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Effect of hydroxyurea on mitotic activity ^{3}H-thymidine and ^{3}H-phenylalanine incorporation in the antheridial filament cells of *Chara vulgaris**

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Abstract

Hydroxyurea inhibits mitotic activity in cells of the antheridial filaments of *Chara vulgaris* by blocking phase S and phase G_2. Blocking of cells in phase G_2 also occurs in the case of the root meristem cells of *Helianthus annuus* and *Vicia faba* var. *minor*. ^{3}H-thymidine incorporation confirmed autoradiographically the blocking of cells of the antheridial filaments in *Chara vulgaris* at phase S and slowing down of the rate of DNA replication. Incubation with ^{3}H-phenylalanine demonstrated that hydroxyurea inhibits protein synthesis.

INTRODUCTION

Hydroxyurea (HU) is a widely used DNA synthesis inhibitor. It has been used until recently in chemotherapy as a cytostatic (Beckloff et al., 1965). The inhibitory influence of HU on DNA replication was noted in viruses (Bell and Maassab, 1968), in the cells of *Prokaryota* (Rosenkranz et al., 1966) and *Eukaryota* (Sinclair, 1967; Cameron and Jeter, 1973; Rennert, 1977 a, b). Moreover, HU is used for synchronization of cells, although it is a mutagenic agent.

The molecular mechanism of HU action is still controversial. It is supposed that it consists in blocking reductase activity of ribonucleoside diphosphatase (Young et al., 1967). The nucleus is deprived either of all desoxyribonucleotides (Plagemann, and Erbe 1974), or according to other authors, only of desoxythymidylic acid (Habdas,

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1977). As consequence of this, the pool of endogenic desoxyribonucleotides is soon exhausted and this can be observed as a slowing down of the rate of synthesis of a new DNA chain (Ramseier et al., 1977). The action of HU seems to be more extensive since an inhibitory effect on RNA and protein synthesis was also observed (Maciejewska-Potapczyk et al., 1970; Butler and Mueller, 1973; Ruderman and Gross, 1974; Bilecka, 1975a, b).

Phase S of the cell cycle is particularly susceptible to HU. The cell nucleus ultrastructure is then considerably modified, accumulation of chromatin present at the nuclear envelope occurs, the chromocentres are joined by chromatin bridges with the nuclear envelope (Nagl, 1973).

The object investigated in the present study were the antheridial filaments of *Chara vulgaris* L., arising as the result of successive spontaneously synchronized divisions of one cell. In 64-cell filaments differentiation of spermatides occurs. This material is characteristic for the cell cycle of type $S+G_2+M$, and the cell length is strictly correlated with the interphase period (Olszewska and Godlewski, 1972).

Primarily the aim of the present paper was an attempt to achieve complete synchronization of division in the antheridial filaments of *Chara vulgaris* within the antheridium or even in the whole plant, and study of the influence of HU on mitotic activity, radioactive thymidine and phenylalanine incorporation and on interphasal cell length. Synchronization of the whole population of antheridial filaments could not be achieved because part of them is blocked in phase S and part in phase G_2. Blocking in phase G_2 also occurs in the root meristems of *Helianthus annuus* and *Vicia faba*.

MATERIAL AND METHODS

Chara vulgaris plants from the pond in the village Pełczyska (Łódź district) were used for the experiments. The water from this pond body is characterized by a high content of salts (ca. 0.3%, pH 7.45) (Godlewski, 1973). Therefore the plants in the laboratory were kept in water from the natural environment and under artifical illumination (4000 lux) with a normal photoperiod (14 : 10) at 24°—26°C.

HU was applied in 10^{-3} M concentration. Water for dissolving HU and the radioactive compounds were also taken from the same pond.

Five development stages were investigated: 2-, 4-, 8-, 16- and 32-cell filaments. Only the generation dominating in the antheridium was analysed (Olszewska, 1978). The means were calculated on the basis of 6-8 antheridia from 4-5 plants.

Influence of HU on mitotic activity

The apical parts of the alga thallus were incubated in the HU solution for 12, 24, 48 and 72 h. The thallus fixed in a mixture of ethanol and glacial acetic acid (3 : 1) for 30 min was stained by the acetoorcein method. Squashes were prepared from antheridia and from them the mitotic index was calculated.

