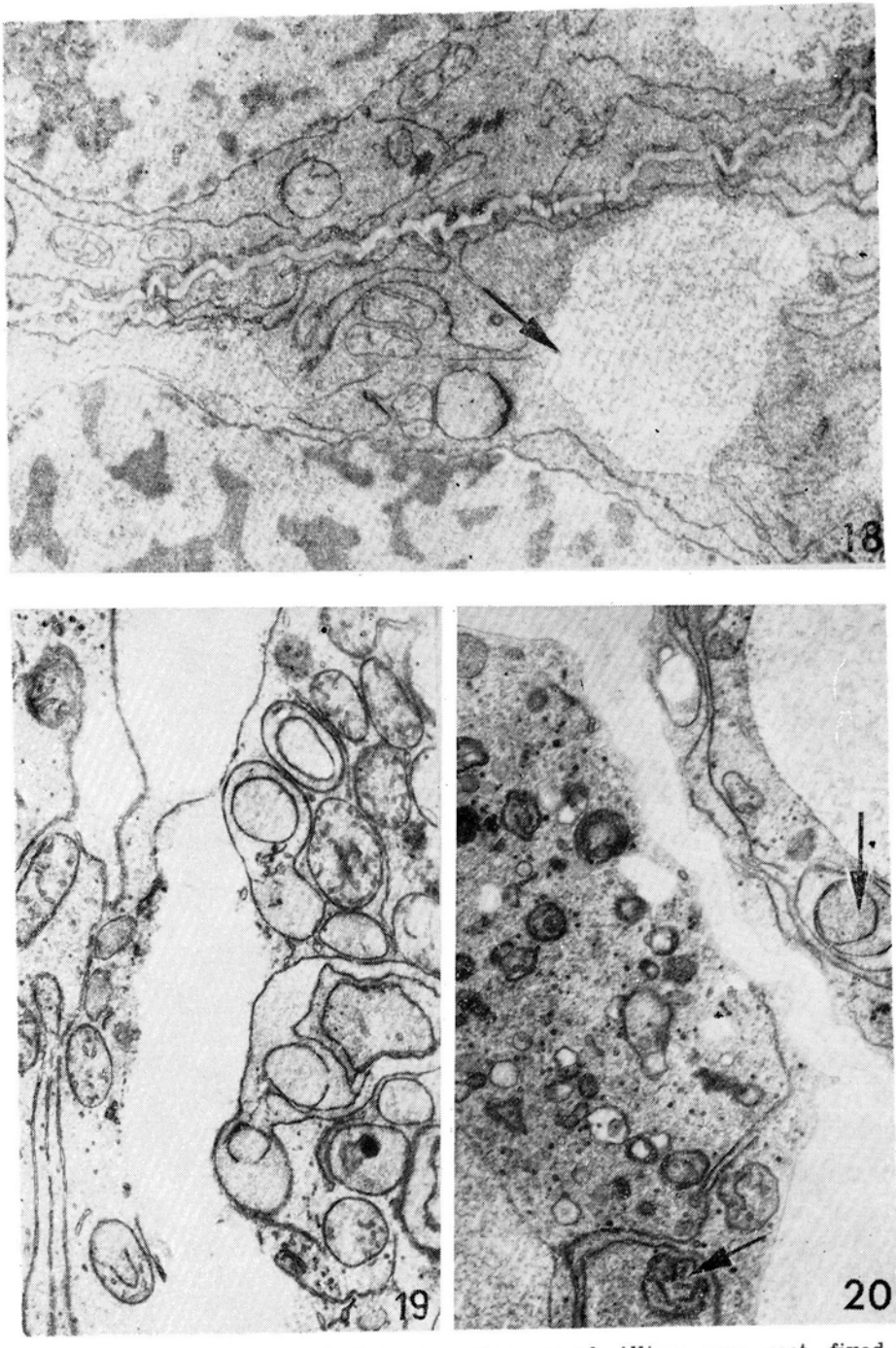


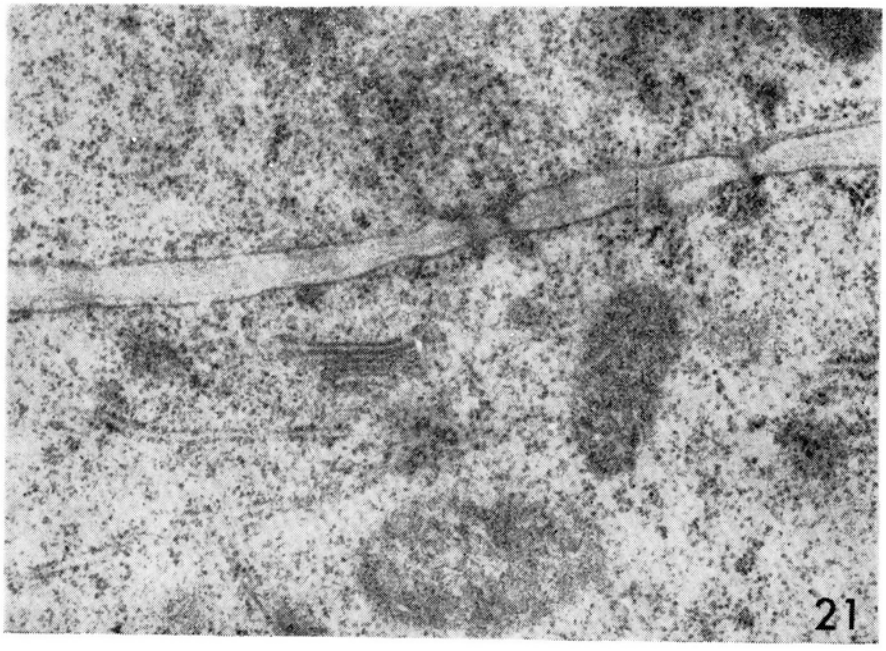

Figs 14, 15. Meristematic cells from growth apex of *Allium cepa* roots treated with 0.4% cyclophosphamide for 3 h; fixed in $KMnO_4$; ER cisternae, mitochondria, dictyosomes with typical structure. Fig. 14 × ca. 14 000, Fig. 15 × ca. 35 000

