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Studies on Cytokinin-Controlled Bud Formation in Moss Protonemata

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Abstract. **Application of cytokinins to moss protonemata of the proper physiological age causes bud formation on specific cells (caulonema). During the early stages of their development, buds revert to protonemal filaments if the cytokinin has been removed by washing the protonemata. This indicates that the hormone is not acting as a "trigger" but has to be present during a critical period of time until differentiation is stabilized. Autoradiographs of protonemata treated with a labeled cytokinin, benzyladenine-benzyl-7-^{14}C, show a striking accumulation of the radioactivity in caulonema cells which are in the stage of bud formation, and in the buds themselves. Cells which did not react to the hormone contained very little radioactivity. The accumulation of benzyladenine in the "target cells" may be due to the presence of binding sites which, in turn, may distinguish responding cells from non-responding ones.**

Cytokinins applied at a certain stage in the development of moss protonemata cause profuse formation of so-called buds, which are the initials of the moss shoots or gametophores (6, 10, 11, 19, 20, 21, 22, 25, 26, 27, 28). The differentiation leads from the 2-dimensional protonema stage to the 3-dimensional tissue stage. Occurrence of an adenine-type cytokinin (="bryokinin") in mosses was demonstrated by Bauer (3), and Klein (24) showed that a hormonal substance ("factor H"), produced by the protonema itself, is regulating bud formation in *Funaria hygrometrica*. Studies on the morphogenetic effects of applied cytokinins are, thus, relevant to the endogenous regulation of bud formation in mosses.

The moss protonema system offers certain advantages for studying the action of cytokinins. Unlike most other cases where cytokinin effects are being investigated, one is not dealing with tissue cultures or isolated organs but with an intact organism. The developmental steps of the filamentous system can be followed microscopically without fixation or sectioning. Cytokinin activity is restricted to a specific morphogenetic change during protonema development, the formation of buds on certain caulonema cells (6, 22). The lag period between the application of a cytokinin and the response of the cell is short, the first visible signs of cytokinin-caused bud formation becoming detectable by vital staining as early as 10 hours after the application of the hormone (12). The first morphological change, *i.e.* swelling of side branch initials on caulonema filaments, can be observed 16 to 18 hours after the start of the hormone treatment.

We expanded and quantitized earlier work on the moss protonema system and followed the fate of a labeled cytokinin (benzyladenine-benzyl-7-^{14}C) as a first step towards determining the site of action of this hormone.

Materials and Methods

Culture Methods and Application of the Cytokinin. Single spores of the moss *Funaria hygrometrica* (L.) Sibth (capsules collected on the campus of Michigan State University) were transferred under sterile conditions to cellophane discs (diameter 20 mm) which were placed on the surface of an agar medium. The cellophane discs were pre-sterilized by autoclaving in distilled water (7). Knop agar (100 ml/petri dish of 14 cm diameter) was used as the basal nutrient medium [1.0 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 g KH_2PO_4, 0.25 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 43 mg Sequestrene NaFe, Heller's microelements (29), 10 g glucose, 15 g Bacto-Agar (Difco), 1000 ml distilled water]. The protonemata were cultured at an 18 hour photoperiod and 24° in an air conditioned room. The light intensity at culture level was 400 ft-c (Westinghouse, cool white fluorescent lamps, F40CW). Usually, the protonemata were precultured on cellophane discs on basal medium for 14 days and then transferred with the cellophane disc to basal medium containing the cytokinin. In certain experiments, cytokinins were removed from the protonemata by the following "washing" procedure. The protonemata were transferred on the cellophane discs to fresh basal medium. They were left there for 20 minutes and this process was repeated 3 more times. After the fourth transfer, the protonemata were left on basal medium until

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the end of the experiment. If low benzyladenine concentrations (0.1 μM) were used during the pulse period, fewer transfers were required. The hormone could also be withdrawn by dipping the protonemata for a few minutes into sterile water.