Part of the plants incubated for 24 h in the HU solution were postincubated in water for 24 or 48 h. The mitotic index was calculated from the squashes (prepared as mentioned above).

Two-day-old *Helianthus annuus* seedlings were incubated in HU solution (10^{-3}M dissolved in distilled water) for 3 h (duration time of phase G_2) or for 8 h (joint time of $S+G_2$ — Marciniak, unpublished data). The roots fixed (as described above) were stained by the Feulgen method. Squashes were made from apexes 1 mm long. The mitotic index was calculated from 8-10 roots after counting more than 200 cells.

Vicia faba var. *minor* seedlings 5-days-old were incubated in HU solution (10^{-3}M) dissolved in distilled water for 4.5 h (duration of phase G_2) or for 9 h (joint $S+G_2$ time — Osiecka, unpublished data). The preparations and analyses were made as for *Helianthus annuus*.

Influence of HU on ^{3}H-thymidine and ^{3}H-phenylalanine incorporation

The apical part of the *Chara vulgaris* thallus, after 22-h incubation in HU (conc. 10^{-3}M) were incubated for 2 h in HU solution containing ^{3}H-thymidine of 60 μCi/ml concentration (spec. act. 5 Ci/mM) or DL/G ^{3}H-phenylalanine of 7.5 μCi/ml concentration (spec. act. 4.8 Ci/mM). They were fixed as described above. The squashes were coated with liquid Ilford L4 emulsion. The exposure lasted 8 month for the material incubated in radioactive thymidine or 11 days for material incubated with ^{3}H-phenylalanine. The autoradiograms were stained with the Unna's mixture. The per cent of labelled nuclei and the level of their radioactivity were calculated according to a 5-grade scale of increasing intensity 1 to 5.

In the autoradiograms with radioactive phenylalanine the number of dark emulsion grains over the nucleus and cytoplasm was counted by means of an ocular grid micrometer. The successive steps of interphase were determined on the basis of the cell size (Olszewska and Godlewski, 1972).

Influence of HU on cell length

The autoradiograms with ^{3}H-thymidine served for measuring the length of telophase cells, whereas the distribution of this length and the frequency of occurrence of cells of a define length were analysed on preparations stained by the aceto-orcein method.

RESULTS

Influence of HU on mitotic activity

HU has an inhibitory influence on the mitotic activity in antheridial filaments of *Chara vulgaris*. The data shown in table 1 indicate that the restriction of the mitotic index is correlated with the time of action of this compound. After 12 h of exposure to HU the mitotic activity in 16- and 32-cell filaments was slightly changed or not at all, whereas some few cells in the same filaments underwent division after 72 h of exposure. The effectiveness of the HU inhibitory action is thus closely related to the development stage of the antheridial filaments. Cells of young generations are more susceptible to this compound as compared with 16- and 32-cell filaments.

Table 1

Effect of hydroxyurea on the mitotic activity (%) in successive 2-,4-,8-,16- and 32-celled generations of antheridial filaments of *Chara vulgaris*

Stage of development / HU, h	2-cell	4-cell	8-cell	16-cell	32-cell
Control	20.6 ±2.2	21.5 ±2.6	22.8 ±2.6	23.2 ±2.1	24.3 ±3.7
12	8.7 ±2.2	11.0 ±3.8	15.5 ±2.3	16.6 ±3.2	22.9 ±3.5
24	0.9	0.7	1.9 ±0.7	6.4 ±2.8	16.1 ±3.9
48	0.0	0.0	1.6 ±0.8	5.0 ±1.1	9.2 ±1.7
72	0.0	0.0	0.0	2.0 ±1.3	4.2 ±1.9

An exposure of 12 h to HU comprises various phases of the cell cycle of the 5 studied stages of development of the antheridial filaments (Fig. 1, arrows). On the basis of the previously obtained data (Godlewski and Olszewska, 1973; Godlewski, 1977; Maszewski, 1977) it may be assumed that this period includes more than half of the duration time of phase G_2 in 2-cell filaments and part of phase S in 32-cell ones. Nevertheless, the mitotic index is most limited in the 2-cell filaments where inhibition reaches 58 per cent (Fig. 1). In 32-cell filaments it does not change, if we take into account the standard error.

