

Autoradiographic analysis of the effect of cytokinin on protein and RNA syntheses in the *Ceratodon purpureus* protonema

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Abstract

In the protonema of *Ceratodon purpureus* (Hedw.) Brid., apical parts of the protonemal filaments (apical cells, initials of protonemal side branches and of gametophore buds) proved to be preferential sites of [¹⁴C]-leucine incorporation into proteins. In some filaments, a similar preference for [³H]-uridine incorporation into RNA was observed, whereas in others there was a rather uniform distribution of label over all cells. A short (0.5-2 h) treatment with cytokinin (N⁶-2-isopentenyladenine) enhanced [¹⁴C]-leucine incorporation, without changing the relative distribution of label. No such enhancement, as well as no change in label distribution could be observed in [³H]-uridine incorporation. No direct relationship seems to exist between the early promotion of protein synthesis by cytokinin in the protonema and cytokinin induction of gametophore buds.

INTRODUCTION

In the protonema of mosses, cytokinins enhance cell division (Szweykowska et al. 1971, 1972) and chloroplast development (Woźny 1978) and they specifically induce mass formation of gametophore buds (Gorton et al. 1957, Hahn and Bopp 1968). Experiments with specific inhibitors showed a dependence of the bud induction response on RNA and protein syntheses (Brandes and Bopp 1965, Szweykowska and Schneider 1967), and investigations with labelled precursors demonstrated stimulation of protein synthesis by cytokinin (Spychała and Szweykowska 1981, Gwóźdz and Szweykowska 1982) and a promotion of synthesis of a fraction of polydisperse RNA (Spychała et al. 1982). The protonema,

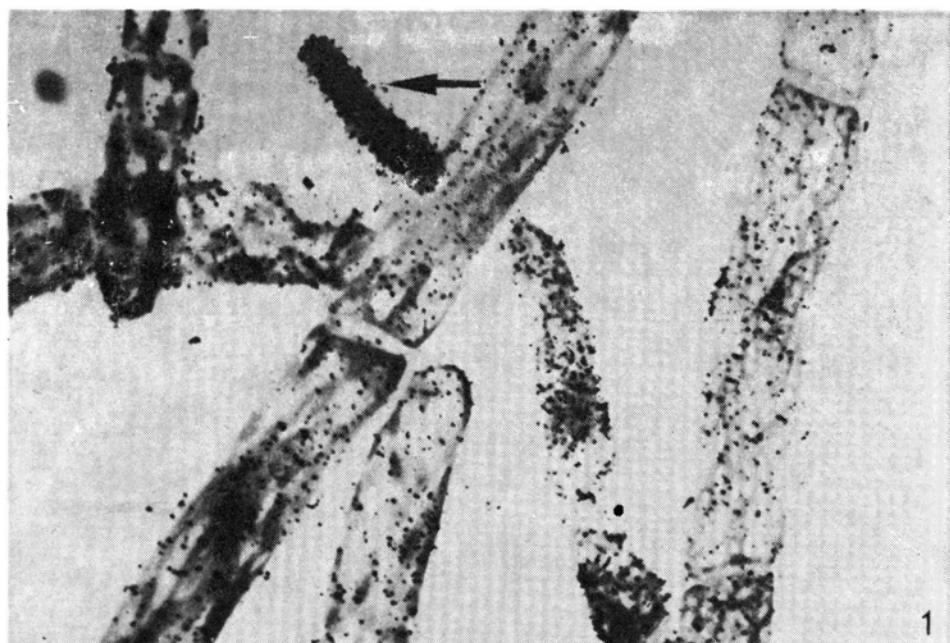
though apparently simple in its morphological structure, is in fact composed of several cell types and undergoes differentiation during its development. The cytokinin-induced gametophore buds appear at a definite stage of the protonema development (Bopp 1957) and at a definite distance (30-40 cells) from the filament tip (Idzikowska and Szweykowska 1978a). It was, therefore, interesting to establish the distribution of the protein and RNA syntheses in the protonema filaments and the response of these syntheses in various cells to cytokinin treatment.

