

Effects of 5-fluorouracil on the mitotic activity of onion root tips apical meristem*)

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Abstract

The effects of various concentrations of 5-FU on the mitotic activity of onion root tips apical meristem were investigated during 24-hour incubation in 5-FU and postincubation in water. The incubation in 5-FU caused a reversible inhibition of mitotic activity, and waves of the partially synchronised mitoses were observed during the period of postincubation. The most pronounced synchronisation of mitoses was obtained after incubation in 100 mg/l. 5-FU but the mitotic index of the resumed mitotic activity amounted to only one half of the control value. 5-FU was found to cause some cytological changes in meristematic cells such as enlargement of the nucleoli, change in the interphasic nuclei structure, appearance of subchromatid and chromatid aberrations and micronuclei. The effects of 5-FU on nucleic acids and the cell division cycle are discussed and compared with the effects of 5-FUdR.

INTRODUCTION

5-Fluorouracil (5-FU) has been widely used in investigations on cell growth and plant development (Key, 1969). It is known to inhibit DNA and normal r-RNA synthesis and to partly disturb the functioning of t-RNA, although it only slightly affects of not all m-RNA activity (Cohen et al., 1958; Cherry and van Huystee, 1965; Key, 1966; Key and Ingle, 1968; Mandel 1969). Studies performed on plant material on the influence of 5-FU on cell division demonstrated that continuous incubation in 500 mg/l. of this substance completely inhibits division in emerging oat roots (Masuda et al., 1966). A depression of the mitotic index was also found in isolated pea embryo rootlets (Paranjothy and

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Raghavan, 1970) as well as inhibition of cell division in soybean roots (Lin and Key, 1968) and in wheat coleoptile (Rose et al., 1970).

The mechanism of DNA synthesis inhibition has been explained at the molecular level: 5-FU undergoes in the cell partial transformation to 5-fluorodeoxyuridine (5-FUdR) and phosphorylation to 5-fluorodeoxyuridinemonophosphate (5-FdUMP) which causes a block of thymidyl acid synthesis by inactivating thymidylate synthetase (Cohen et al., 1958; Balis, 1968; Harbers et al., 1968).

5-FU does not directly inhibit RNA synthesis. It was found, however, that, after transformation to nucleosidetriphosphate, it is incorporated into RNA, substituting uracyl in 20—80 per cent of the newly synthesized ribosome fractions (Key, 1966; Mandel, 1969). The forming FUR-RNA prevents the production of normal ribosomes (Mandel, 1969; Rose et al., 1970; Rose and Setterfield, 1971). The changes in the structure of nucleoli described by numerous authors and the considerable increase in their volume in 5-FU-treated cells are interpreted as the consequence of synthesis of abnormal FUR-RNA accumulated in the nucleoli (Willen and Stenram, 1967; Simard, 1970; Rose et al., 1970). It was, moreover, found that 5-FU is incorporated into m-RNA and t-RNA so that it may disturb their normal function, since 5-FU forms more readily a complementary pair with guanine than with the normal partner of uracyl — adenine (Mandel, 1969). This would lead to serious disturbances in the translation process.

5-FU is, thus, not a specific inhibitor of DNA synthesis, since it acts in a characteristic way on the production and function of RNA. So far special investigations have not been performed on the course of inhibition of mitotic activity under the action of various 5-FU concentrations and on the degree of recovery after removal of the inhibitor. This task was undertaken in the present study which envisages the utilization of 5-FU as a manysided inhibitor.

MATERIAL AND METHODS

1. As outset material served adventitious roots of onion (*Allium cepa* L. variety 'Wolska') of 4—5 cm length.

2. 5-FU (Fluka AG Buchs, Switzerland) was used for the experiment. The solutions were prepared in distilled spring water of concentration: 1 mg/l, 10 mg/l and 100 mg/l.

3. The onion bulbs with roots 4—5 cm long were incubated singly in 75-ml flasks for 24 h in unaerated 5-FU solution: 100 g/l (bulbs A and B), 10 mg/l (bulbs C and D) and 1 mg/l (bulb E).

Simultaneously a culture of control bulbs was run in water under the same conditions. In the course of incubation 2 roots from each onion were

fixed after 0, 4, 8, 10, 12 and 24 h. After thorough washing of the roots in several waters, the bulbs were transferred for the postincubation period to a glass container of 3 l. volume. During postincubation the water was continuously aerated and changed every 12 h. Samples were taken after 4, 8, 10, 12, 14, 16, 18, 20, 24, 28, 34, 36 and 48 h, two roots being fixed from each bulb. The experiment was run in darkness at 24° C.

4. The root tips collected were at once fixed in acetoalcohol (1:3) for 2 h, washed with 96 per cent ethanol for 1/2 h and with 80 per cent ethanol in which the material was stored. The tip 0.3 cm in length was stained and macerated in 2 per cent acetoorcein with 1 N HCl (9:1) added for 1 h at room temperature and then a permanent squash was prepared by the dry-ice technique.

5. Microscopic analysis of the squashes was performed at: $\times 1000$ magnification. The mitotic index was calculated for 1000 meristematic cells in each root. On the basis of the sum of mitoses in both roots and the frequency of occurrence of the successive mitose phases the phase index was calculated for each bulb in each sample.