Postincubation of plants treated previously for 24 with HU did not change the mitotic activity of the 2- and 4-cell filaments (Table 2). The antheridial filaments in later developmental stages showed an increase of mitotic activity, higher after a longer postincubation time (48 h). Thus, synchronization of division was not observed within the thallus.

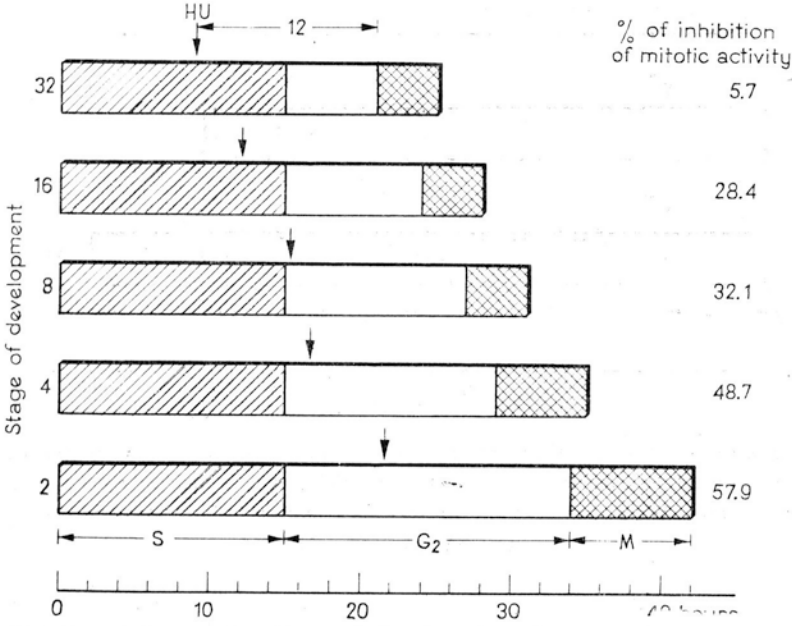

Fig. 1. Relation between the time of treatment with HU (12 h) and inhibition of mitotic activity in 2-, 4-, 8-, 16- and 32-cell antheridial filaments of *Chara vulgaris*. Arrows indicate beginning of incubation with HU

Table 2

Mitotic activity (%) in successive 2-,4-,8-,16- and 32-celled generations of antheridial filaments of *Chara vulgaris* incubated for 24 h in hydroxyurea solution and subsequently transferred into water for 24 or 48 hours

Stage of development / Postincubation, h	2-cell	4-cell	8-cell	16-cell	32-cell
24	0.4	1.4	5.5 ±2.7	10.7 ±2.6	16.3 ±3.2
48	0.3	0.8	8.7 ±2.1	12.4 ±3.2	17.5 ±3.5

The relation between the time of HU action in the cell cycle and the mitotic activity of root meristems is shown in table 3 and fig. 2. When the time of action of this compound was equal to phase G_2