MATERIAL AND METHODS

A sterile, vegetatively propagated clone culture of *Ceratodon purpureus* (Hedw.) Brid. protonema was grown on cellophane discs placed on the surface of a mineral medium (Kofler 1959, Szweykowska et al. 1971) solidified with 0.8% agar, under continuous white fluorescent light of ca. 1.4 Wm^{-2} and at a temperature of ca. 25°C . The protonemata were divided in as many equal parts as there were experimental variants and were pre-incubated for 12 h in the basal incubation medium (mineral solution of the same composition as that used for growing the protonemata, without agar) in order to habituate them to the liquid medium. Experimental incubations were carried out in the basal incubation medium (control) or with the addition of $0.5 \mu\text{M i}^6\text{ade}$.

AUTORADIOGRAPHIC DETERMINATION OF PRECURSOR INCORPORATION INTO PROTEIN AND RNA

$[^{14}\text{C}]$ -Leucine (7.8 GBq/mmol) at 185 kBq/ml or $[^3\text{H}]$ -leucine (1.96 TBq/mmol) at 74 kBq/ml were used as precursors of protein synthesis, and $[^3\text{H}]$ -uridine (37 GBq/mmol) at 2.96 MBq/ml as a precursor of RNA synthesis. Precursors were added either some time before the end of incubation proper, or, to eliminate the effect of the cytokinin on precursor uptake, the protonemata were first incubated with the labelled precursor, and after washing it out with the basal incubation medium containing a cold, non-radioactive precursor (0.01 M ^{12}C , ^1H -leucine or ^1H -uridine), they were incubated with $0.5 \mu\text{M i}^6\text{ade}$ or without it in the control. The protonemata were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8, for 24 h, washed several times with the same buffer in order to eliminate the non-incorporated precursor, placed on glass slides and coated with Ilford K₅ or L₄ liquid emulsion. They were exposed in darkness at 4°C , for 3 days in the case of leucine, and 21 days in the case of uridine. The radioautographs were developed, fixed, and washed by a standard method (Herrmann and Abel