Synthesis of Radioactive Benzyladenine. Radioactive benzyladenine (6-benzylaminopurine) was prepared according to the method of Daly and Christensen (17). Benzylamine-7-^{14}C (82 μmoles, specific radioactivity 12.2 mc/mmole, I.C.N. Corp.) was refluxed with 6-chloropurine (82 μmoles, K and K Laboratories, Inc.) in 50 ml *n*-butanol for 24 hours. The butanol was evaporated and the residue taken up in water. Benzyladenine was isolated and purified from the aqueous solution by partitioning into diethylether. Residual benzylamine stayed in the aqueous phase. The ether was evaporated, the residue taken up in water and partitioning with ether was repeated in order to remove remaining traces of benzylamine. The purity of benzyladenine-benzyl-7-^{14}C was verified by comparing it to an authentic sample, using thin layer chromatography, UV-spectrophotometry and biological assays. Scanning of the thin layer chromatograms with a Packard Radiochromatogram Scanner showed that all the radioactivity was associated with the benzyladenine spot. The labeled benzyladenine was stored in aqueous solution in a freezer and was added to the agar medium prior to autoclaving. No breakdown of benzyladenine occurred during autoclaving.

Autoradiography. Protonemata were precultured on basal medium and then transferred to basal medium containing the radioactive hormone. Filaments were isolated after different times of treatment and were quickly dried on microscope slides in an oven at 100°. Freeze- or air-drying at room temperature gave the same quantitative results but the cells were more distorted. The filaments were covered with "dry" photographic emulsion (Kodak NTB3) using the loop technique of Caro (15), and the preparations were developed after 1 to 3 weeks of exposure. In spite of all precautions and employment of different techniques of fixation, leakage of radioactivity from the filaments could not be entirely prevented. This led to an increased background in the vicinity of those cells which accumulated much of the label. The grain count around the cells which did not concentrate the hormone was, however, low. Only dry autoradiographic procedures gave positive results; no label could be detected in the filaments when liquid fixation techniques were employed. The size, deformability, and high water content of the protonema cells are some of the disadvantages of the moss protonema system for autoradiographic studies. The large cells (*ca.* 250 $\mu \times 40\ \mu$) curled and folded during the drying procedure. Most filaments did not lie flat on the microscope slides and often caused the presence of air bubbles in the emulsion.

Results

Bud Formation in Protonemata. The development of moss protonemata has been described in detail by Bopp (5,9). Figure 1 summarizes the growth of the protonema and the effect of benzyladenine on bud formation. The germinating moss spore gives rise to a filamentous structure, the chloronema, which is characterized by cells with perpendicular cross walls and a high content of chloroplasts. After approximately 8 to 9 days the growth pattern changes, the new cells having oblique cross walls and small spindle-shaped chloroplasts. These cells, called the caulonema, will begin to form buds approximately 15 days after spore germination. Bud formation in the caulonema is restricted to cells of a certain physiological stage. No buds develop on the apical 3 cells and on old caulonema cells (6,13,22). Added cytokinins can cause budding in caulonema cells before natural bud formation takes place (5). The effect of the cytokinin is again restricted to caulonema cells of the proper physiological stage. The apical 3 cells of the caulonema filaments and the chloronema cells will not react to the added hormone. This cytokinin response has been found in many other moss species as well (11). The morphological appearance of caulonema cells with and without buds is shown in figure 2.

The dependence of bud formation on benzyladenine concentration is shown in figure 3. Optimal bud formation was obtained at a 1 μM concentration of benzyladenine. The best time for studying the effect of cytokinins on bud development is 1 to 2 days prior to the onset of spontaneous bud formation (fig 1). Other adenine-type cytokinins such as kinetin (6-furfurylaminopurine), 6-(γ,γ-dimethylallylamino)purine, and SD 8339 [6-benylamino-9-(tetrahydropyran-2-yl)-9H-purine] showed comparable hormonal activity. The diphenyl urea deriva-

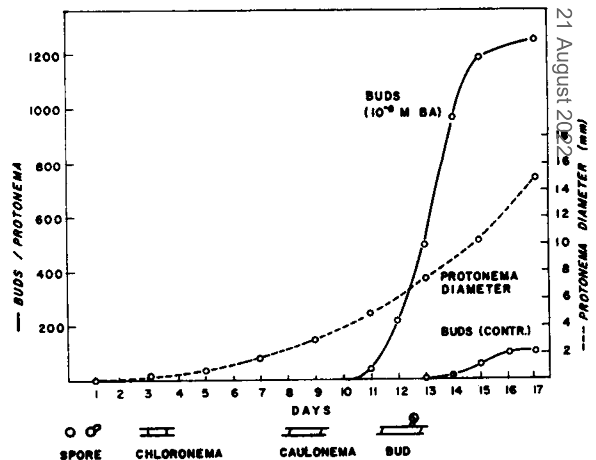

FIG. 1. Growth curve of a moss protonema and spontaneous and benzyladenine-caused (1 μM) bud formation.