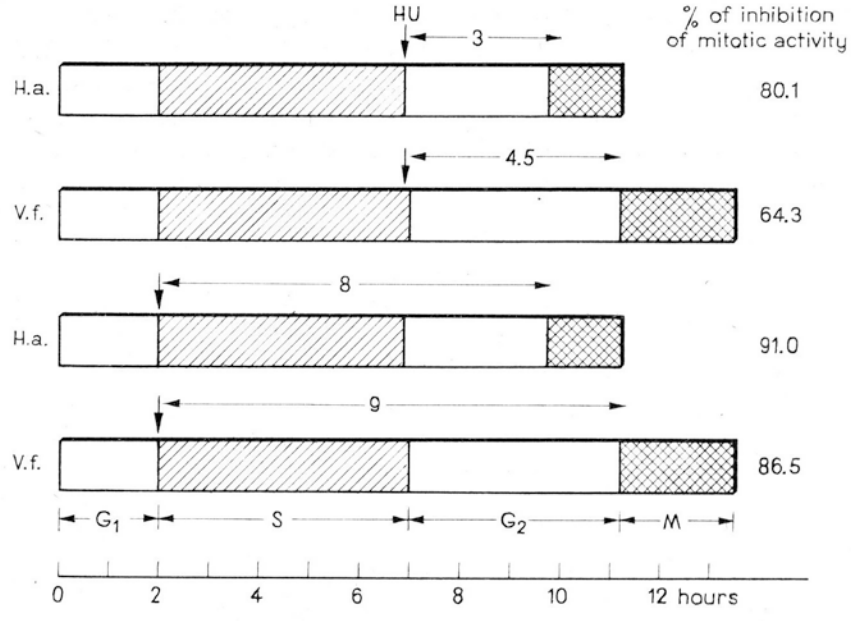

Fig. 2. Relation between time of HU treatment (including phase G_2 or $S+G_2$) and inhibition of mitotic activity in the meristematic zone of *Helianthus annuus (H.a.)* roots and *Vicia faba* var. *minor (V.f.)*. Arrows indicate the beginning of incubation with HU

Table 3

Effect of hydroxyurea on the mitotic activity (%) in root meristems

Species \ Cell cycle phase	Control	G_2 treatment with HU	$S+G_2$ treatment with HU
		3 h	8 h
Helianthus annuus	8.2 ±0.5	1.6 ±0.2	0.7 ±0.2
		4.5 h	9 h
Vicia faba var. *minor*	6.4 ±0.5	2.3 ±0.5	0.9 ±0.3

duration, a strong inhibition of mitotic activity, reaching for *Helianthus annuus* more than 80 per cent was observed, for *Vicia faba* it exceeded 64 per cent. The inhibitory effect is enhanced with prolongation of exposure to HU; when it comprises phases $S+G_2$ inhibition attains 91 and 85 per cent, respectively.

Influence of HU on ^{3}H-thymidine incorporation

In the presence of HU the per cent of nuclei labelled with ^{3}H-thymidine increases (Fig. 3). This effect is correlated with the stage of development of the antheridial filaments and consists in an increase as compared with the control of the number of labelled nuclei: in 2-cell filaments by 12.9, in 4-cell ones by 17.5, in 8-cell ones by 20.2, in 16-cell ones by 22.7 and in 32-cell ones by 20.3 per cent.

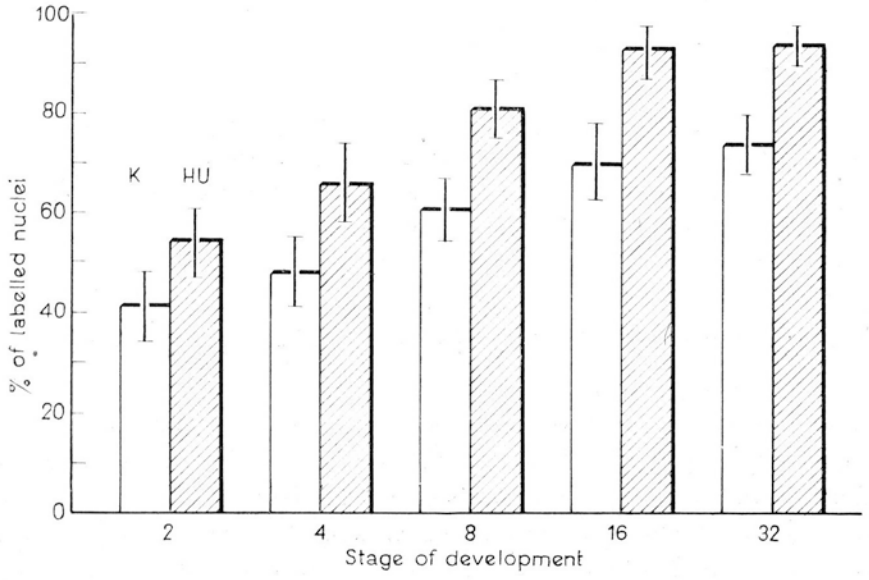

Fig. 3. Influence of HU (24 h) on the number of nuclei incorporating ^{3}H-thymidine in the cells of antheridial filaments of *Chara vulgaris*
K — control, HU — hydroxyurea

