Effect of kinetin on the course of cell cycle in successive developmental stages of the antheridial filaments of *Chara vulgaris* L.

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Abstract

Effects of kinetin on the course of cell cycle in successive developmental stages of the antheridial filaments of *Chara vulgaris* L. were investigated. A shortening of the duration of cell cycles has been observed, particularly in initial and final developmental stages. S phase shortened in all stages whereas G_2 phase+mitosis shortened in early but become longer in late developmental stages of filaments. Incorporation of ^{14}C-adenine into cell nuclei increased after kinetin treatment in 4- and 8-celled filaments whereas that of ^{3}H-phenylalanine increased in 8- and particularly 16-celled ones. This plant growth regulator stimulated also the ^{3}H-thymidine incorporation into cells in studied developmental stages of filaments. The stimulation of radioactive phenylalanine incorporation into nucleus and cytoplasm was stronger in late G_2 phase. A participation of cytokinins in the control of cell cycle in relation to process of differentiation of antheridial cells is discussed. A possibility of changes in the cytokinin content in antheridia and antheridial filament cells during their development has been postulated.

INTRODUCTION

The highest content of free cytokinins occurs in tissues characterised by high mitotic activity (M i l l e r 1961, W e i s s and V a a d i a 1965, L e t h a m and W i l l i a m s 1969, B l u m e n f e l d and G a z i t 1970, S h o r t and T o r r e y 1972). This fact as well as the lack of proliferation of cells cultivated *in vitro* in the absence of these substances (M i l - l e r et al. 1956, D a s et al. 1956) suggest an important role of cytokinins in the control of the course of cell cycle. When an *in vitro* culture is transferred into a cytokinin deprived medium, the cell cycle is retarded. This retardation occurs in G_2 phase or on the transition from G_2 phase into mitosis (D a s et al. 1958, P a t a u and D a s 1961, J o u a n - n e a u and T a n d e a u de M a r s a c 1973, F o s k e t and S h o r t

1973, F o s k e t 1976). It has been shown recently on synchronized, cyto-kinin independent cell cultures of tobacco that the content of free cyto-kinins increases manyfold during mitosis (N i s h i n a r i and S y o n o 1980a and b).

If one wants to draw conclusions concerning the participation of cytokinin in the development of normal undamaged plants, the observa-tions from cytokinin-dependent *in vitro* cultures need to be completed by data from *in vivo* experiments. The scantiness of information con-cerning the participation of cytokinins in the control of the processes of growth and differentiation during cell cycle in *in vivo* experiments follows from: a) a limited possibility of referring data obtained with the use of biochemical methods to processes occuring in particular cells, and the more so, to successive stages of cell cycle; b) a small number of cytophysiological methods; and c) difficulties in the choice of an ex-perimental plant model suitable for this type of investigations. The basic condition determining the usefulness of an object for investigations on the course of cell cycle is the possibility of referring the course of the studied process to stage of interphase. This condition is fulfilled by the cells of antheridial filaments of *Chara vulgaris* L. in which the stage of interphase may be determined precisely on the basis of the length of cells. The precision is enhanced by the spontaneous within a given filament synchronicity of the course of cell cycles. The investigations with the use of labelled thymidine have shown that the cell cycle of cells from antheridial filaments of this alga is of $S+G_2+M$ type (O l s z e w s k a and G o d l e w s k i 1972).

The choice of antheridial filaments as the model for investigations on the participation of cytokinins in the control af cell cycle is also justified by the fact that the relationship was studied between many processes of the cell cycle in this alga (O l s z e w s k a and G o d l e w-s k i 1972, G o d l e w s k i and O l s z e w s k a 1973, O l s z e w s k a 1974, K u r a n 1975, K w i a t k o w s k a and M a s z e w s k i 1978, 1979). More-over the modifications were found in course of the cell cycle caused by IAA, GA_3 and CCC (G o d l e w s k i 1973, 1977, 1978, 1980a and b) and by the light factor (M a s z e w s k i 1977).

The present work concerns the effect of kinetin on the duration of cell cycle and on its successive stages as well as on the intensity of incorporation of radioactive precursors of nucleic acids and proteins in the course of interphase in antheridial filament cells of *Chara vulgaris* L.

MATERIAL AND METHODS

The experiments were performed on plants collected from a pond in Pełczyska, Łódź district. The plants were cultivated under laboratory conditions (4 klux, L:D=16:8, 23-26°C, ph 7.4) in water from the same

pond. The content of mineral salts in the water was 0.3% and it was similar as in previous experiments (G o d l e w s k i 1973).