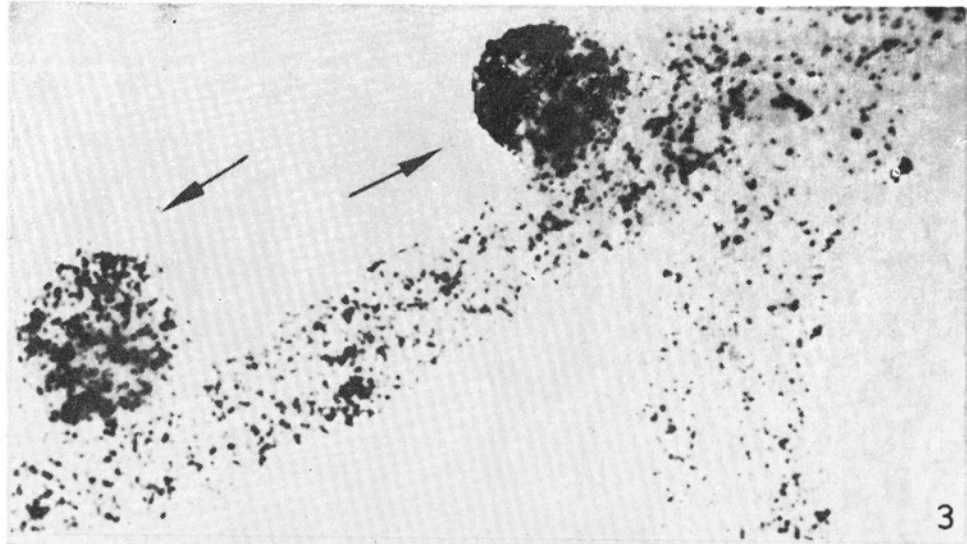


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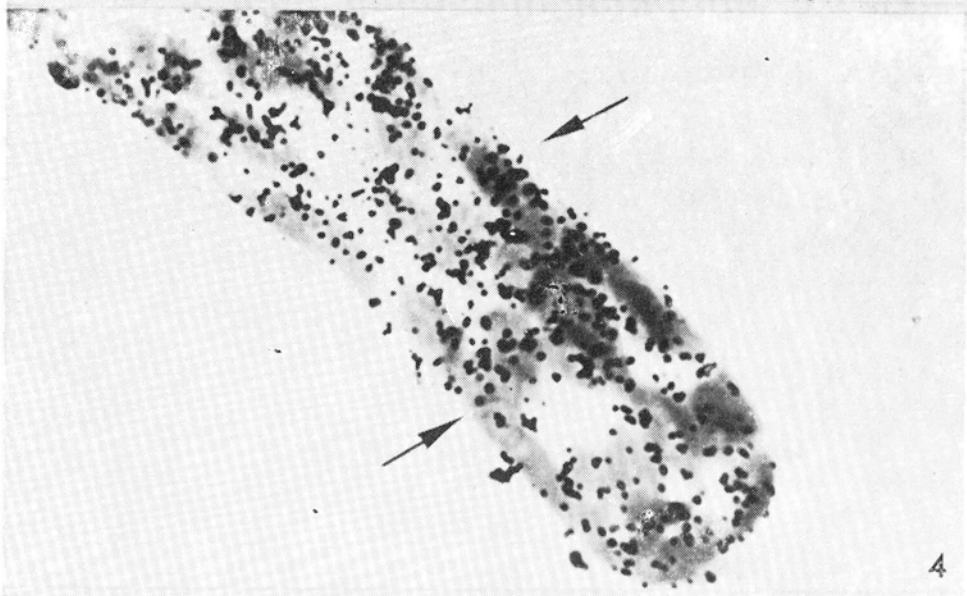


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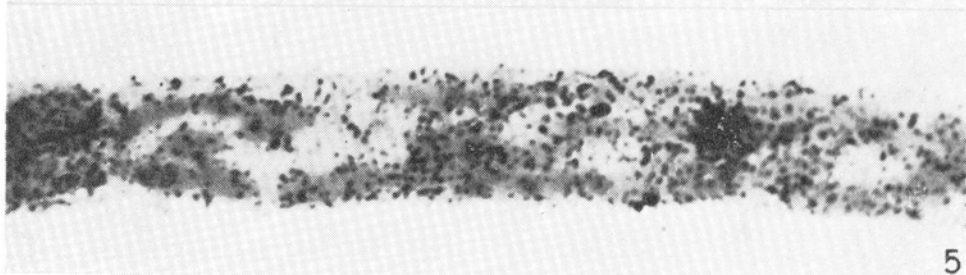
Figs. 1-2. Effect of *iade* on the distribution of $[^3\text{H}]$ -leucine incorporation in protonema cells. Protonemata were incubated for 1 h in the basal incubation medium (control — Fig. 1) or in the same medium with the addition of $0.5\ \mu\text{M}$ *iade* (Fig. 2). The precursor at 74 kBq/ml was added 0.5 h before the end of incubation. Arrows indicate apical cells with a high concentration of silver grains. $\times 1440$



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4



5

Figs. 3-5. Effect of i^6ade on the distribution of $[^3H]$ -leucine incorporation in protonema cells. Protonemata were incubated with $0.5 \mu M$ i^6ade . The precursor at 74 kBq/ml was added 0.5 h before the end of incubation

Fig. 3. Incubation with i^6ade for 1 h . A high concentration of silver grains above bud initials (arrows). $\times 1440$. Fig. 4. Incubation with i^6ade for 1 h . Silver grains localized mainly above chloroplasts (arrows). $\times 3500$. Fig. 5. Incubation with i^6ade for 12 h . A high concentration of silver grains above nucleus. $\times 1440$

1972) and stained in Unna mixture. Label incorporation was determined by counting silver grains above specified cells of the protonemata. Photographs were made in the Reichert light microscope with a Reichert camera and various filters.

The distribution of the protein content in the protonemata was estimated cytochemically in material fixed with a mixture of equal parts of 1.5% formaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, and stained in mercuric bromophenol blue according to Mazia et al. (1953).