The intensity of labelling of the nuclei incorporating ^{3}H-thymidine diminishes under 24-h exposure to HU (Fig. 4). On the other hand, in cells, the size of which corresponded to phase G_2 in the control material, the nuclei exhibited high radioactivity. Their level of labelling increased proportionally to the progress in the development of the antheridial filaments from the 2-cell generation where it was lowest to the 32-cell one where it reached the highest value. In older developmental stages (16- and 32-cell filaments) nuclear radioactivity in the presence of HU did not change in the course of interphase.

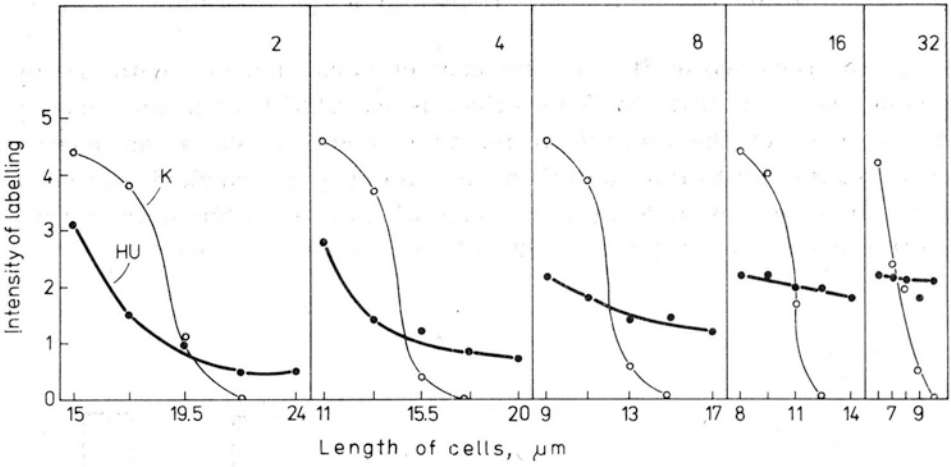

Fig. 4. ^{3}H-thymidine incorporation into the nuclei in the course of interphase in 2-, 4-, 8-, 16 and 32-cell antheridial filaments of *Chara vulgaris* in control material *(K)* and that treated with HU for 24 h

Influence of HU on ^{3}H-phenylalanine incorporation

The changes in radioactivity of nuclei and cytoplasm under the influence of 12- and 24-h exposure to HU are shown in Fig. 5. The inhibitory influence of this compound on ^{3}H-phenylalanine incorporation into the nuclei and cytoplasm is doubtless in all developmental stages of the antheridial filaments. In the case of 12-h action of this compound a drastic diminution of the intensity of nucleus and cytoplasm labelling was observed, falling to the early S phase (smallest cells). Towards the end of interphase, however, a slight increase of radioactivity was noted. The oldest 32-cell stage does not show so marked a reduction of nuclei and cytoplasm labelling as do the younger generations.

Prolonged action of HU deepens the inhibitory effect on ^{3}H-phenylalanine incorporation. After 20 h of treatment with this compound maximal reduction of the radioactivity of nuclei and cytoplasm is observed at the beginning of interphase. The low intensity of labelling persists in the further course of interphase in 2-, 4-, 8-, 16- and 32-cell filaments. In the 32-cell generation the depression of radioactivity after 24 h of treatment with HU is equal to that in young developmental stages.