Apical parts of thalli with nodes and antheridia were placed for 24 h into the solution of kinetin (10^{-6}M) and then, for 2 h more in the same solution with addition of ^{3}H-thymidine (60 μCi/ml; 5 mCi/mM), ^{14}C-8-adenine (6.25 μCi/ml; 54 mCi/mM), or ^{3}H-phenylalanine (20 μCi/ml; 1.53 mCi/mM). Part of the material incubated in the radioactive thymidine was postincubated in the regulator for 15, 20, 25, 30, 35 and 40 h. The material was fixed in ethanol-acetic acid mixture (3:1, v/v). Squash preparations were made from isolated antheridia. They were covered with liquid Ilford emulsion. The exposition time in the case of the material incubated with ^{3}H-thymidine was 8 months, with ^{14}C--adenine — 3 days, with ^{3}H-fenylalanine — 7 days. The autoradiograms were stained with Unna mixture. Plants subjected to the same procedure, but without kinnetin, served as the control material.

Effect of kinetin on the incorporation of labelled adenine and phenylalanine was estimated in five developmental stages of antheridial filaments, namely in the 2-, 4-, 8-, 16- and 32-celled filaments. In all developmental stages studied, five successive stages of interphase were determined on the basis of cell sizes. In the material incubated with ^{14}C-adenine labelling of nuclei was estimated, while in that incubated with ^{3}H-phenylalanine the radioactivity of nuclei and cytoplasm was analysed. The number of grains or traces (depending on whether ^{3}H or ^{14}C was used) was calculated with the use of an ocular grid micrometer. The mean, illustrating the incorporation intensity during each of interphase, was calculated from individual measurements of 35-100 cells from 4-5 antheridia.

The duration of cell cycle was determined with the labelled thymidine method. The radioactive thymidine is incorporated most intensely at the begining of cell cycle i.e. during posttelophase. The postincubation period, after which strongly labelled nuclei appear in cells being in that stage, denotes the duration of the cell cycle (G o d l e w s k i and O l s z e w s k a 1973). The labelling of nuclei was estimated according to a four grade, empiric scale.

Determination of S phase duration was based on calculation of the percentage of cells incorporating labelled thymidine. They were obtained from the material fixed immediately after incubation with the precursor (M o n e s i 1969). Sine the estimated duration of S phase is prolonged by period of incubation with radioactive thymidine, it was diminished by the substraction of a value equal to the ratio of the 2-h incubation period to the duration of the whole cell cycle.

For the estimation of the effect of kinetin on the intensity of ^{3}H--thymidine incorporation the preparations from the material fixed immediately after incubation in this precursor and prepared for the

estimation of the duration of S phase were used. Unfortunately, too strong labelling made it impossible to calculate the number of grains per nuclear surface. Therefore, the labelling was determined with the use of a cytophometric method, employed also by other authors. The labelling of nuclei was determined on the basis of the percentage of the absorption of light by silver grains over the nuclear surface. The surface of the projection cleft was 7.5 μm^2. These measurements were made on unstained preparations.

RESULTS

EFFECT OF KINETIN ON THE DURATION OF CELL CYCLE

In the control material, similarly as it has been found previously (G o d l e w s k i and O l s z e w s k a 1973), the duration of cell cycle shortened gradually in successive developmental stages of filaments. Kinetin shortens the cell cycle in all studied developmental stages (Figs. 1 and 6). The maximum shortening occurs at early stages (2- and 4-celled filaments), and in the last generations of cells preceding the stage of spermatids (32-celled filaments). The mean duration of cell cycle in 2-celled filaments was shortened by about 7 h, in 4-celled by about 5 h and in 32-celled by about 3.5 h. In the case of 8- and 16-celled filaments the shortening was about 2 and 1 h, respectively. In the control material the duration of S phase was similar in all developmental stages and it amounted up to 15-17 h. G_2 phase+mitosis, calculated by the subtraction of the duration of the S phase from the total duration of the cell cycle, shortenes gradually. These observations are consonant with earlier data (G o d l e w s k i and O l s z e w s k a 1973). Kinetin shortens the duration of S phase of cells in all developmental stages of antheridial filaments, this effect being stronger, however in the case of cells from 2- and 32-celled filaments (Figs. 2 and 6). In these developmental stages S phase was shortened by about 4-5 h, whereas in the remaining ones (4-, 8- and 16-celled filaments) it was shortened only by 2.0-2.5 h. In the presence of kinetin G_2 phase+mitosis was slightly shortened in early developmental stages (2- and 4-celled filaments) but prolonged in the remaining ones (8-, 16- and 32-celled filaments).