RESULTS AND DISCUSSION

Demkiv and Lesnyak (1970) showed that a distinct apical dominance, controlled by the apical cell, exists in moss protonemata. Studies of the fine structure (Idzikowska and Szweykowska 1978a and b) revealed in *Ceratodon purpureus* a dense cytoplasm and high concentration of organelles in the apical cells of the protonema and in initials of gametophore buds, whereas intercalary cells were strongly vacuolized and poor in cytoplasmic structures. Demkiv and Fedych (1972) found that concentrations of protein and RNA in *Funaria hygrometrica* were highest in the apical cells of the protonema filaments and decreased abruptly beginning with the first, subapical intercalary cells. A local increase appeared only in those regions of intercalary cells which produced primordia of lateral branches. Autoradiography of [^{14}C]- or [^3H]-leucine incorporation showed a similar distribution of protein synthesis in the protonema. A particular accumulation of silver grains was observed over the apical cells, newly forming branches of the protonema and initials of gametophore buds (Figs. 1-3). In many of the investigated cells a concentration of silver grains was observed over plastids (Fig. 4) and nuclei (Fig. 5). Cytokinin treatment increased the label incorporation in all cell types, with strongest effect after 1 h of incubation with the hormone (Figs. 1, 2 and 6). In the first experiments, [^{14}C]-leucine was added 30 min before the end of protonema incubation with $i^6\text{ade}$. However, as shown in our previous study (Spychała and Szweykowska 1981), cytokinin stimulates [^{14}C]-leucine uptake by the protonema, so in order to eliminate differences in the precursor pool between the control and cytokinin incubations, in further experiments the protonema was pre-incubated with the precursor and then transferred to medium with $i^6\text{ade}$. In these conditions, the pool of [^{14}C]-leucine is the same in both control and cytokinin variants of the experiment. In these conditions, a somewhat smaller, but still distinct stimulation by $i^6\text{ade}$ of label incorporation was also noted, indicating a promotion of protein synthesis in all investigated

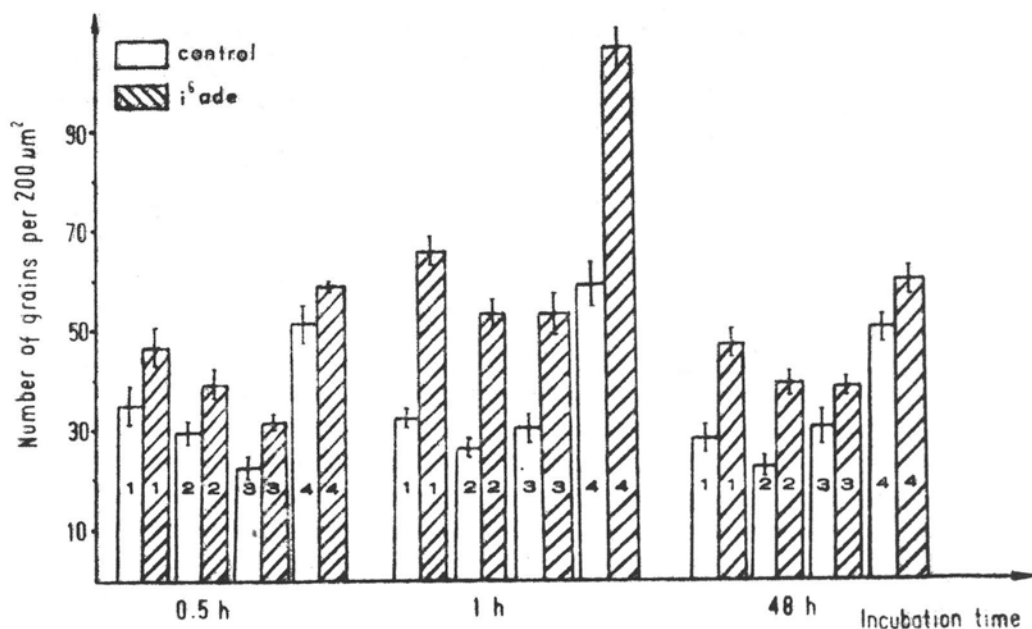


Fig. 6. Effect of i^{ade} on $[^{14}C]$ -leucine incorporation in various cells of the protonema. Protonemata were incubated with i^{ade} (0.5 μM) for 0.5, 1 or 48 h (no cytokinin in controls). The precursor at 185 kBq/ml was added 0.5 h before the end of incubation. 1 — apical cells; 2 — subapical cells; 3 — intercalary cells (the 5th counted from the apex); 4 — one-celled lateral initials