RESULTS

Incubation of 24 h of onion roots in 100 mg/l. 5-FU produces a number of cytological changes in the meristematic cells. The most striking differences are: enlargement of nucleoli and marked thickening and considerable increase in density of the chromatin structure (Plate I). The effect, though less pronounced, appeared when 10 mg/l. 5-FU was used.

It was found that 24-h incubation in 5-FU elicits aberrations. They appear during incubation and occur over the entire period of postincubation. They are most distinct during anaphase and telophase in the form of "bridges" and chromosome fragmentation, the consequence of which is the appearance of micronuclei (Plate II). Aberrations of 1/2 chromatid type were observed only sporadically in the incubation period (Plate II, Photo 1) and chromatid aberrations were noted in the postincubation period. A very characteristic sign of cytological changes under the influence of 100 mg/l. 5-FU is the occurrence within the cytoplasm of numerous grains staining with acetoorceine. They were observed after long (28-48-h) postincubation in meristematic cells both during interphase and mitosis (Plate II, Photo. 8).

In the present study other disturbances making mitosis impossible were not observed. On the other hand, disturbances occurred in the course of cytokinesis under the influence of 100 mg/l. 5-FU, manifested by sporadic appearance of binuclear cells (Plate II, Photo. 7).

The results of calculation of mitotic indices during incubation and postincubation are shown graphically in diagram (Fig. 1) (the percentual values are arithmetic means of mitotic indexes for the 2 roots in the given sample). At the moment of starting incubation the mitotic index in the roots of all onions was rather high (12.7—14.7%). After 24 h incubation it decreased in the roots of the experimental bulbs in dependence

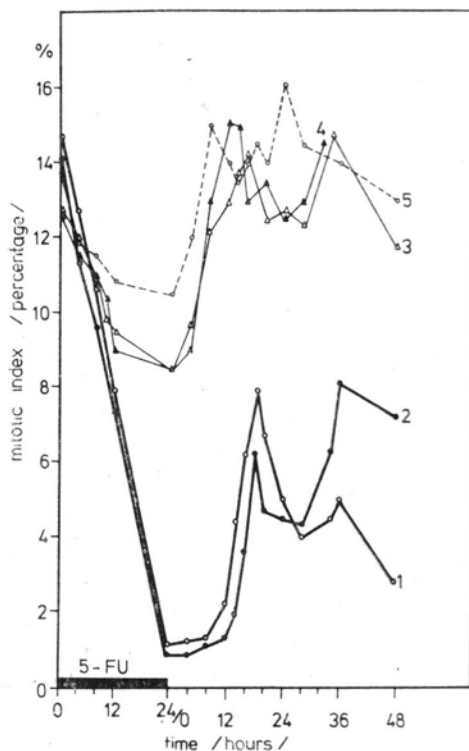
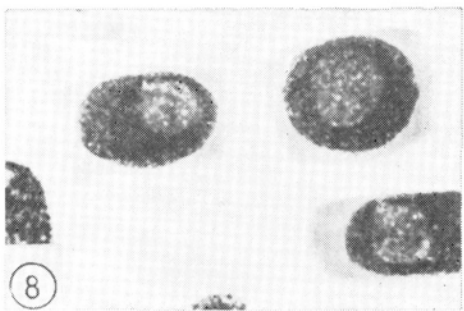
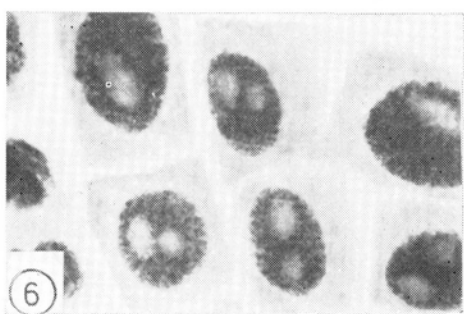
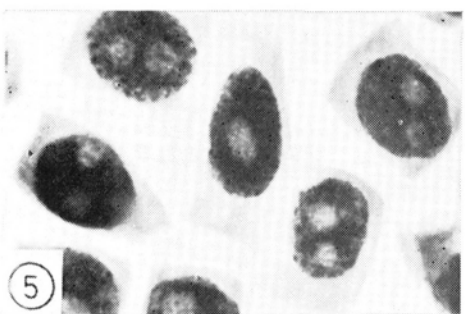
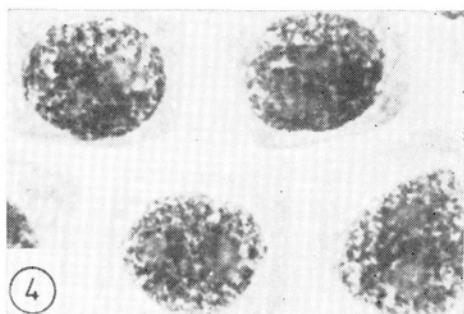
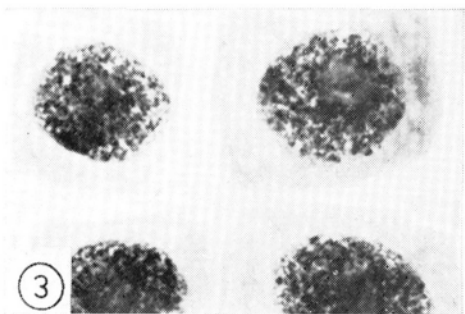
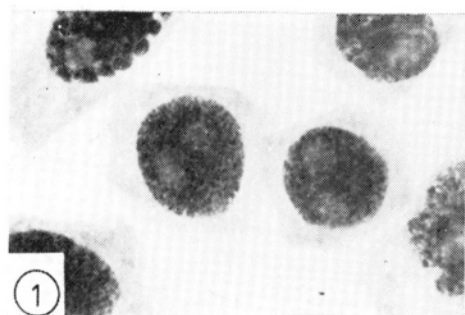