EFFECT OF KINETIN ON THE INCORPORATION OF ³H-THYMIDINE

In the control material, in all developmental stages of filaments radioactive thymidine is incorporated into nuclei at late telophase and this process is continued at the first part of interphase (S phase) (O l s z e w s k a and G o d l e w s k i 1972). Measurements of the in-
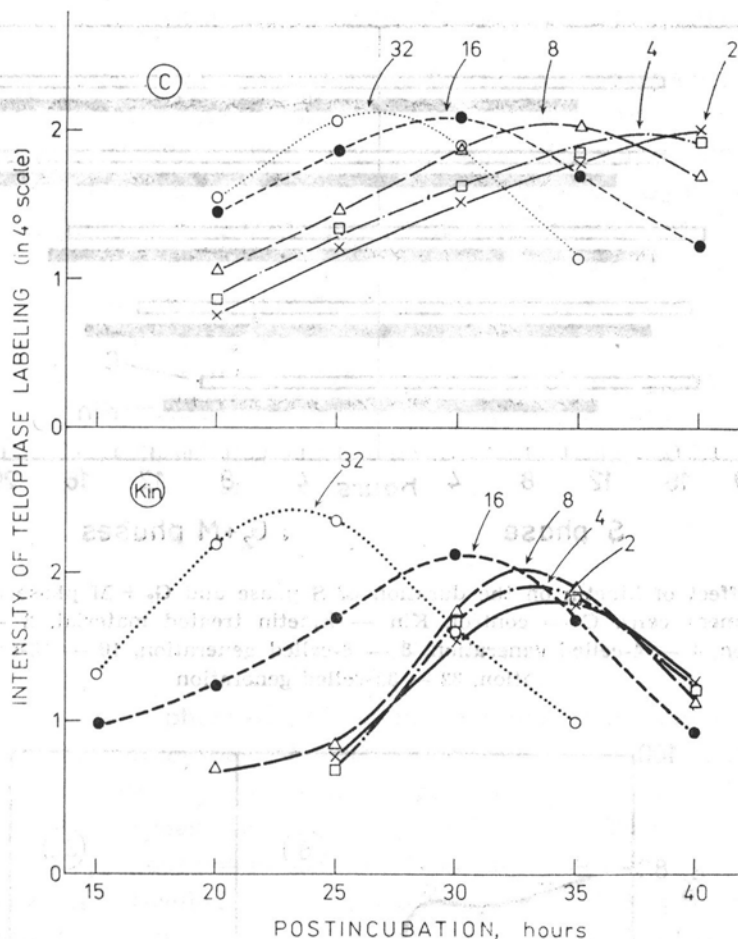
Fig. 1. Effect of kinetin on the duration of cell cycle in antheridial filaments. C — control, Kin — kinetin treated material, 2 — 2-celled generation, 4 — 4-celled generation, 8 — 8-celled generation, 16 — 16-celled generation, 32 — 32-celled generation

corporation intensity have been performed on the kinetin treated and on the control material for the 8- and 32-celled developmental stages of filaments. In these stages the radioactivity of nuclei was higher in plants treated with kinetin (Figs. 3 and 6). The stimulation of incorporation has been found during the whole S phase.

It should be pointed out that the sizes of cells ending S phase are smaller in plants treated with kinetin (Fig. 3). In the studied developmental stages the cells showing the weakest incorporation intensity into nuclei and with the spot-type incorporation into the late replicating heterochromatin are shorter by 0.9 and 0.4 μm in the 8- and 32-celled stage, respectively.
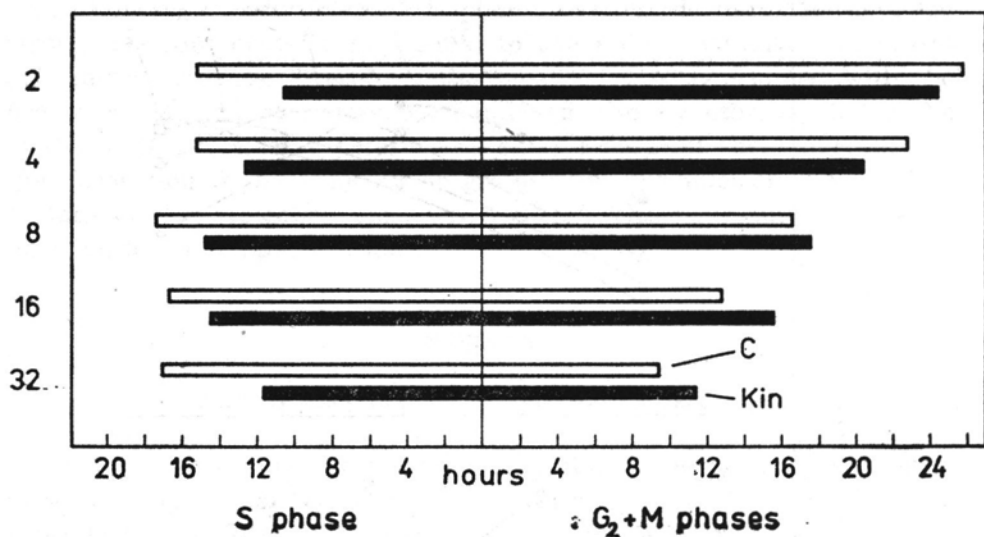
Fig. 2. Effect of kinetin on the duration of S phase and $G_2 + M$ phase in antheridial filament cells. C — control, Kin — kinetin treated material, 2 — 2-celled generation, 4 — 4-celled generation, 8 — 8-celled generation, 16 — 16-celled generation, 32 — 32-celled generation
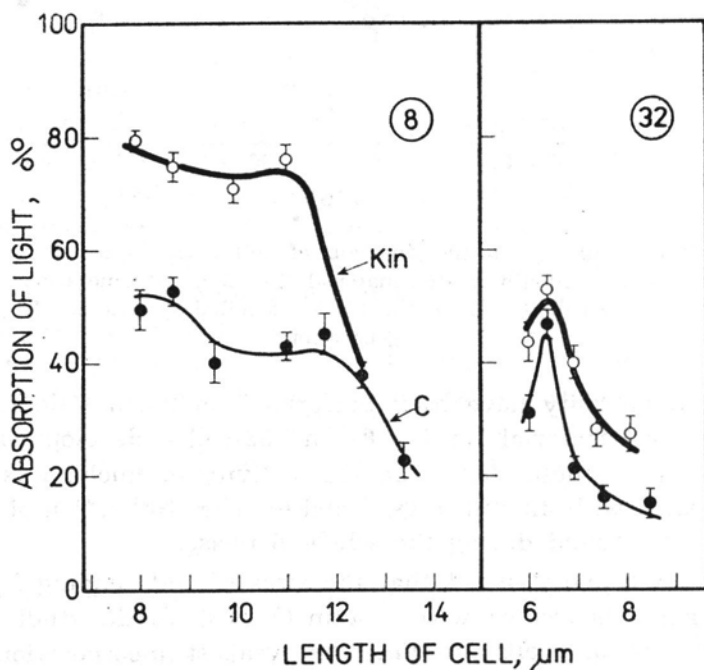