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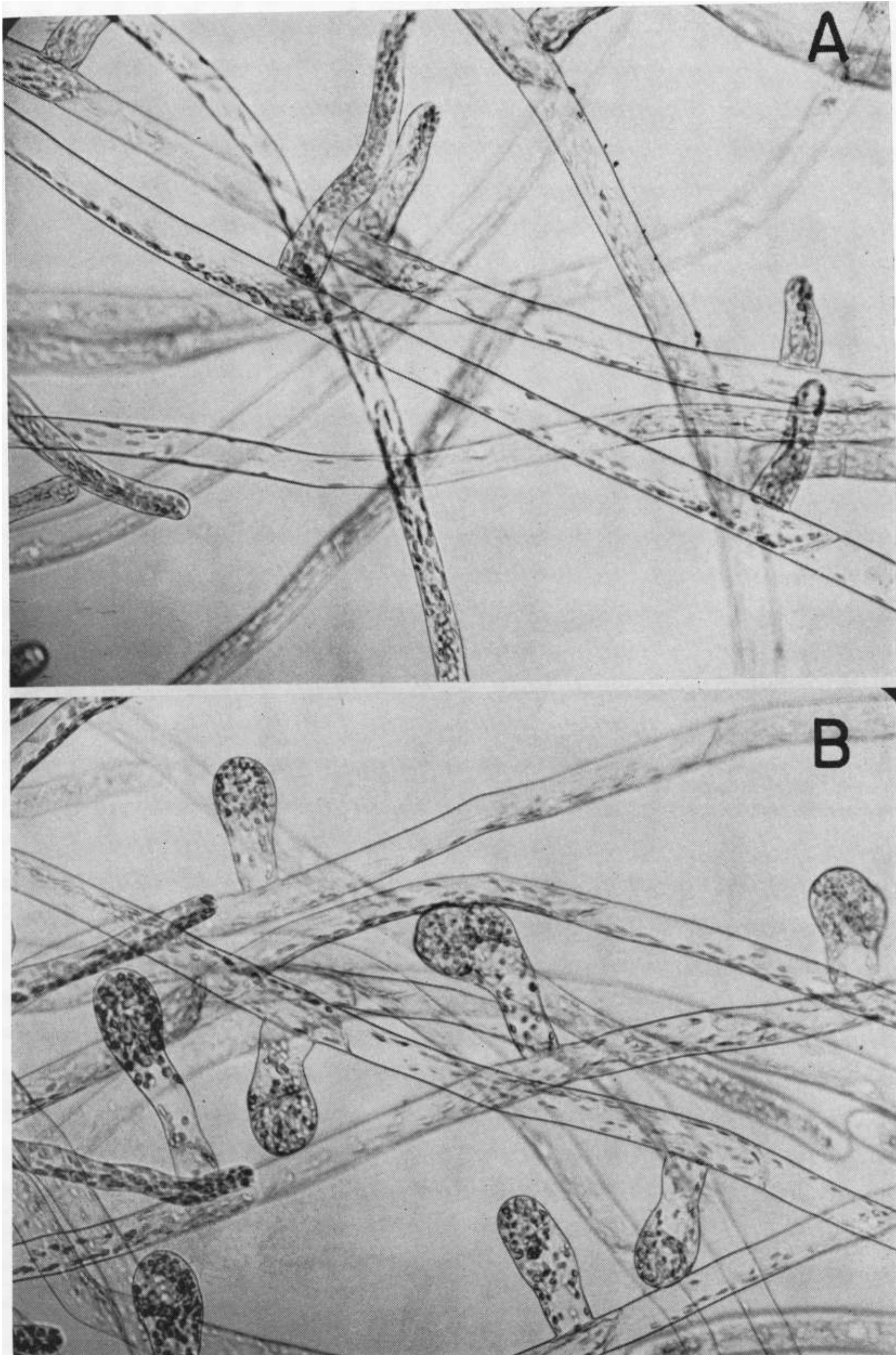

FIG. 2. A) Filaments of a protonema grown on basal medium for 15 days. B) Bud formation on filaments of a protonema which was precultured on a basal medium for 14 days and transferred to basal medium containing benzyladenine (1 μM) for 24 hours.