Fig. 1. Mitotic activity in the roots of experimental onions during 24 hour incubation in 5-FU and in the postincubation period

1—5 average mitotic indices in the roots of onions: 1 — „A” 100 mg/l 5-FU, 2 — „B” 100 mg/l 5-FU, 3 — „C” 10 mg/l 5-FU, 4 — „D” 10 mg/l 5-FU, 5 — „E” 10 mg/l 5-FU

on the 5-FU concentration. Mitotic activity was almost completely abolished only when 100 mg/l. of 5-FU was used (M. I. 0.7—0.9%), partial with 10 mg/l. (M. I. 8.2—8.4%) and only slight in the case of 1 mg/l. (M. I. 10.5%). During postincubation mitotic activity is restored in the form of a wave of partly synchronized mitoses. It was observed that the higher the 5-FU concentration the later did the mitotic index reach maximum during postincubation and the lower was its absolute value (Diagram, fig. 1). In the roots of the control onion the mitotic index fluctuated for 36 h within the limits of 12.0—13.6 per cent (Diagram, fig. 7).



Effect of 24 hour incubation in 100 mg/l 5-FU on the nuclei structure and the nucleolar size in meristematic cells in the roots of onion.

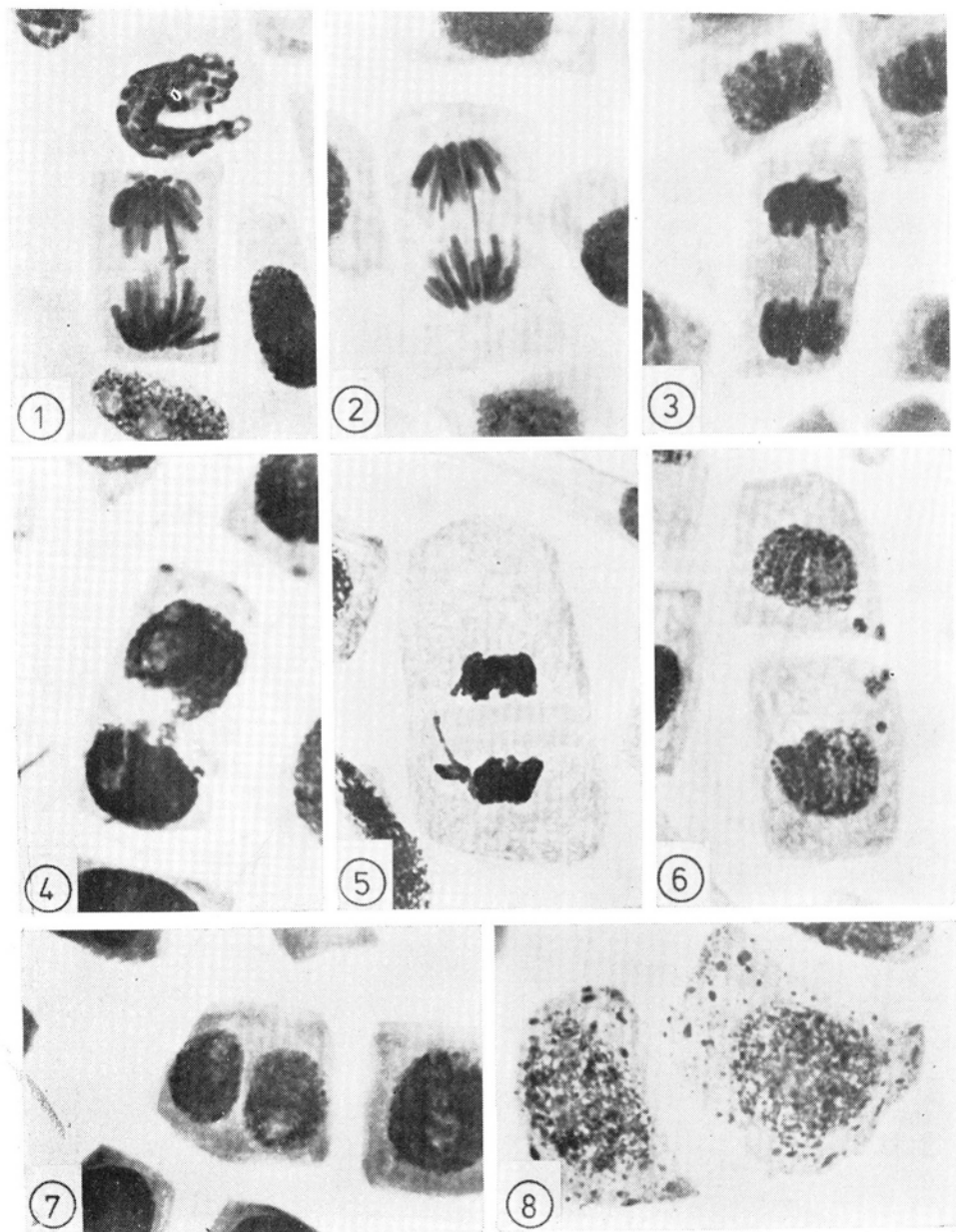
Photos 1, 2. Interphasic nuclei structure in meristematic cells of the control onion roots