Fig. 3. Incorporation of ^{3}H-thymidine into nuclei of antheridial filaments during interphase. C — control, Kin — kinetin treated material, 8 — 8-celled generation, 32 — 32-celled generation

EFFECT OF KINETIN ON THE INCORPORATION OF ^{14}C-ADENINE

After the incubation with radioactive adenine the nuclei of cells from the control material were labelled more intensely during two periods of interphase: at the begining of interphase, when the precursor was incorporated mainly into DNA, and in the middle G_2 phase when it is incorporated into RNA. The level of labelling of nuclei, calculated per a nuclear surface unit, is similar in all developmental stages. In the presence of kinetin an increase of ^{14}C-adenine incorporation (by more than 10 per cent) into nuclei of 4- and 8-celled filaments has been observed. In the remaining developmental stages (2-, 4- and 32-celled filaments) the radioactivity of nuclei was somewhat lower in comparison with the control material. The stimulation effect as well as the inhibition of the precursor incorporation were similar during the whole interphase (Fig. 4).

EFFECT OF KINETIN ON THE INCORPORATION OF ^{3}H-PHENYLALANINE

The radioactive phenylalanine is incorporated during the whole inter-phase of the antheridial filament cells. In the control material the incorporation intensity as calculated per surface unit is higher at the begining of interphase, but it decreases later on. This relationship is much better pronounced in late developmental stages of filaments. The cells of 2- and 4-celled filaments from plants treated with kinetin in-corporate this amino acid less intensely than controls, those of 8- and 16-celled filaments react with a higher and higher stimulation of in-corporation and in cells of 32-celled filaments no effect of the