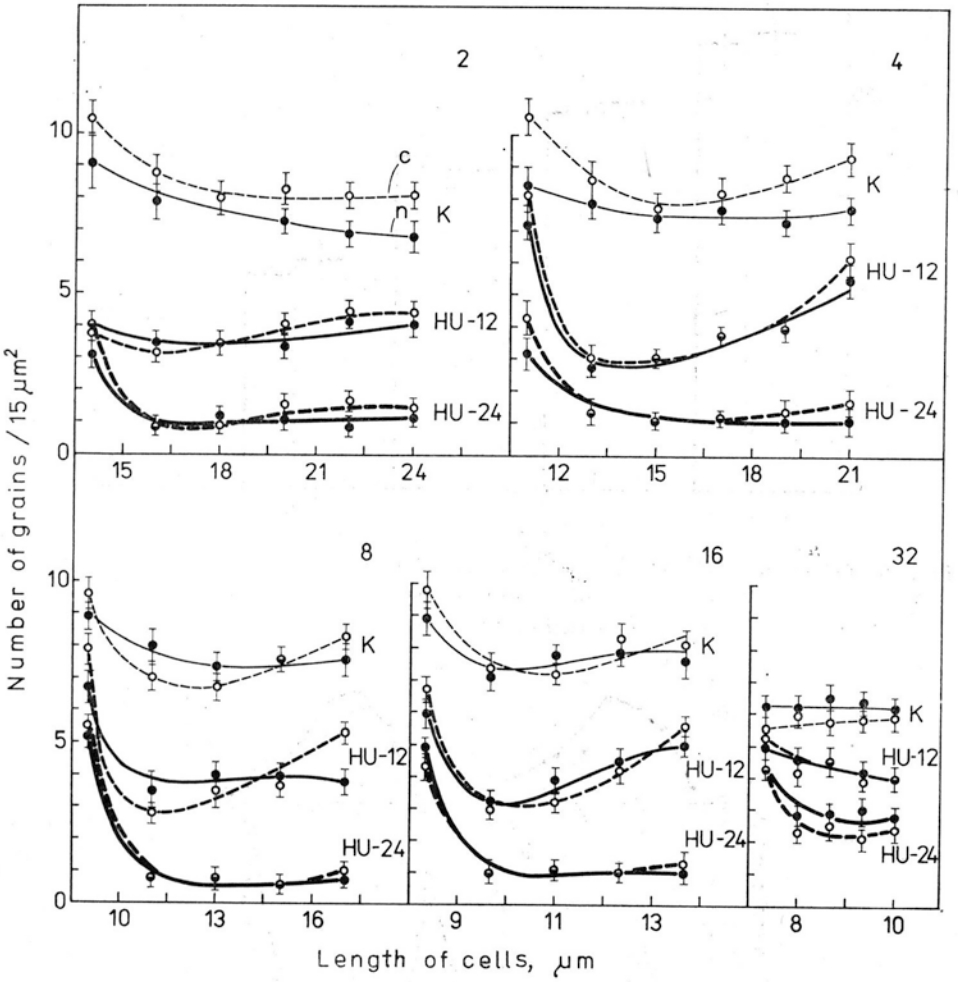

Fig. 5. ^{3}H-phenylalanine incorporation into 2-, 4-, 8-, 16- and 32-cell antheridial *Chara vulgaris* filaments in control material (*K*, *n* — nucleus, *c* — cytoplasm) and in material treated with HU for 12 and 24 h (HU-12, HU-24)

Influence of HU on cell length

HU does not change the length of the telophase cells in the investigated developmental stage of the antheridial filaments in *Chara vulgaris* (Fig. 6). Neither does it change the frequency of occurrence (in %) of interphasal cells of a definite length (Fig. 7). The curves representing these relations for the particular development stages are a similar for the control material and that treated with HU for 24 h.