Photos 3, 4. Interphasic nuclei structure in meristematic cells of the experimental onion roots incubated 24 hour in 100 mg/l 5-FU

Photos 5, 6. Nucleolar sizes in meristematic cells of the control onion roots

Photos 7, 8. Nucleolar sizes in meristematic cells of the experimental onion roots incubated 24 hour in 100 mg/l 5-FU

Photos 1—8 — stained with acetoorceine, 1000 \times ; photo 7 — phase contrast, \times contrast, \times 1000



Cytological changes in meristematic cells of onion roots incubated in 5-FU

Photo 1. 1/2 chromatid aberration in telophase, 100 mg/l 5-FU, 24 + 0

Photos 2—5. Chromatid aberrations in telophase, 10 mg/l 5-FU, 24 + 14

Photo 6. Cells with micronuclei, 100 mg/l 5-FU, 24 + 18

Photo 7. Binuclear cell, 100 mg/l 5-FU, 24 + 18

Photo 8. Grains in cytoplasm in meristematic cells of the experimental onion roots after long postincubation 100 mg/l 5-FU, 24 + 28

Photos 1-8 — stained with acetoorceine, $\times 1000$

Diagrams (figs 2—7) present graphically the mitotic index and phase indices in the roots of experimental onions and of the control one during incubation and postincubation. The phase indices in the control onion roots were more or less stable over 48 h, varying within the limits of several per cent. Fluctuation of the prophase index in these roots reflects variations in the mitotic index, and the decrease in the per cent of prophases was accompanied by an increase in the per cent of telophases and vice versa. Such relations are to be expected when the time of the successive mitosis phases does not change and the variations of the mitotic index are the consequence of changes in the per cent of cells entering mitosis.

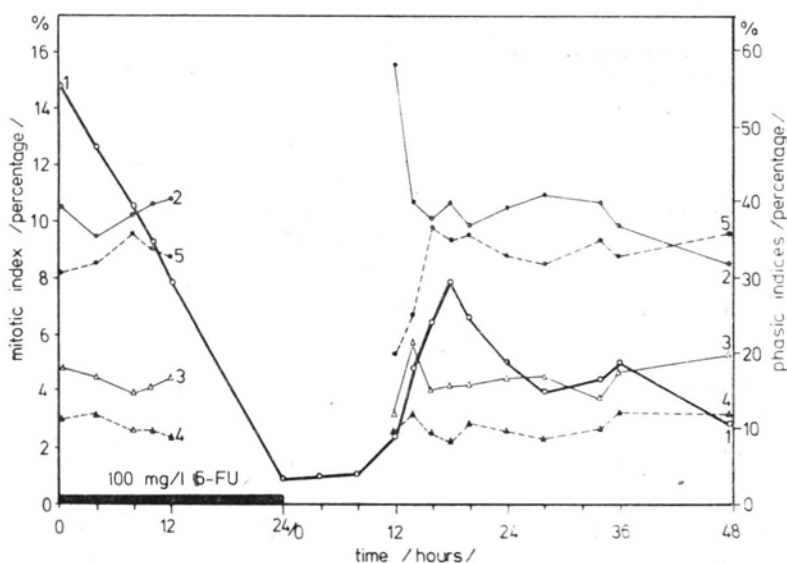


Fig. 2. Mitotic index and phase indices in the roots of onion "A" during 24 hour incubation in 100 mg/l 5-FU and in the postincubation period in water

1 — mitotic index, 2 — prophase index, 3 — metaphase index, 4 — anaphase index, 5 — telophase index

In the period of incubation in 5-FU there was no fall of the prophase index in roots of the experimental onions, in spite of a drastic decrease of the mitotic index. In the course of the first 12 h of incubation even a rise of the prophase and metaphase index was noted in all the concentrations tested. This points to a considerable prolongation of the duration of prophases and metaphases under the direct action of 5-FU.

In the postincubation period the phase balance is disturbed owing to partial synchronization of mitoses. The increase in the mitotic index starts with the prophase wave. The metaphase, anaphase and telophase waves are correspondingly shifted in time in relation to the prophase wave. The