cytokinin treatment on the precursor incorporation is observed (Figs. 5 and 6). The strongest effect of the treatment with kinetin was observed in cells of 16-celled filaments where the incorporation intensity during the whole interphase was about 2 times higher than in the control material. In cells of plants incubated with this cytokinin the relationship between the stage of interphase and the intensity of incorporation of this amino acid changed, a clear-cut decrease in the incorporation intensity during G_2 phase was not found. In the premitotic period, in cells of 4- and 8-celled filaments, the precursor incorporation was similar or even higher as at the begining of interphase. In cells of 2-, 4-, 8- and 16-celled filaments from plants treated with kinetin the radioactivity of cytoplasm was higher than that of the nucleus. This difference was not so clear in the control material.
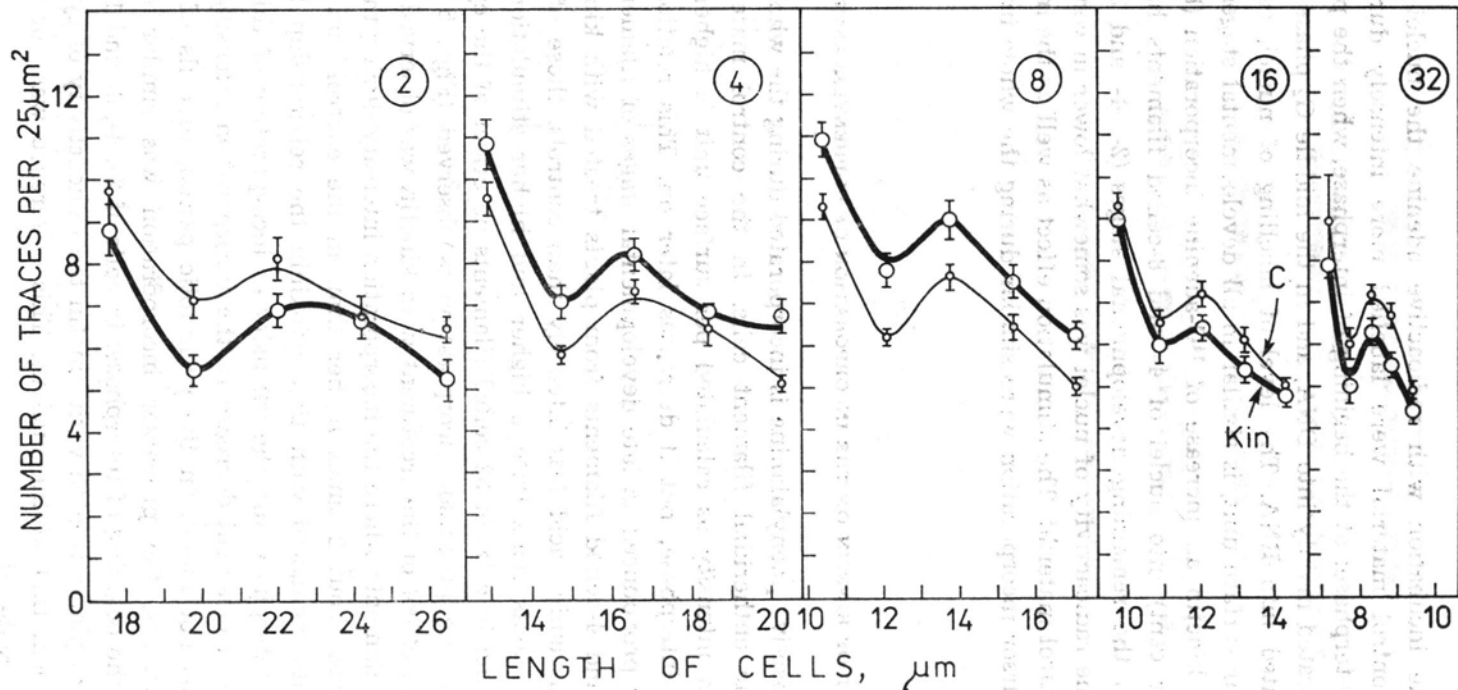
Fig. 4. Incorporation of ^{14}C-adenine into nuclei of antheridial filaments. 2 — 2-celled generation, 4 — 4-celled generation, 8 — 8-celled generation, 16 — 16-celled generation, 32 — 32-celled generation
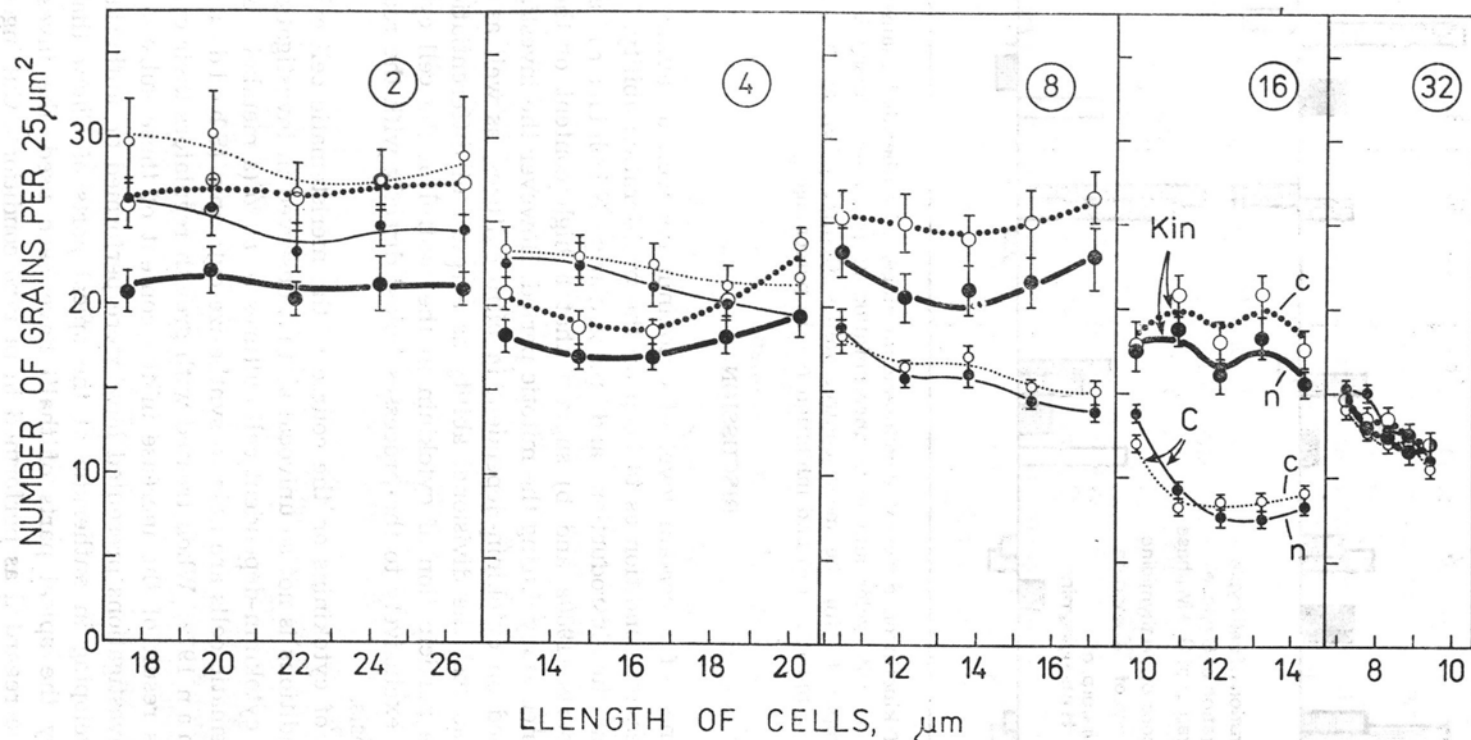
Fig. 5. Incorporation of ^{3}H-phenylalanine into nuclei (n) and cytoplasm (c) of antheridial filaments during interphase. C — control, Kin — kinetin treated material, 2 — 2-celled generation, 4 — 4-celled generation, 8 — 8-celled generation, 16 — 16-celled generation, 32 — 32-celled generation
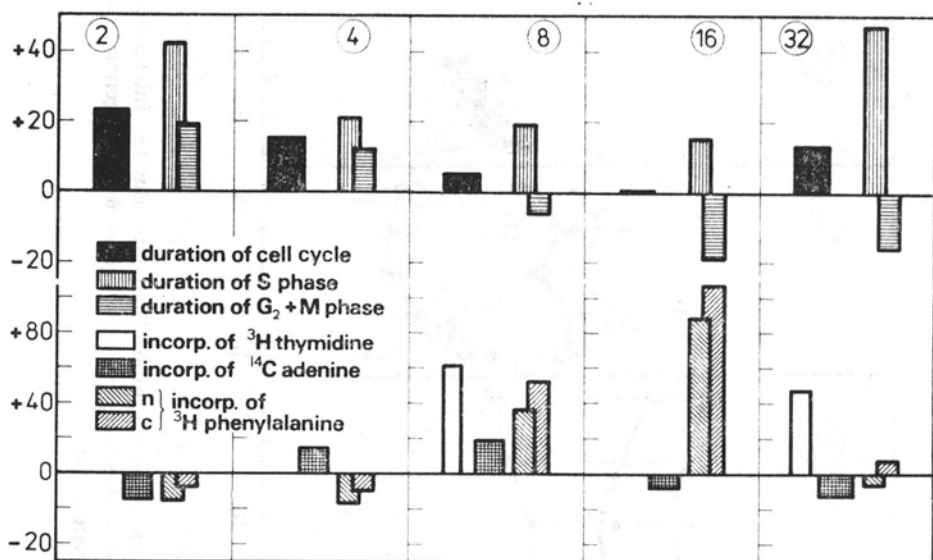
Fig. 6. Effect of kinetin on different developmental stages of antheridial filaments. Mean duration of cell cycles and mean incorporation of precursors during inter-phase were compared with the mean control values. Control taken as "O", sti-mulation (+) and inhibition (−), in percentage