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tives (N-3-chlorophenyl-N′-phenylurea and N-4-chlorophenyl-N′-phenylurea) of Bruce and Zwar (14) were inactive in this system (Brandes and Kende, unpublished data).

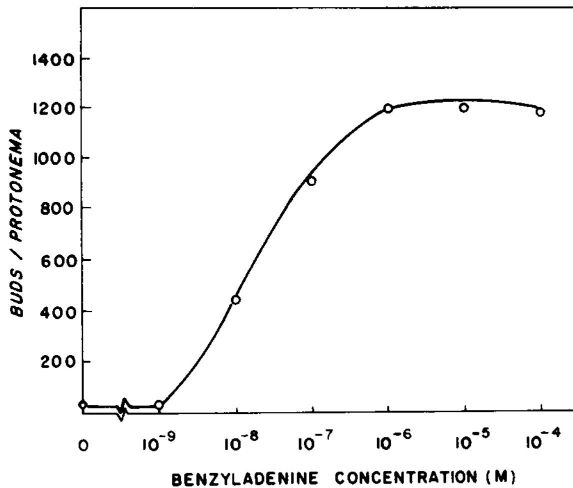

FIG. 3. Dose response curve of benzyladenine on bud formation in moss protonemata.

Reversal of Initiated Buds. The differentiation of buds is reversible during the early stages of bud development. Reversibility of bud formation was previously demonstrated by raising the IAA concentration in the medium (4), by treatment with an extract from bacteria (1, 2), or by subjecting the protonemata to sub-optimal growth conditions (5). When bud formation is caused by applied cytokinins, reversal can be brought about by removal of the hormone. The morphology of bud reversal is shown in figure 4. The protonemata were precultured on basal medium and were then transferred to medium containing benzyladenine (0.1 μM) for 24 hours. Many buds were induced during this treatment but the buds reverted to filamentous growth after the protonemata were transferred to basal medium for another 48 hours.

A quantitative analysis of de-differentiation is shown in figure 5. Control protonemata did not form buds within the first 72 hours of the experiment, while protonemata treated with 0.1 μM benzyladenine developed many buds during the same period. If benzyladenine was removed at early stages of development, the buds reverted to protonemal fila-

FIG. 4. Morphology of bud reversal. Buds were initiated during a benzyladenine treatment (0.1 μM) of 24 hours. The protonema was then washed and grown on basal medium for additional 48 hours.

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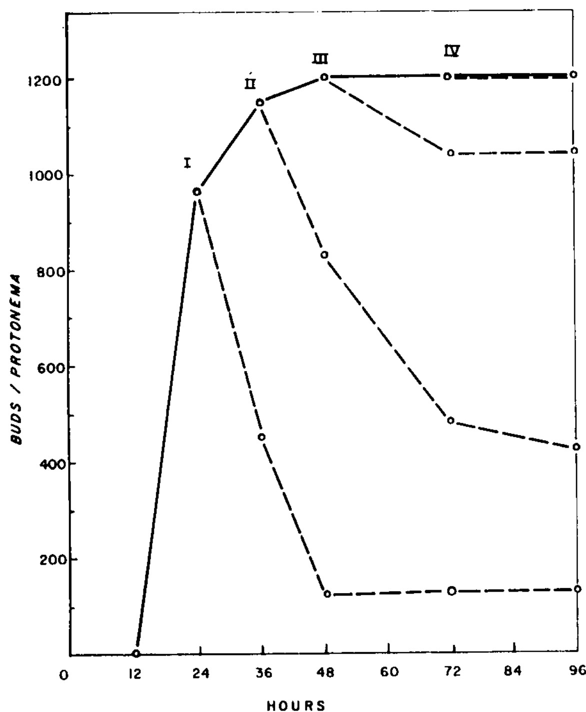

FIG. 5. Kinetics of bud reversal. Protonemata were grown on benzyladenine (0.1 μM) for 24 (I), 36 (II), 48 (III), and 72 (IV) hours. After these periods, the protonemata were washed and transferred to basal medium (broken lines).

ments (fig 5, I) but withdrawal of the hormone at later stages caused less and less de-differentiation. No reversal could be obtained after the protonemata had been treated with the cytokinin for 72 hours (fig 5, IV).