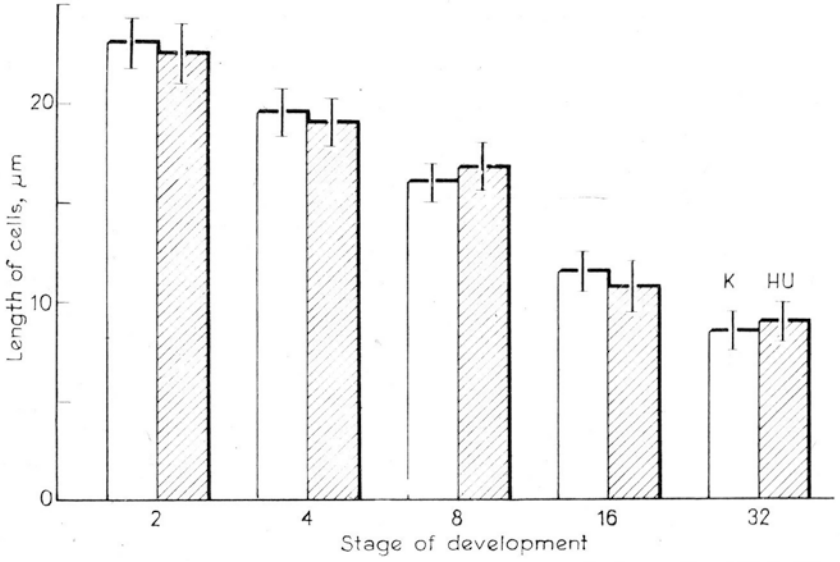

Fig. 6. Effect of HU (24 h) on telophase cell length in antheridial filaments of *Chara vulgaris*
K — control, HU — hydroxyurea

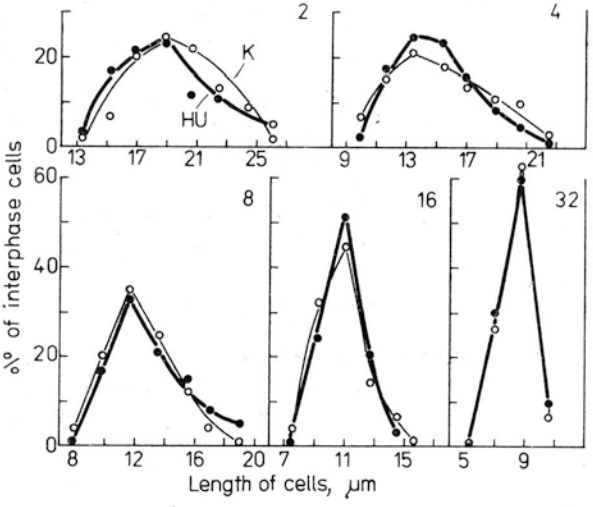

Fig. 7. Effect of HU (24 h) on the frequency of occurrence of cells of a given length (in %) in the antheridial filaments of *Chara vulgaris*
K — control, HU — hydroxyurea

DISCUSSION

Earlier investigations of the same authors on tissues of *Nicotiana tabacum* cultured *in vitro* demonstrated that the action of HU is not limited to inhibition of DNA replication, but it also diminishes RNA

and protein synthesis (Maciejewska-Potapczyk et al., 1970; Bilecka, 1975a, b). The present studies of antheridial filaments of *Chara vulgaris* demonstrated that HU has an inhibitory effect on mitotic activity as well as on incorporation of ^{3}H-thymidine and ^{3}H-phenylalanine. Cells objected to the action of HU are arrested in phase S and phase G_2, after shorter exposure only in phase G_2.

The cell cycle in the antheridial filaments is of type $S+G_2+M$, and DNA synthesis starts as early as the telophase of the preceding generation. The time of duration of S phase amounting to 16 h is a constant value for the cells of all developmental stages (Godlewski and Olszewska, 1973; Godlewski, 1977; Maszewski, 1977).

Analysis of the results concerning the influence of HU on radioactive thymidine incorporation seems to indicate that this compound slows down the rate of DNA replication, since it increases the number of labelled nuclei as compared with the control. Labelling of the nuclei of the antheridial filaments treated with HU continues over the entire interphase. The maximal increase of the number of labelled nuclei in cells corresponding in size to the G_2 phase in the control was observed in older developmental stages. It results therefrom that with development of the antheridial filaments the rate of DNA replication is slowed down more and more by HU. This supposition is confirmed by analysis of the intensity of labelling of the nuclei (Fig. 4). The cell nuclei treated with HU exhibit a lower radioactivity as compared with those of the control material in which maximal radioactivity is noted in the nuclei of the early S phase. Thus, the cells are blocked in phase S, their growth continuing normally (Figs 6 and 7). The action of HU on phase S is in agreement with numerous reports concerning both animal and plant cells (Fouquet et al., 1975; Diment and Blinova, 1976; Cernavali and Mariotti, 1977; Hecker, 1978).