DISCUSSION

The presence of a certain level of cytokinin (as well as auxin) in cells is a necessary condition as to keep up the proliferation ability. The data cited in the introduction and particularly N i s h i n a r i and S y o n o's reports (1980a and b) suggest that a high content of these substances is necessary during the mitotic period. However the investigations performed on cytokinin-dependent in vitro cultures, as well as on cells which had lost the divisional ability as a result of differentiation, show that the participation of cytokinin in the control of the cell cycle is not limited exclusively to the processes linked directly with the realization of mitosis.

The effect of cytokinins on the course of the meristematic cell cycle in in vivo conditions is not so univocal as that observed in investigations performed on cytokinin-dependent cell cultures in vitro (a metabolic blokade). Meristematic cells are able to synthesize cytokinin (S h e l d r a k e 1973, F e l d m a n 1979). When treated with growth regulators their cycle is modified as result of the increase in the content of these substances inside. The investigations presented have were performed on antheridial filaments developing in antheridia of the apical parts of Chara thalli. Although only the apical parts of thalli have been used, the investigations may be regarded as performed in in vivo conditions. Cutting off

the lower part of the thallus have no effect on the development of antheridial filaments (Kwiatkowska and Godlewski, unpublished data). Investigations performed on isolated antheridia have shown an at least partial autonomy of these organs in relation to growth regulators. In an environment deprived of these substances the antheridial filament cells in isolated antheridia pass through several cell cycles (K u r a n and M a r c i n i a k 1981).