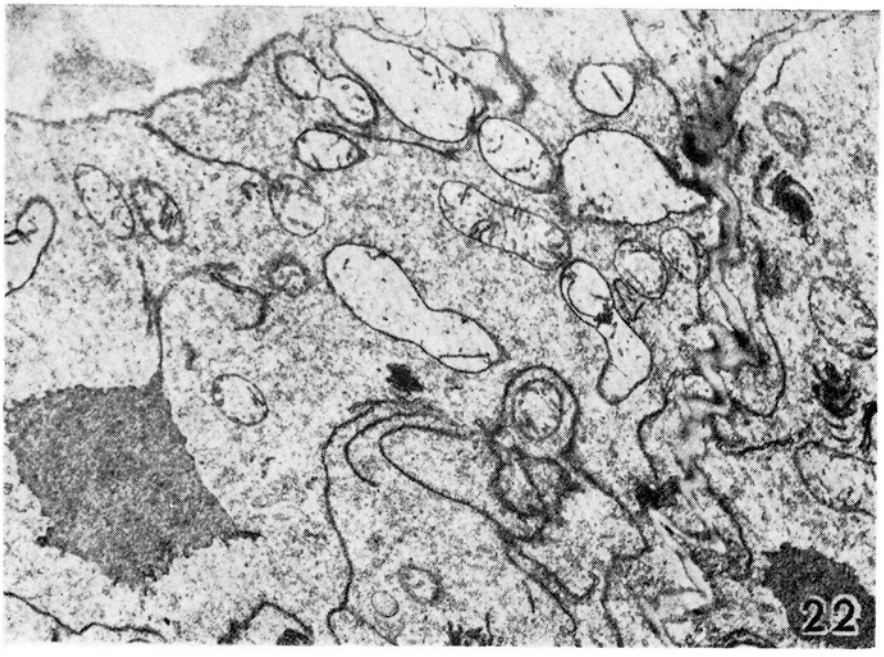



Figs 16, 17. Meristematic cells from growth apex of *Haemanthus albiflos* root treated with 0.4% cyclophosphamide for 12 h fixed with 2% $KMnO_4$, Figs 16, 17 — ER arranged parallelly and spherically, at the end of cisternae vesicle-like distentions. Fig. 16 × ca. 12 000, Fig. 17 × ca. 16 000