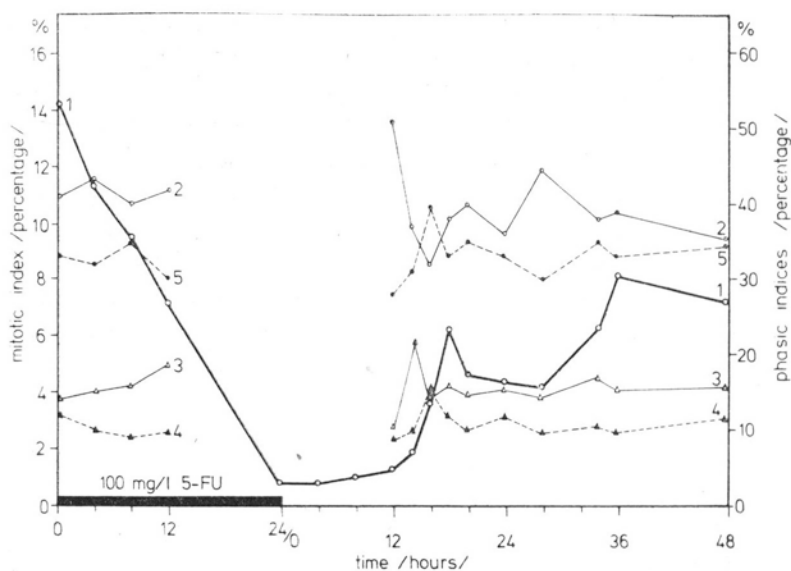


Fig. 3. Mitotic index and phase indices in the roots of onion "B" during 24 hour incubation in 100 mg/l 5-FU and in the postincubation period in water (Explanations as in Fig. 2)

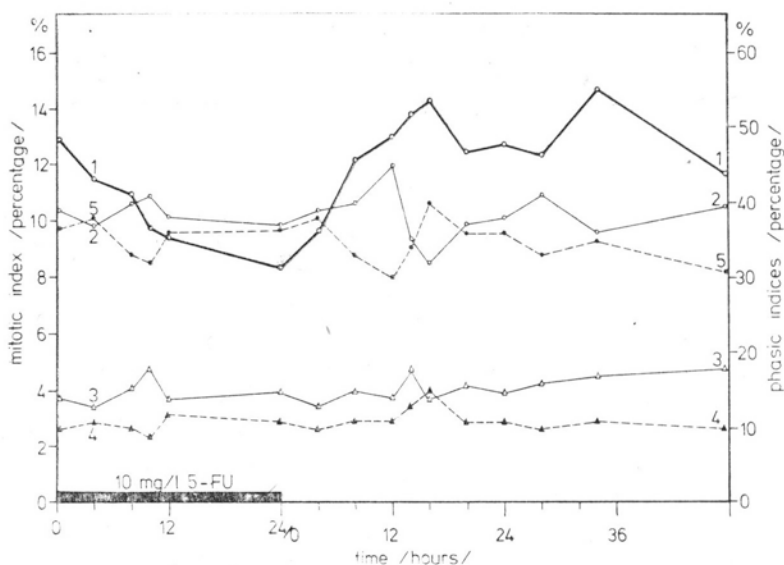


Fig. 4. Mitotic index and phase indices in the roots of onion "C" during 24 hour incubation in 10 mg/l 5-FU and in the postincubation period in water (Explanations as in Fig. 2)

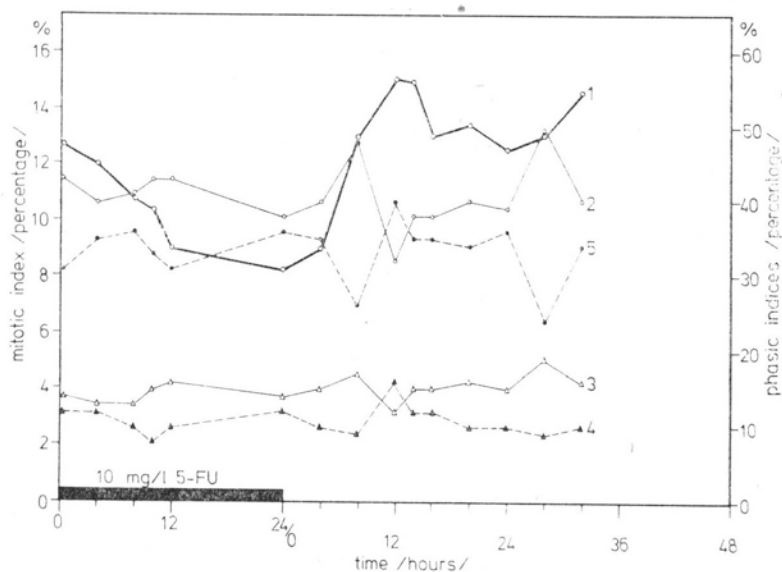


Fig. 5. Mitotic index and phase indices in the roots of onion "D" during 24 hour incubation in 10 mg/l 5-FU and in the postincubation period in water (Explanations as in Fig. 2)