The development of antheridial filaments is connected with differentiation towards smaller and smaller sizes of cells until a critical size is attained at which they loose the divisional ability and become spermatids. The diminution in size of cells runs in parallel with shortening of the cell cycle, limitation of the dry mass increment, and a reduction in the binding ability of actinomycin D by nuclei as well as with a reduced incorporation of adenine and phenylalanine (O l s z e w s k a and G o d l e w s k i 1972, G o d l e w s k i and O l s z e w s k a 1973, O l s z e w s k a 1974, K u r a n 1975). A similar course of the process of decrease of cell sizes was observed in *in vitro* cultures as the auxin and cytokinin contents in the environment increased (F o s k e t 1976). Meristematic cells are able to synthetize fitohormones. During the development of filaments the cells with divisional ability increase in number from 150 to several thousands. It allows one to expect an increased content of these substances in antheridia. Determination of these changes might verify this assumption. This however necessitates the use of microquantitative methods owing to the small sizes of antheridia.

The performed investigations in which the effects of kinetin were analysed after 24 h incubation have shown that this regulator modifies the processes connected with the cell cycle course, and that these effects are dependent, in majority of instances, on the stage of development of antheridial filaments (Fig. 6). Kinetin, exerting no perceptible effects on the size of mitotic cells, shortens clearly the duration of the cell cycle particularly in early and late developmental stages of filaments. S phase shortened in all studied developmental stages of filaments, and G_2 phase+mitosis shortened in early but lengthened in late developmental stages. It appears then that cells from successive developmental stages reacted differentially in response to the increased cytokinin content. If one assumes the possibility of changes in the cytokinin content during the development of antheridia, the observed effect of kinetin on the duration of S phase in different developmental stages may suggest either a small specificity or a high tolerance of processes connected with DNA replication with respect to the cytokinin content in cells. On the other hand the dependence of the effect of kinetin on the duration of G_2 phase+mitosis on the developmental stages of fila-

ments suggest a specific action of this cytokinin in this part of the cell cycle. The lengthening of the duration of G_2 phase+mitosis in the late developmental stages of filaments might result from too high concentration of this regulator in kinetin treated cells.

In different experimental sets it has been shown that cytokinins are not necessary for the initiation of S phase. No effect of these substances on the intensity of DNA replication has been found (S z w e y-k o w s k a 1974, F o s k e t 1976). In some instances however, it has been observed that apart from auxin, the presence of cytokinin was necessary for initiation of DNA replication. The data obtained by T o r r e y on pea root sections might be pointed out as an example (L i b b e n g a and T o r r e y 1973, P h i l l i p s and T o r r e y 1973). Stimulation of radioactive thimidine incorporation into DNA was obtained in experiments performed on 2iP (6-(3-methyl-2-butenyl-amine)purine) treated cells of *Funaria hygrometrica* protonema (Spychała et al., unpublished data). A similar effect has been obtained in the present work. The shortening of S phase of antheridial filament cells is connected with stimulation of ^{14}C-adenine incorporation into nuclei (in 4- and 8-celled filaments) as well as of ^3H-thymidine. This effect does not seem to be caused by an increased activity of DNA polymerase. It comes from autoradiographic *in situ* investigations on the activity of this enzyme, performed on antheridial filament cells of *Chara* (Godlewski, in preparation). The effect observed in this work was obtained after 24 h treatment with kinetin. The stimulation of ^3H-thymidine incorporation in protonema cells was also seen not before 24 h of cytokinin treatment. It may by suspected then that it was not a result of a specific action of cytokinin on the process of replication itself. It seems, that the stimulation of DNA synthesis may result from a direct or indirect influence of this regulator on chromatin proteins. Such a possibility, at least with respect to the process of transcription, was shown in investigations on the labelled cytokinin binding (M a t-t h y s e and A b r a m s 1970). A loosening of chromatin has also been found in the presence of this substance (F e l l e n b e r g 1969). In investigations performed on antheridial filament cells it was shown that ^3H-actinomycin D labelling of nuclei was doubled during the whole interphase (Olszewska and Godlewski, unpublished data). The increased template accessibility during S phase and particularly during its early part, when the intensity of replication is the highest, is connected primarily with DNA synthesis.

In plants with cell cycle of G_1+S+G_2+M type the processes occuring till S phase inclusive, occur in the absence of cytokinin. In cytokinin--dependent *in vitro* cultures of tobacco (J o u a n n e a u and T a n d e a u de M a r s a c 1973) and soybean (F o s k e t and S h o r t 1973) cells

transferred into cytokinin deprived medium still incorporated thymidine, giving rise to poliploidal cells. Thus, replication cycles of DNA proceeded without cell divisions for which cytokinins are necessary. The control of the processes occuring during G_2 phase and during the initiation of mitosis is linked primarily with an effect of cytokinin on the synthesis of proteins. However, the increase in the rate of protein accumulation and initiation of mitosis resulting from the attainment of certain cytoplasm/nucleus mass ratio does not seem to be so important as Trombett's hypothesized (1942, cit. in F o s k e t and S h o r t 1973). Cells in which cell cycle has been inhibited by the lack of cytokinin, continue the synthesis of proteins and the protein content may exceed significantly the amount of proteins in dividing cells (M i t h e l 1968, F o s k e t and S h o r t 1973). Thus, a sequential synthesis of specific proteins is necessary for the continuation of cell cycle. It seems to be particularly important during the premitotic period. This is suggested by investigations on cytokinin treated cells, performed with the use of protein synthesis inhibitors (J o u a n n e a u 1975). An increase in number of polyribosomes has also been found during this period (S h o r t et al. 1974, M u r e n and F o s k e t 1977, F o s k e t and T e p f e r 1978). Investigations on the composition of proteins synthesized in the presence as well as in the absence of cytokinin have shown that at least one specific protein appears before the cell division (J o u a n n e a u 1970).