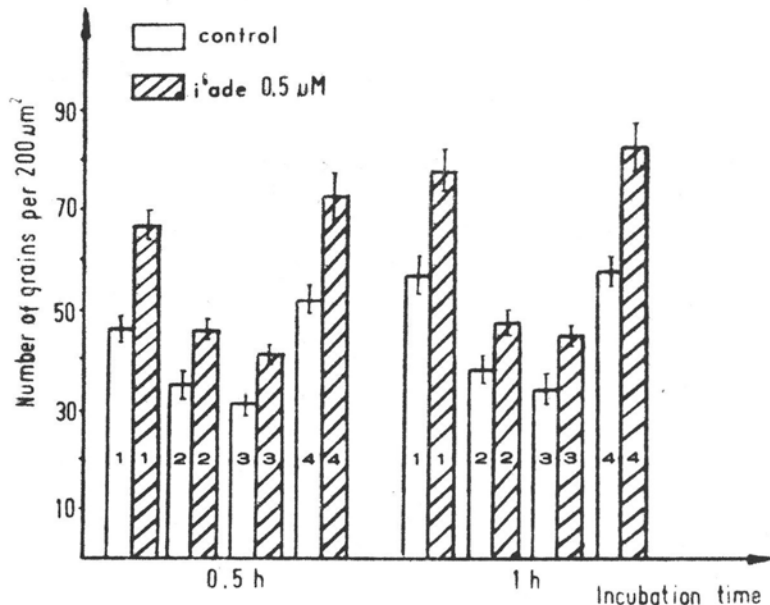
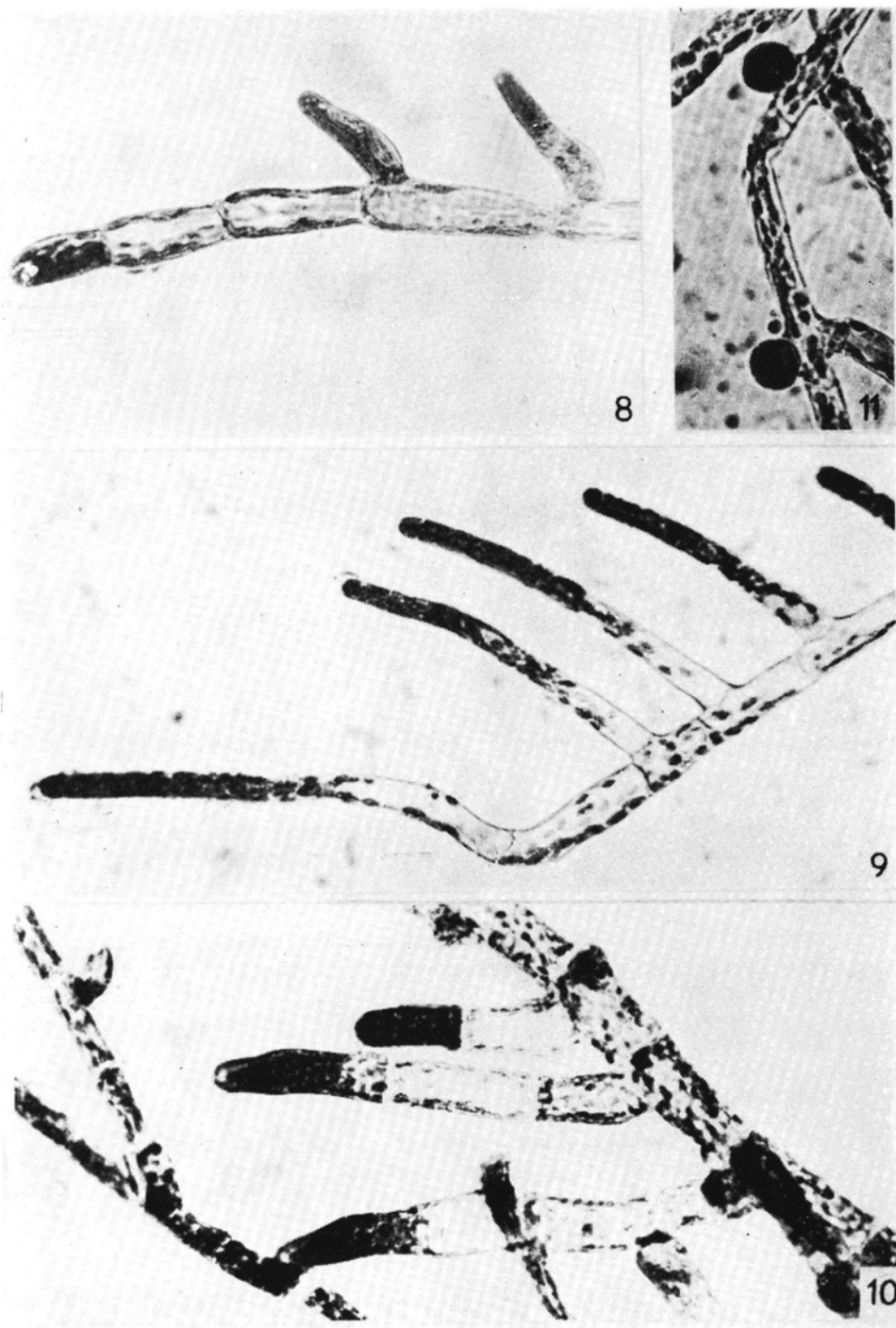
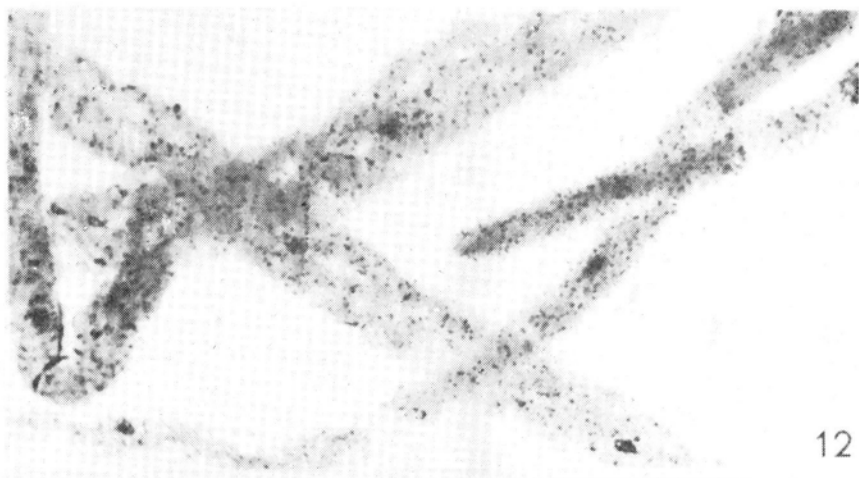