The greatest depression of ^{3}H-phenylalanine incorporation in all the studied developmental stages is observed in cells, the size of which corresponds to phase S cells in the control material (Fig. 5). In the presence of HU histone synthesis is inhibited, and this process is coupled with DNA replication (Butler and Mueller, 1973). A considerable diminution of ^{3}H-phenylalanine incorporation at the beginning of interphase is, therefore, probably due to the slowing down of the rate of DNA replication (cf. Figs. 4 and 5).

Analysis of the influence of 12-h exposure to HU on mitotic activity of antheridial filaments seems to indicate that HU also interferes with phase G_2. This may be concluded from the fact that the period of action of HU in 2-, 4-, and 8-cell generations, bringing about inhibition of activity by 58, 49 and 32 per cent, respectively, falls to phase G_2 (Fig. 1).

Postincubation of plants (Table 2) leads to an increase of mitotic activity of the cells only in older generations of the antheridial filaments, while the young developmental stages continue to exhibit inhibited mitotic activity. Synchronization was not observed either within the rosette or within the thallus. This is understandable in the light of the results obtained which point to the interference of HU both with phase S and phase G_2.

It seem that the influence of HU on phase G_2 is not only limited to the cells of the antheridial filaments, but it also involves cells of higher plants with a typical cell cycle G_1+S+G_2+M. This supposition finds support in the results of experiments performed on the root meristems of *Helianthus annuus* and *Vicia faba* var. *minor*. The mitotic activity of the root meristems was greatly inhibited (more than 80 and 64%) even when the time of action of HU included only phase G_2. Protraction of the time of exposure to this compound over a period including phases $S+G_2$ only produced a further depression of the mitotic index (in *Helianthus* to 91 and in *Vicia* to 86.5%). Habdas (1977) obtained similar results in investigations on the influence of HU on the root meristem of onion.

Initiation of mitosis is known to depend on the synthesis of specific proteins. The interference of HU with protein synthesis in phase G_2 manifested by a lowered radioactivity of the nuclei and cytoplasm of antheridial filament cells in *Chara vulgaris* incubated in a medium with ^{3}H-phenylalanine for 12 h seems to indicate that the process of synthesis of the proteins responsible for mitosis initiation is inhibited.

The susceptibility of the antheridial filament cells to inhibitory agents, decreasing as the plants develop, remains an open question. Since this is true both for HU and for lack of light (Maszewski, 1977), it does not seem possible to attribute this effect to, for instance, the greater permeability of the membranes in the young developmental stages.

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Added in proof:

Schneiderman M. H., Kimler B. F., Leeper D. B. and Dewey W. C. (Exp. Cell Res. 1978, 115: 465-467) demonstrated that the treatment of monolayer cultures of Chinese hamster ovary cells with 1.0 mM hydroxyurea reduced the rate at which G_2 cells entered mitosis.

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Wpływ hydroksymocznika na aktywność mitotyczną, włączanie ^{3}H-tymidyny do DNA oraz ^{3}H-fenyloalaniny do komórek nici spermatogenicznych Chara vulgaris

Streszczenie

Stwierdzono, że HM hamuje aktywność mitotyczną komórek nici spermatogenicznych *Chara vulgaris*. Obniżenie indeksu mitotycznego jest tym większe, im dłuższy był czas działania tego związku. W zależności od czasu działania hydroksymocznika, komórki są zablokowane w fazie G_2 bądź w fazie G_2 i w fazie S. Wykazano, że blokowanie komórek w fazie G_2 dotyczy nie tylko nici spermatogenicznych *Chara*, lecz występuje również w merystemach korzeniowych. Po 24 i 48 godz. postinkubacji, nici spermatogeniczne traktowane uprzednio HM, wykazują pewien wzrost indeksu mitotycznego.

Metodą autoradiograficzną, poprzez włączanie ^3H tymidyny wykazano że HM powoduje obniżenie syntezy DNA, oraz zwolnienie tempa tego procesu, ponieważ zwiększa się liczba komórek włączających radioaktywną tymidynę.

Opierając się na analizie wpływu HM na włączanie ^3H-fenyloalaniny stwierdzono, że po 12 godz. działania zablokowanie komórek w fazie G_2 może być spowodowane zablokowaniem białek.