Increased ^{3}H-phenylalanine incorporation in the presence of kinetin has also been observed in antheridial filament cells. The stimulatory effect was stronger in the premitotic period, in G_2 phase. However, an interpretation of the phenomena occuring during G_2 phase of the cell cycle is difficult if some additional investigations are not performed. Kinetin exerts a clear stimulatory effect on the incorporation of this amino acid into cells of 8- and 16-celled filaments in which the duration of cell cycle has been shortened to a lesser degree than in other developmental stages (Fig. 6). Possibly, the increased synthesis of proteins does not concern the proteins linked specifically with the initiation of mitosis. Also, a possible effect of cytokinin on the isotope uptake from the incubation medium cannot be exclused. It might be due to a certain cytokinin content in cells from these developmental stages of filaments after the kinetin treatment.

The stimulation of synthesis of the proteins which makes possible a reactivation of the proliferation ability after the cytokinin treatment, may be caused by the formation of polyribosomes owing to mmRNA activation (M u r e n and F o s k e t 1977, F o s k e t and T e p f e r 1978) or by activation of polyribosomes (G w ó ź d ź 1979). The stimulation of the formation of polyribosomes and protein synthesis precedes the stimulation of RNA synthesis. It speaks in favour of a direct control

of translation by cytokinins with omission of transcription. On the other hand, however, an interaction of cytokinin with chromatin seems to be out of question (J a c o b s e n 1977). Although numerous attempts have not revealed an univocally specific, with respect to the action of cyto-kinin, fraction of RNA and particularly mRNA responsible for the synthesis of proteins necessary for the realisation of mitosis, a certain effect of cytokinins may be expected also on the level of transcription. Kinetin stimulates the incorporation of radioactive adenine into RNA in G_2 phase of 4- and 8-celled antheridial filaments of *Chara*, whereas the maximum incorporation of ^{3}H-phenylalanine occurs in 16-celled* filaments. This shift in effect suggests an independent action of kinetin on transcription and translation and is connected probably with a change in the concentration of cytokinins in antheridia during the development of antheridial filaments.

The relatively small effect of kinetin on the incorporation of radio-active adenine is rather unexpected, if one takes into account the stimulatory effect of this regulator on the course of cell cycle. A de-creased radioactivity of nuclei has been found also in S phase of cells from 2-, 16- and 32-celled filaments where a stimulation of ^{3}H-thymidine incorporation and a shortening of S phase were observed. An interpreta-tion of this result should take into account the possibility of a secondary isotope thinning resulting from the competition between both com-pounds — adenine and kinetin (O l s z e w s k a 1959).

The investigations on the course of cell cycle in successive develop-mental stages of *Chara* as well as the cited reports suggest, that the participation of cytokinins in the control of the differentiation processes in plants may be realized, among others, through an effect on the divisional ability of cells as well as through modifications of the course of cell cycle.

Acknowledgment

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Wpływ kinetyny na przebieg cyklu komórkowego w kolejnych stadiach rozwoju nici spermatogenicznych Chara vulgaris L.

Streszczenie

Badano *in vivo* wpływ kinetyny na przebieg cyklu komórkowego w kolejnych stadiach rozwoju nici spermatogenicznych *Chara vulgaris* L. Stwierdzono skrócenie czasu trwania cykli komórkowych, szczególnie w pierwszych i końcowych stadiach rozwoju nici. Faza S uległa skróceniu we wszystkich stadiach rozwojowych, natomiast faza G_2+mitoza — skróceniu we wcześniejszych i wydłużenie w późniejszych stadiach rozwoju nici. Traktowanie kinetyną zwiększa, w badanych stadiach rozwojowych, włączanie ^{3}H-tymidyny, ^{14}C-adeniny do jąder komórek nici 4- i 8-komórkowych, zaś fenyloalaniny do 8- a szczególnie 16-komórkowych. Stymulacja włączania radioaktywnej fenyloalaniny do jądra i cytoplazmy była silniejsza w późnej fazie G_2. Dyskutowana jest regulacja przebiegu cyklu komórkowego przez cytokininy w aspekcie różnicowania komórek nici spermatogenicznych oraz możliwość zmiany zawartości cytokinin w plemniach i komórkach nici spermatogenicznych w czasie ich rozwoju.