Fig. 7. Effect of i^{ade} on $[^{14}C]$ -leucine incorporation in various cells of the protonema. Protonemata were pre-incubated for 0.5 h with the precursor at 185 kBq/ml, washed and incubated for the next 0.5 or 1 h in medium containing 0.5 μM i^{ade} (no cytokinin in controls). Other details as in Fig. 6

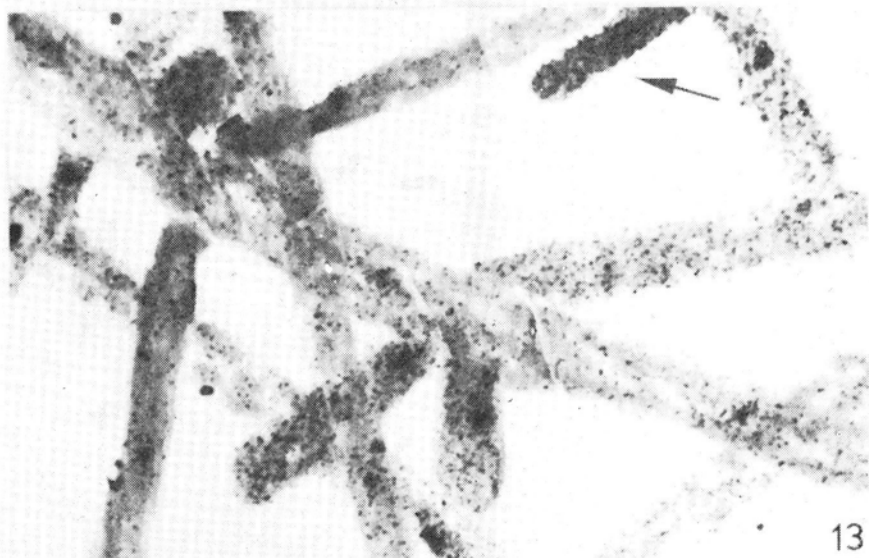


Figs. 8-11. Effect of i^6ade on distribution of protein content in protonema cells. After incubating the protonema with i^6ade (no cytokinin in control incubations), proteins were stained by the mercuric bromophenol blue reaction according to Mazia et al. (1953)