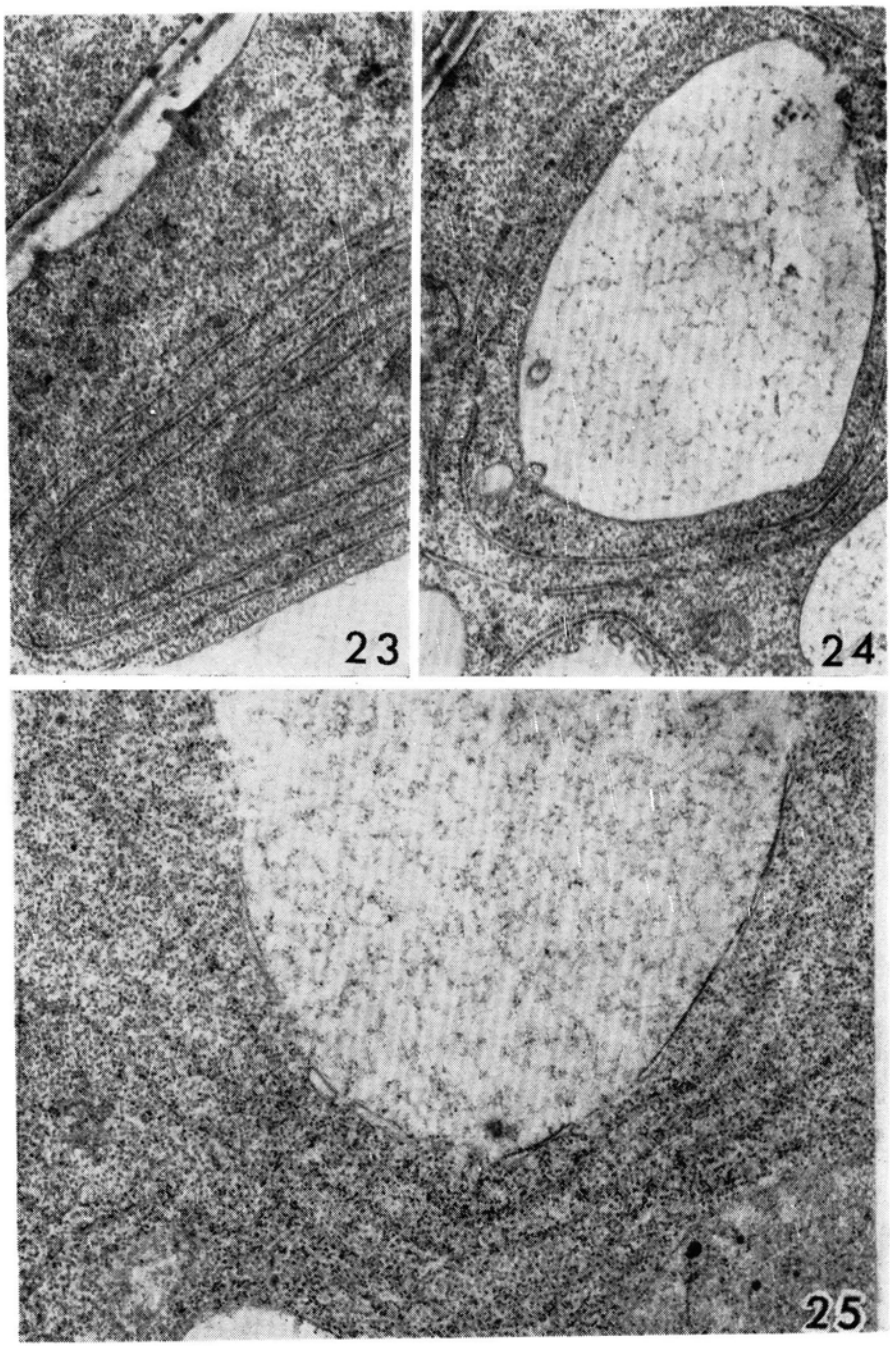



Figs 18—20. Meristematic cell from growth apex of *Allium cepa* root, fixed in 2% $KMnO_4$. Fig 18 — cells treated with 0.4% cyclophosphamide for 12 h, vacuole forming from ER; Figs 19, 20 — treated with 0.1% cyclophosphamide for 3 h, changes in mitochondrial membranes structure. Fig. 18 × ca. 12 000, Fig. 19 × ca. 14 000