Localization of Labeled Benzyladenine in Protonema Cells. The labeled cytokinin was preferentially accumulated by caulonema cells which were in the responsive stage of development, and by the newly-formed buds (fig 6C, D). Very little label was detectable in the chloronema and caulonema cells which did not react to the hormone. Since the apical cells divide once every 6 to 7 hours, 2 new cells were formed on the main filaments during the 12 hours of benzyladenine treatment. All other cells of the main filaments were laid down before the hormone treatment was started. The third cell (fig 6B) has thus been in contact with the radioactive hormone for the same period as the bud forming cells (fig 6C, D) but no hormone accumulation could be observed in cell 3 (fig 6B). The same holds for cell 2 and the tip cell of the caulonema filament.

Because of leakage of labeled material from the filaments, it was not possible to determine accurately the distribution of radioactivity among the various cells. The differences were, however, evident even if the radioactivity which diffused from the cells was not taken into account. The grain count in cell 3 (fig 6B) gave, after substraction of the general background in the emulsion, a specific activity of 13.0 grains per unit area. The specific activity in cell 5 without the bud (fig 6C) was 95.1 grains per unit area.

As described earlier, washing of the filaments caused de-differentiation of young buds. It also removed the radioactivity from cells which originally accumulated labeled benzyladenine. Protonemata were treated with radioactive benzyladenine as in the experiment shown in figure 6. After 10 hours of ^{14}C-benzyladenine treatment, the protonemata were rinsed in water for 3 minutes, and autoradiographs were prepared from these washed filaments. Figure 7 shows that the accumulation of label in the responding cell No. 6 had disappeared as a consequence of the washing.

Discussion

The protonema system provides a good example for the notion that responsiveness to a hormone depends on prior differentiation of specific, reactive cells. Under certain conditions, caulonema cells de-differentiate to chloronema cells and, consequently, loose their sensitivity to cytokinins (8).

The reversal of buds after withdrawal of the cytokinin shows that the hormone does not act as a "trigger." It needs to be present for a critical period of time during which the differentiation is "stabilized." A similar observation was made by Chrispeels and Varner (16) who showed that gibberellin-controlled α-amylase synthesis in aleurone layers was reduced after the withdrawal of the hormone. If, in the moss system, the cytokinin was removed during a washing period but if the protonemata were transferred back to nutrient agar containing benzyladenine, bud formation proceeded normally. This proved that it was the cytokinin itself which was removed from the cells, and not some other factor(s) required for bud formation.

Our experiments lead to some predictions on the nature of the bond between the hormone and its site of action. The major part of the hormone can be removed from the filaments by washing while a small fraction remains bound to macromolecular components of the cells (Brandes and Kende, in preparation). The physiologically active hormone is then either the portion which is released during washing or the small fraction which is tightly bound. If the latter is true, the hormone-receptor complex must have a fast turn-over rate because of the reversibility of the hormonal response, and a large pool of free hormone is required for continuous formation of that complex. Autoradiography of washed filaments showed that the label accumulated by "target cells" was loosely bound as it was removable by rinsing with water. The same washing procedure also caused de-differentiation of the young

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buds. These data suggest that the easily removable, probably non-covalently bound hormone is the physiologically active one. Under natural conditions, "factor H" is released into the medium, and continuous removal of this hormone retards and reduces bud formation (24). Evidence for non-covalent attachment of a gibberellin to its site of action has been recently presented (18, 23).

A striking feature of the protonema system is the accumulation of the hormone in the "target cells," *i.e.* those caulonema cells which react to the hormone by bud formation, and in the newly formed buds. How can the concentration of the hormone by certain cells be explained? One possibility would be selective uptake of the hormone by the "target cells," perhaps due to permeability differences. However, general permeability differences cannot be the reason for the concentration of the hormone in the "target cells," since radioactive adenine was taken up rapidly by all cells, the highest concentration of label being found in tip cells. Tip cells, however, showed no accumulation of benzyladenine. The accumulation of the hormone in the "target cells" may, on the other hand occur because the target cells contain binding sites for the hormone. Presence and absence of such binding sites may also be the biochemical basis for the difference between responding and non-responding cells. In other words, cells which have become responsive to the hormone may have attained that state, at least partly, by the formation of some sites at which the hormone attaches, this attachment being the premise for its action.

The features mentioned above, the rapidity of the reaction, the reversibility of the hormonal effect, and the localization of the hormone in "target cells", make the protonema system a suitable object for studies on the site of action of cytokinins.