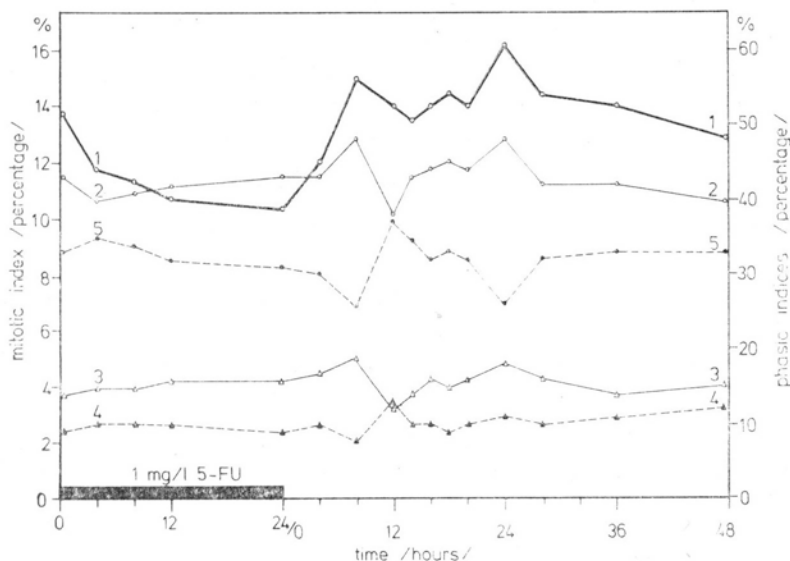


Fig. 6. Mitotic index and phase indices in the roots of onion "E" during 24 hour incubation in 1 mg/l 5-FU and in the postincubation period in water (Explanations as in Fig. 2)

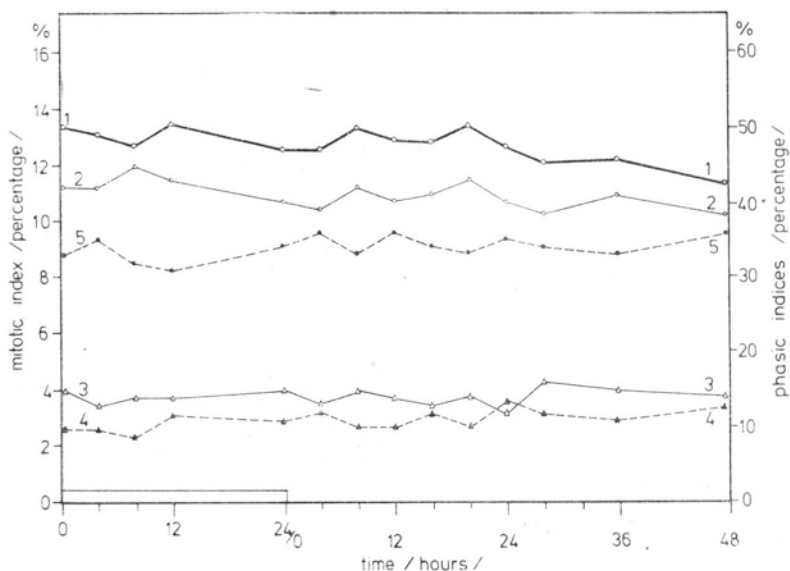


Fig. 7. Mitotic index and phase indices in the roots of the control onion.
(Explanations as in Fig. 2)

peak of the first telophase wave was shifted in relation to the prophase peak by 4 h in all cases.

In the roots of all experimental bulbs, with the exception of bulb A, a distinct second mitosis wave appeared and prophase, metaphase, anaphase and telophase waves shifted accordingly. This made possible approximate determination of the duration of the first mitotic cycle in the post-incubation period (Tab. 1). It amounts after incubation in 100 or 10 mg/l 5-FU to 18 (20) hours and after incubation in 1mg/l. 5-FU to 16 hours.

Table 1

Duration of first mitotic cycle in the postincubation period, after 24 hour of incubation in 5-FU, in the onion root apical meristem

Onion	5-FU concentration	Mitotic cycle calculated on the basis of the phase index	Mitotic cycle calculated on the basis of the mitotic index
A	100 mg/l	T=18 h	T \geq 18 h
B	100 mg/l	T=18 h	T \geq 18 h
C	10 mg/l	T \geq 16 h	T=18 h
D	10 mg/l	T=20 h	T=20 h
E	1 mg/l	T=16 h	T=16 h