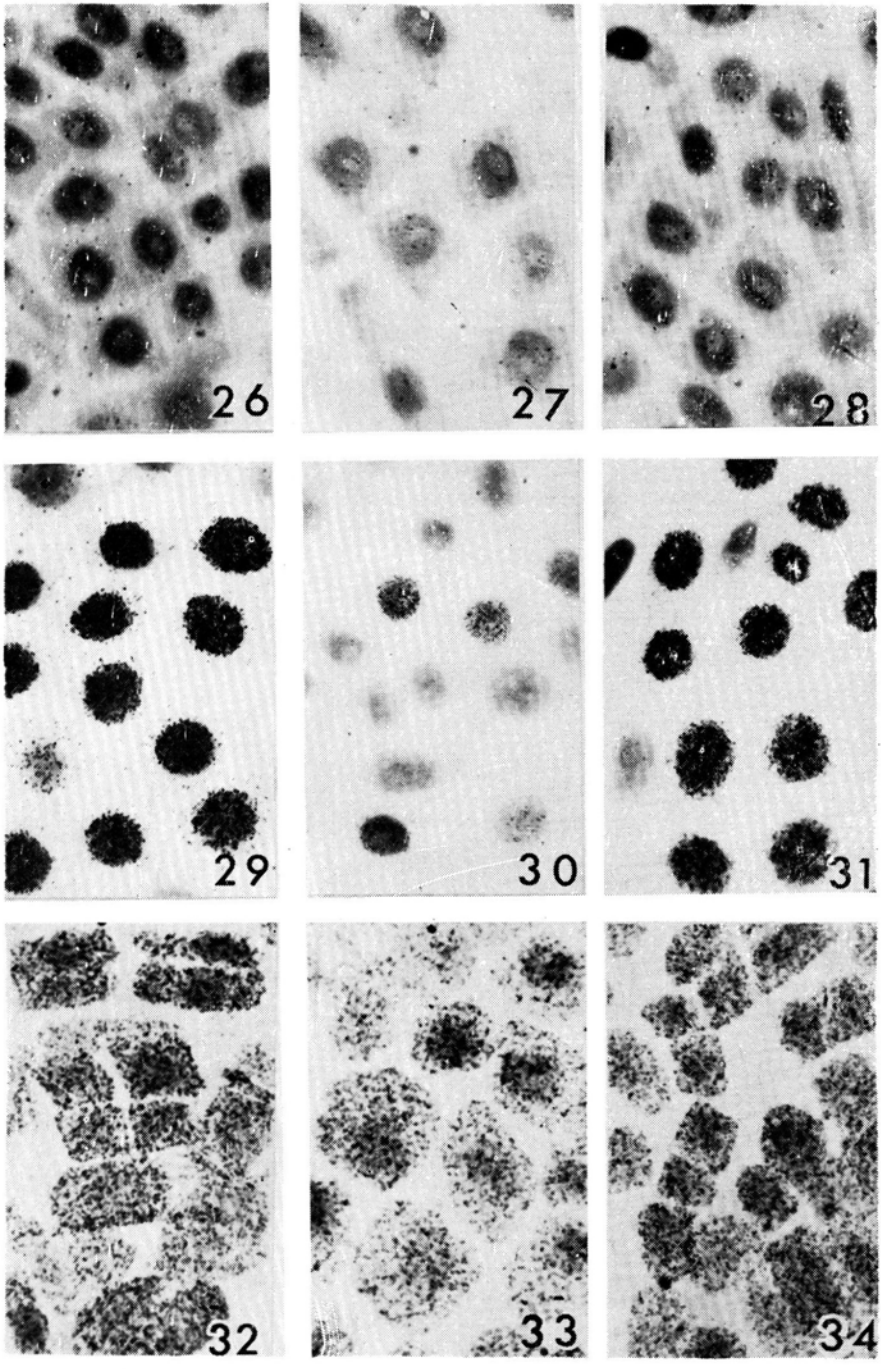



Figs 21, 22. Meristematic cells from growth apex of *Allium cepa* root. Fig. 21— treated with 0.4% cyclophosphamide for 12 h, fixed in GA after Karnowsky; ribosomes in ground cytoplasm; Fig. 22 — control fixed in 2% $KMnO_4$. Fig. 21 × ca. 20 000, Fig. 22 × ca. 12 000