Fig. 8. Control protonema, accumulation of protein in apical cells. $\times 600$. Fig. 9. Control protonema. An accumulation in the tip regions of longer apical cells. $\times 500$. Fig. 10. Treatment with i^6ade ($1 \mu M$) for 12 h. High content of protein in short apical cells, soon after division. $\times 900$. Fig. 11. Treatment with i^6ade ($0.5 \mu M$) for 6 h. High accumulation of protein in initials of gametophore buds. $\times 900$



12



13

Figs. 12-13. Distribution of [^3H]-uridine incorporation in protonema cells. The protonema was pre-incubated with the precursor at 2.96 MBq/ml for 2 h, washed and incubated in the basal medium for the next 2h

Fig. 12. Similar concentration of silver grains above the apical and intercalary cells. Accumulation of label in nuclei. $\times 800$. Fig. 13. Concentration of silver grains above some apical cells (arrow). $\times 800$

cells independently of precursor uptake (Fig. 7). The stimulation was higher in the growing parts of the protonema (apical cells and 1-celled initials of lateral branches or gametophore buds) than in intercalary cells. No difference, however, appeared between the apical cells and 1-celled lateral initials which included initials of gametophore buds.

As a result of promotion of protein synthesis, an increase in protein content, as estimated by the reaction with bromophenol blue, was observed in cells of the $i^{6}ade$ -treated protonema as compared with the untreated control (Figs. 8-10). The increase was a function of cytokinin concentration and the time of treatment. With 0.5-1 μM $i^{6}ade$ concentrations, it was noticeable only after 12 h of treatment (Fig. 10), although cytokinin-induced bud initials were already present after 6 h (Fig. 11). At 2 μM , it could be observed as early as after 6 h, and at 10 μM after 4 h. However, at higher $i^{6}ade$ levels, degeneration symptoms appeared in the cells, the sooner the higher was the concentration; with 10 μM , they were observable in some cells already after 4 h, and after 12 h all cells degenerated. Cytokinin did not affect the distribution of the protein content in the protonema. In the control, as well as in $i^{6}ade$ -treated protonemata, protein accumulated in the growing parts of filaments: in short apical cells, shortly after division (up to 50 μm long), in terminal parts of long apical cells (80-250 μm long), in short subapical cells (up to 60 μm) adjacent to short apical cells, soon after division, in initials of lateral branches and in bud initials (Figs. 8-11).

Using staining with acridine orange and a fluorescence-microscopic analysis of the protonema in *Funaria hygrometrica*, Brandes (1967) found an increase in the cytoplasmic RNA content in bud initials as compared with that in the initials of ordinary protonemal branches. There was no difference in this respect between spontaneous and kinetin-induced buds, though the number of buds was much higher in the kinetin-treated protonema. In Brandes' experiments, kinetin-induced bud initials, with their high RNA content, were found as early as after 10 h of kinetin treatment. In our study, autoradiographic estimations of [3H]-uridine incorporation into the protonema cells were carried out after 1 and 2 h of experimental incubation, when a considerable stimulation of protein synthesis by cytokinin was observed. On the average, the number of silver grains was higher in apical parts of the protonema and decreased in the successive subapical and intercalary cells (Fig. 14). Analysis of autoradiograms, however, showed differences in the distribution of the label incorporation between individual filaments. In many filaments, cells were uniformly labelled, with no distinction between the apical and intercalary cells, whereas in others uridine incorporation was similar as in the case of leucine incorporation, with apical cells, and initials of lateral branches and of gametophore buds appearing as

preferential sites of RNA synthesis (Figs. 12 and 13). It seems that the enhancement of RNA synthesis in apical cells is limited either to particular phases of the cell cycle or to particular filaments of the protonema.