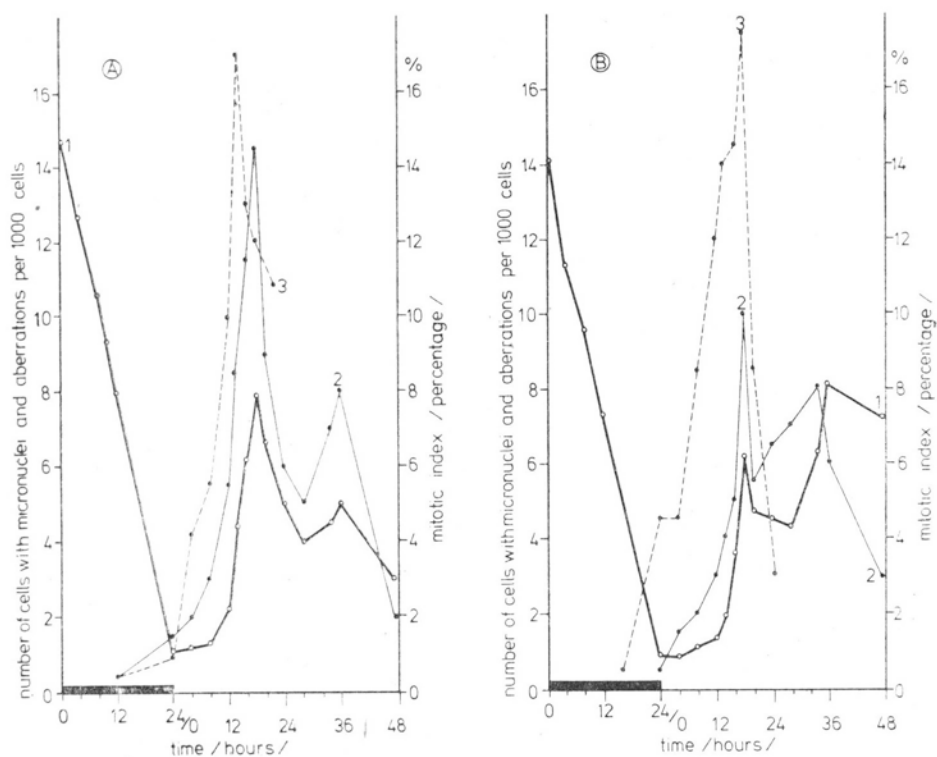


Fig. 8. Effect of 24 hour incubation in 100 mg/l 5-FU on the frequency of occurrence of chromatid aberrations and micronuclei per 1000 meristematic cells

A — onion „A”, B — onion „B”

1 — mitotic index, 2 — number of cells with aberrations per 1000 meristematic cells, 3 — number of cells with micronuclei per 1000 meristematic cells

DISCUSSION

A characteristic manifestation of the action of 5-FU is the considerable increase in the nucleolus size, as earlier noted both in plant and animal material. Analogous results were obtained in the present study. It is generally considered that the enlargement of the nucleoli is the consequence of accumulation in them of abnormal FUR-RNA (Simard, 1970; Rose et al., 1970).

Striking is the change in chromatin structure. Nuclei with a denser structure appear in large numbers after 24 h of incubation and in the early period of postincubation. In the cells of the control roots nuclei with such a structure occur much less frequently. Probably nuclei with structure changed under the influence of 5-FU correspond to those in late G_1 phase or at transition of G_1/S phases.

5-FU, as demonstrated during the present investigations, provokes chromosome aberrations manifested both during incubation and postin-

cubation. The long, 24-h, period of incubations does not allow to establish the relation between the action of 5-FU on a definite phase of the mitotic cycle and the type of aberration arising. Characteristic, however, is the finding of a type 1/2 chromatid aberration after 24 h of incubation in 100 mg/l. 5-FU. Aberrations of this type are evoked by 5-FUdR as the direct consequence of its action on G_2 , whereas aberrations evoked by influence on phase S preceding the occurrence of aberrations mitosis are of chromatid type (Kihlman, 1962; Taylor et al., 1962; Bell and Wolff, 1964). Such chromatid aberrations occur under the influence of 5-FU in the postincubation period. Thus, the mechanism evoking aberrations through 5-FU is the same as that acting through 5-FUdR and it is associated with inhibition of DNA synthesis.

A sequel of the occurrence of chromosome aberrations is the appearance of micronuclei in the cells, arising from eliminated acentric chromosome fragments not incorporated into the nuclei. It results from diagram 8 that in the postincubation period the number of micronuclei increases for a certain time and then decreases, whereas the frequency of occurrence of cells with micronuclei is higher than that of the occurring aberrations. This seeming contradiction can be explained if we consider that the increasing number of micronuclei is, up to a certain moment, the result of summation of all the earlier formed ones. The decrease of the number of micronuclei after 14–18 h of postincubation is the result of their disappearance. In the period of interphase between the first and second mitotic cycle the micronuclei dissolve in the process of autolysis.

The sporadically occurring disturbances in the cytokinesis process, leading to the formation of binucleate cells are a direct or indirect consequence of disturbances in RNA biosynthesis.

It has been demonstrated in numerous papers that 5-FU causes blocking of mitoses in the root meristem. Analysis of the mechanism of action of 5-FU (inhibition of thymidylic acid synthesis) leads to the conclusion that abolition of the mitotic activity is a consequence of the action of 5-FU in the intermitotic period on the S phase. It has been demonstrated in the present study that 5-FU in a 100 mg/l. concentration almost completely inhibits the mitotic activity of onion root meristems without arresting the cells in G_2 phase, as indicated by the gradual depression of mitotic activity during incubation. The blockade of thymidylic acid synthesis leads therefore, finally to an arrest of most cells in phase G_1 , part of them being in various stages of phase S. Even the highest concentration applied in the study did not have a lethal effect on the onion root meristem in the 24-h period of incubation. This is indicated by the fact of renewal of mitotic activity in the roots of all experimental bulbs in the postincubation period. This recovery, was, however, not complete in all cases (return to the state of the control). In the roots of bulbs A and B (100 mg/l. 5-FU) in which almost complete abolition of mitotic

activity was observed after 24 h of incubation renewal of this activity started latest. This may be evidence of a prolonged action of 5-FU due to the longer lasting washing out of the inhibitor from the cells. It was also noted that the meristematic cells of these bulbs exhibited the most enlarged nucleoli what may be considered as a symptom of inhibition of normal ribosome synthesis. It is not known exactly how this inhibition of ribosome production in interphase affects the course of the subsequent mitosis. The few studies dealing with this problem seem to indicate that normal metabolism in the nucleolus is indispensable. McLeisch (1964) demonstrated that the micronuclei obtained as the result of action of maleic hydrazide on the meristematic cells of field bean roots are only capable of mitosis if they possess nucleoli. Investigations on animal embryogenesis suggest that the nucleolus is not indispensable for cell division only when the cells are rich in ribosomes (Hay, 1968).