Figs 23—25. Meristematic cells from growth apex of *Allium cepa* root. Figs 23, 25 — cells treated with 0.4% cyclophosphamide for 12 h, fixed in GA after Karnowsky, rough ER in spherical arrangement; Fig. 24 — treated with 0.1% cyclophosphamide for 3 h, fixed in GA after Karnowsky, rough ER in neighbourhood of vacuoles, Figs 23, 24 × ca. 14 000, Fig. 25 × ca. 20 000




Figs 26—34. Maristematic cells from growth apex of *Allium cepa* root, fixed in AA; acetoorcein. Fig. 26 — RNA labelled with $^3$H-uridine — control; Fig. 27 — RNA labelled with $^3$H-uridine, 0.4% cyclophosphamide, incubation 24 h; Fig. 28 — RNA labelled with $^3$H-uridine, 0.4% cyclophosphamide, postincubation 72 h; Fig. 29 — DNA labelled with $^3$H-thymidine — control; Fig. 30 — DNA labelled with $^3$H-thymidine, 0.4% cyclophosphamide, incubation 24 h; Fig. 31 — DNA labelled with $^3$H-thymidine, 0.4% cyclophosphamide, incubation 24 h, postincubation 72 h; Fig. 32 — protein labelled with $^3$H-leucine — control; Fig. 33 — protein labelled with $^3$H-leucine, 0.4% cyclophosphamide, incubation 24 h; Fig. 34 — protein labelled with $^3$H-leucine, 0.4% cyclophosphamide, incubation 24 h, postincubation 72 h

was visible (subchromatids; Figs 2, 4). The degree of their shortening is directly proportional to the time of exposure to cyclophosphamide solution. In the light microscope, in preparations stained with iron haematoxylin changes were observed in the arrangement of the fibrous structures of the mitotic spindle, increasing with the drug concentration. The chromosome fibres from the fan arrangement typical for normal cells change to an almost parallel position characteristic for karyokinesis disturbances (Figs 13a, b). Variously modified anaphases were observed in dependence on concentration and the time of action of cyclophosphamide. Most deformed were the patterns analogous do c-anaphases appearing after 0.4 per cent cyclophosphamide (Fig. 6) and telophases at lower concentrations (0.1 and 0.2%) with chromatid bridges formed, owing to the increased viscosity of the telomeres. At a higher concentration (0,4%) coalescing groups of telophase chromosomes were observed (Figs 8, 9). As the result of disturbances in the course of cytokinesis 2- or 3-nucleate cells, were formed (Figs 9, 10). The mitosis disturbances are reversible only to some small degree. During postincubation division of 2-nucleate or polyploid cells forming as the consequence of anaphases were not observed.

It results from light microscope observations that cyclophosphamide also causes distinct changes in the protoplast morphology. Meristematic cells after 12 h of exposure to the action of the drug become vacuolised, the cytoplasm showing no signs of degeneration or disorganisation (Fig. 11). Mitochondria become polymorphic after 3 h of exposure and after 12 h they have a spherical shape and are slightly swollen. Membraneous structures were noted within the cytoplasm in spherical and parallel arrangement similar to ER (Figs 11, 12), which form themselves after 12 h of treatment with 0.4 per cent and 3 h of 1 per cent cyclophosphamide solution.

Observations in the electron microscope demonstrated an increase in the number of cell organelles, among them of mitochondria, dictyosomes, plastids as the result of a short (3-h) exposure to 0.4 per cent cyclophosphamide (Figs 14, 15). After 12 h of incubation with the drug an increase of the number of ER cisternae was observed, forming usually parallel (Fig. 16) or spherical patterns (Figs 17, 23). The ends of these cisternae, frequently characteristically distended (Figs 15, 17) may be closed and detached, giving rise to vesicular or canalicular forms of ER. ER cisternae also often give rise to vacuoles (Fig. 18). The number of ribosomes is large. They were observed both within the ground cytoplasm, occurring singly or as polyribosomes (Fig. 21) and in the neighbourhood of ER membranes (rough ER, Fig. 25). The ultrastructure of mitochondria, plastids and dictyosomes is similar to that in the control (Fig. 15). After 3 h of treatment with 1 per cent cyclophosphamide a grouping of ER membranes was observed in the region of the vacuoles

(Fig. 24), the number of which markedly increased. It was noted that mitochondria and plastids underwent deformation (Figs 19, 20) as compared with the control (Fig. 22). It was manifested in the membrane configuration, both of the outer and the inner one (Figs 19, 20). The mitochondrial cristae were reduced, shortened, and sometimes spherical bodies appeared within the mitochondria as if formed from disintegrating inner membranes.