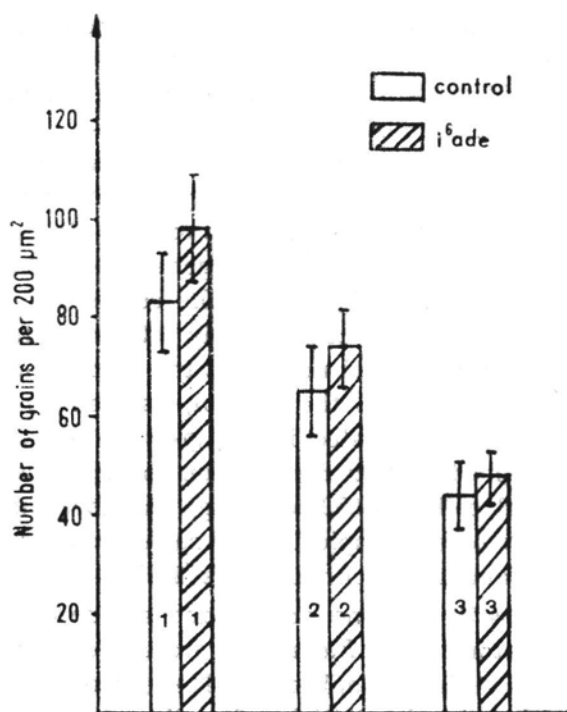


Fig. 14. Effect of i^6ade on $[^3H]$ -uridine incorporation in various cells of the protonema. Protonemata were pre-incubated for 2 h with the precursor at 2.96 MBq/ml, washed and incubated for the next 2 h in medium with 0.5 μM i^6ade (no cytokinin in control incubation). 1 — apical cells; 2 — subapical cells; 3 — intercalary cells (the 5th counted from the apex)

In contrast to leucine incorporation, differences in the quantity of incorporated $[^3H]$ -uridine between the control and i^6ade -treated protonema were small and insignificant (Fig. 14). Neither could differences be observed in the distribution of label in the protonema between the control and the cytokinin variant.

Autoradiographic estimations of the cytokinin effect on label incorporation into proteins and RNA in the protonema of *Ceratodon purpureus* confirmed our previous results of biochemical estimations: a promotion of protein synthesis and no distinct promotion of total RNA synthesis after 1-2 h of cytokinin treatment (Spychała and Szwejkowska 1981, Spychała et al. 1982). However, early stages of

gametophore buds did not appear as specific centres of protein or RNA synthesis. Early promotion of protein synthesis occurred to a similar extent in apical cells and in lateral initials. Thus, quantitative analysis of the distribution of this promotion gave no evidence that it is directly connected with cytokinin induction of gametophore buds. The problem of induction may lie in qualitative changes of RNA and protein syntheses. In this respect, a finding of S p y c h a ł a et al. (1982) of a transient enhancement of the synthesis of a fraction of polydisperse RNA after 1-2 h of cytokinin treatment may be of significance and will be the subject of further investigations.

Acknowledgments

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Autoradiograficzna analiza wpływu cytokininy na syntezę białka i RNA w spletku *Ceratodon purpureus*

Streszczenie

Włączanie [^{14}C]-leucyny do białka było najsilniejsze w szczytowych partiach nici spletku (komórki szczytowe, zawiązki odgałęzień bocznych spletku i pąków gametoforowych). W niektórych niciach podobną preferencję partii szczytowych obserwowano we włączaniu [^3H]-urydyny do RNA, w wielu innych jednakże było ono mniej więcej równomierne we wszystkich komórkach. Krótkie (0,5-2 godz.) działanie cytokininy (N^6 -izopentenyladeniny) zwiększało włączanie [^{14}C]-leucyny, nie zmieniając przy tym względnego rozmieszczenia piętna radioaktywności w niciach. Nie stwierdzono natomiast takiego zwiększenia, jak również zmiany rozmieszczenia piętna w przypadku włączania [^3H]-urydyny. Wydaje się, że nie ma bezpośredniego związku między wczesną cytokininową stymulacją syntezy białka w spletku a cytokininową indukcją pąków gametoforowych.