The mitotic activity restored in the postincubation period is of undulatory character owing to partial synchronization of mitoses. On this basis the approximate duration of the first mitotic cycle in the roots of the experimental onions was calculated. As compared with the data of López-Sáez et al. (1966) and González-Fernández et al. (1966) (mitotic cycle at 25°C 12-14 h), this would mean a prolongation of the duration of the life cycle of meristematic onion root cells incubated in 10 and 100 mg/l. 5-FU by about 4-6 h and of those incubated in 1 mg/l. by 2-4 h. The causes of protraction of prophase and metaphase duration during incubation in 5-FU, of prolongation of duration of the mitotic cycle and of the relatively low level of the renewed mitotic activity should be sought in the disturbances of normal protein biosynthesis due to incorporation of 5-FU into RNA.

This conclusion is confirmed by reports on the influence of 5-FUdR on mitotic activity. This compound has long been used as a specific DNA biosynthesis inhibitor, it is commonly utilized in animal tissue cultures for synchronization of cell populations at a definite phase of the mitotic cycle (Ruecker and Mueller, 1960; Littlefield, 1962; Epifanova et al. 1969). After 16 h of acting with 1.5×10^{-6} M 5-FUdR, the mitotic activity of HeLa cells is reduced to zero per cent, and 6-10 h after elimination of the blockade and addition of thymidine 70-95 per cent of the cells enter mitosis (Rueckert and Mueller, 1960). Epifanova et al. (1969) demonstrated that in cultures of Chinese hamster cells there appears after 8-12 h from the moment of complete blocking of the mitotic activity by 10^{-6} M 5-FUdR, a distinct wave of synchronized mitoses exceeding (43-46%) that in the controls (33-37%):

5-FUdR is thus effective in concentrations 100 times lower than those of 5-FU, and within the concentration range 10^{-6} - 10^{-7} M the former might be considered as a specific DNA synthesis inhibitor. Van Hofsten (1964), however, while investigating the influence of FUdR on the growth

and morphogenesis of *Ophiostoma multiannulatum* remarked a considerable enlargement of the nucleoli under the influence of 10^{-6} – 10^{-4} M 5-FUdR, which could be evidence of inhibition of normal ribosome production. It is known that 5-FUdR can be partially transformed in the cell to 5-FU. Thus 5-FUdR can disturb normal biosynthesis and functioning of RNA as well, and in turn normal protein synthesis.

The results of the present study indicate that the influence of 5-FU on DNA synthesis is reversible. When 5-FU is eliminated from the medium, and after a delay period during which the 5-FU concentration falls below the threshold value, thymidylc acid synthesis is resumed. Evidence of this is found in the renewed mitotic activity. The consequences of 5-FU incorporation into RNA are more durable and for a longer time period normal metabolism of the cell is disturbed.

Acknowledgement

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*Wpływ 5-fluorouracylu na aktywność mitotyczną merystemu
wierzchołkowego korzeni cebuli.*

Streszczenie

W pracy tej zbadano wpływ różnych stężeń 5-fluorouracylu (5-FU) na aktywność mitotyczną merystemu wierzchołkowego korzeni cebuli (*Allium cepa* L.) w okresie inkubacji w 5-FU oraz postinkubacji na wodzie. Wykazano, że 24-godzinna inkubacja korzeni cebuli w 100 mg/l 5-FU całkowicie ją wygasza, przy czym efekt ten jest odwracalny. W czasie postinkubacji aktywność mitotyczna wznowia się w postaci fali częściowo zsynchronizowanych mitoz. Najwyraźniej zaznaczoną synchronizację mitoz uzyskano pod wpływem 100 mg/l 5-FU, lecz indeks mitotyczny wznowionej aktywności mitotycznej był o połowę niższy od wartości kontroli.

Synchronizacja mitoz umożliwiła obliczenie w sposób przybliżony czasu trwania pierwszego cyklu mitotycznego. Stwierdzono, że cykl mitotyczny merystematycznych komórek korzeni cebuli inkubowanych w 10 i 100 mg/l 5-FU ulega przedłużeniu o 4-6 godzin a inkubowanych w 1 mg/l 5-FU o około 2-4 godz. Stwierdzono też, że 24-godzinna inkubacja w 5-FU wywołuje szereg zmian cytologicznych w merystematycznych komórkach korzeni cebuli. Zmianie ulega struktura chromatyny jąder interfazowych a jąderka znacznie się powiększają. Obserwowano występowanie aberracji 1/2-chromatydowych i chromatydowych w okresie inkubacji w 5-FU oraz aberracji chromatydowych w czasie postinkubacji.

Zmiany zachodzące w merystemie korzeniowym cebuli w trakcie inkubacji w 5-FU oraz przyczyny wydłużenia się czasu trwania cyklu mitotycznego i względnie niskiego poziomu wznowionej aktywności mitotycznej podczas postinkubacji przedyskutowano w oparciu o molekularne mechanizmy działania 5-FU na syntezę kwasów nukleinowych i porównano je z wpływem 5-FuDR.