## DISCUSSION AND CONCLUSIONS

Under the conditions of the experiment a mitostatic action of cyclophosphamide was demonstrated. It appeared at the same time as an important factor contributing to the mitodepression effect of the concentration of the solution. The dependence of the cytotoxic effect of the drug on the dose was demonstrated by Carmel and Brown (1977). This phenomenon is based on the specific action of the tested compound on phase S in the cell life cycle. It results, namely, from autoradiographic investigations that cyclophosphamide inhibits DNA synthesis. This finding is in agreement with the observations of Palme et al. (1963), who established that the site of action of the products arising from cyclophosphamide decomposition in the human organism is the DNA chain susceptible to alkylation.

The described here disturbances in the course of mitosis under the influence of the drug prove both its action in phase M itself and in phase $G_2$. This finds its morphological expression in numerous loosened prophases observed independently of the time of action and concentration of cyclophosphamide solutions. The metaphase chromosomes, dispersed and abnormally migrating to the poles, observed after treatment with the drug and the blockade of metaphases may be the result of disorganisation within the mitotic apparatus (spindle fibres), according to the observations of Östergren (1944, 1950) and Tarkowska (1977) concerning disturbances in the movements of chromosomes as the result of injury to the mitotic spindle. The change in arrangement of the spindle fibrous structures, observed in the present study in the light microscope, from fan-shaped to parallel confirms this conclusion. The restitution nuclei arising as the result of transformations analogous to c-anaphases are not capable to divide. This consequence of cyclophosphamide treatment distinguishes essentially its action from that of colchicine.

An interesting phenomenon seems to be the reorganization of ergastoplasm under the action of cyclophosphamide. Hypertrophy and also characteristic configurations of rough ER membranes have been described in various cases. Such changes accompany both the dominance of ana-

bolic and of catabolic processes in the cell (Dereuddre, 1971, 1973; Duckett, 1972; Begfeld and Falk, 1968; Dexheimer, 1966). It is also known that accumulation of ER cisternae in the cell is the result of transformation within the already existing membranes (Whaley et al., 1964).

Submicroscopic pictures analogous to those described after treatment with cyclophosphamide were also noted as the consequence of treatment with specific respiratory inhibitors, particularly those of the respiratory chain (Podbielkowska and Borys, 1975, Podbielkowska et al., 1975).

A positive correlation was also demonstrated between membranisation of protoplast of meristematic cells and the rise of the glycolysis rate induced by anaerobic conditions — $N_2$ atmosphere (Podbielkowska and Kupidłowska, 1976). If energy deficit may be the cause of ER hypertrophy, it may be supposed that the development of the characteristic configuration of mature ER under the influence of cyclophosphamide may be the result of action of this compound on processes connected with energy supply to the cell.

In this connection it would seem desirable in further experimental steps to solve the problem whether and in how far cyclophosphamide inhibits the process of cellular respiration, and how is the influence of this drug manifested in the protoplast structure of glycolytically respiring cell, thus in the way most tumour cells respire (Bernhard, 1969).

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## *Skutki działania cyklofosfamidu na merystematyczne komórki roślinne*

Streszczenie

Wykazano aktywność cyklofosfamidu (preparat onkostatyczny) w odniesieniu do merystematycznych komórek roślinnych. Stwierdzono cytostatyczny efekt działania tego związku wyrażający się spadkiem liczby mitoz w merystemie korzeniowym oraz specyficzną inhibicją syntezy DNA. Opisano zaburzenia w przebiegu mitozy polegające na stopniowej dezorganizacji wrzeciona podziałowego (figury podobne do zaburzeń kolchicynowych oraz powstawanie komórek 2-jądrowych). Stwierdzono charakterystyczną reorganizację ergastoplazmy, którą, ze względu na morfologiczną analogię, uznano za obraz przemian zachodzących w komórce przy deficycie energetycznym.

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