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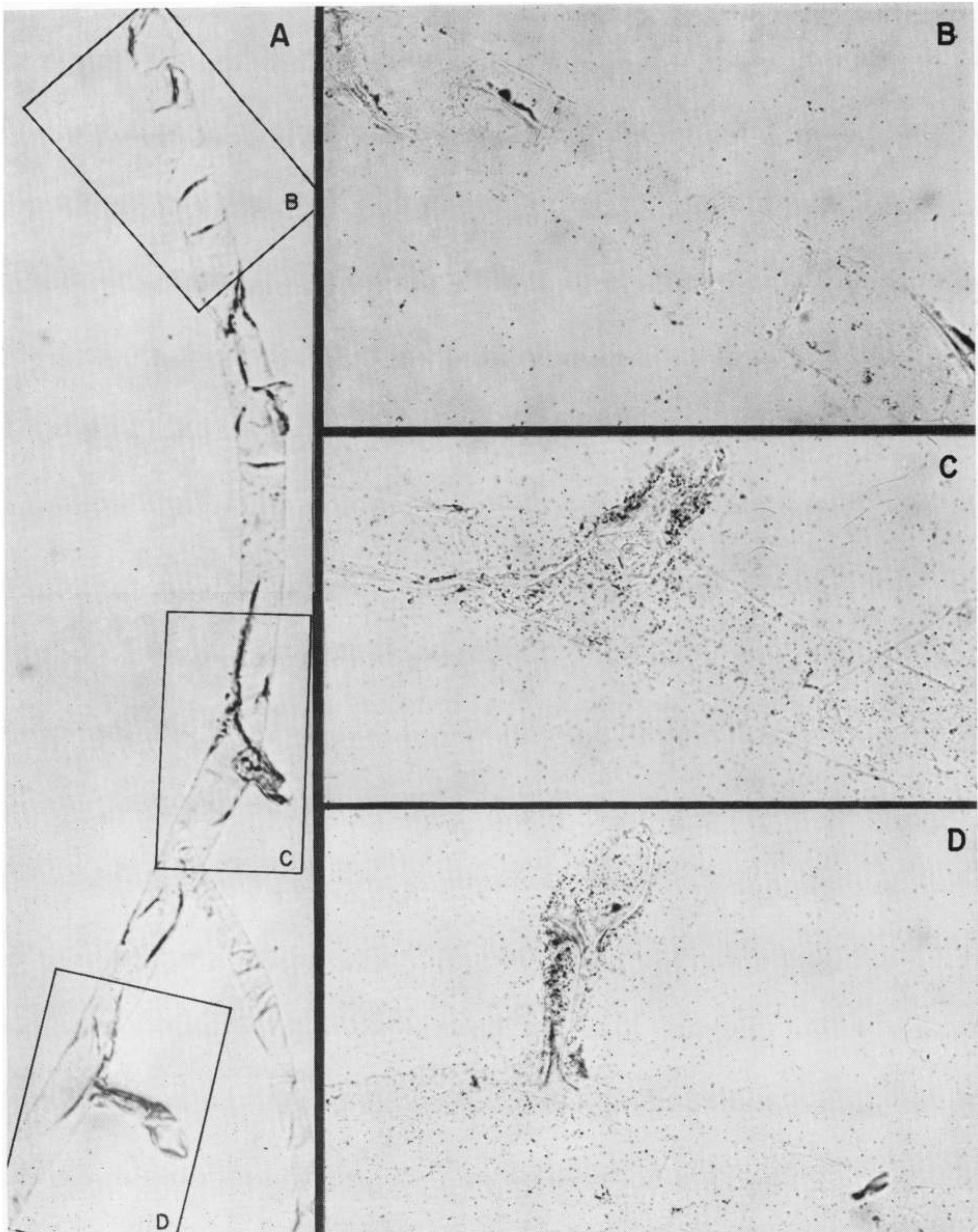

FIG. 6. Autoradiography of caulonema filament after 12 hours of ^{14}C-benzyladenine treatment. In this particular experiment, a drop of ^{14}C-benzyladenine (0.1 mM) was placed on the agar surface under the cellophane disc. A) Caulonema filament with 4 cells (cells No. 3-6, counted from the tip). B) Cell No. 3. C) Cell No. 5 and bud initial. D) Cell No. 6 and bud initial.

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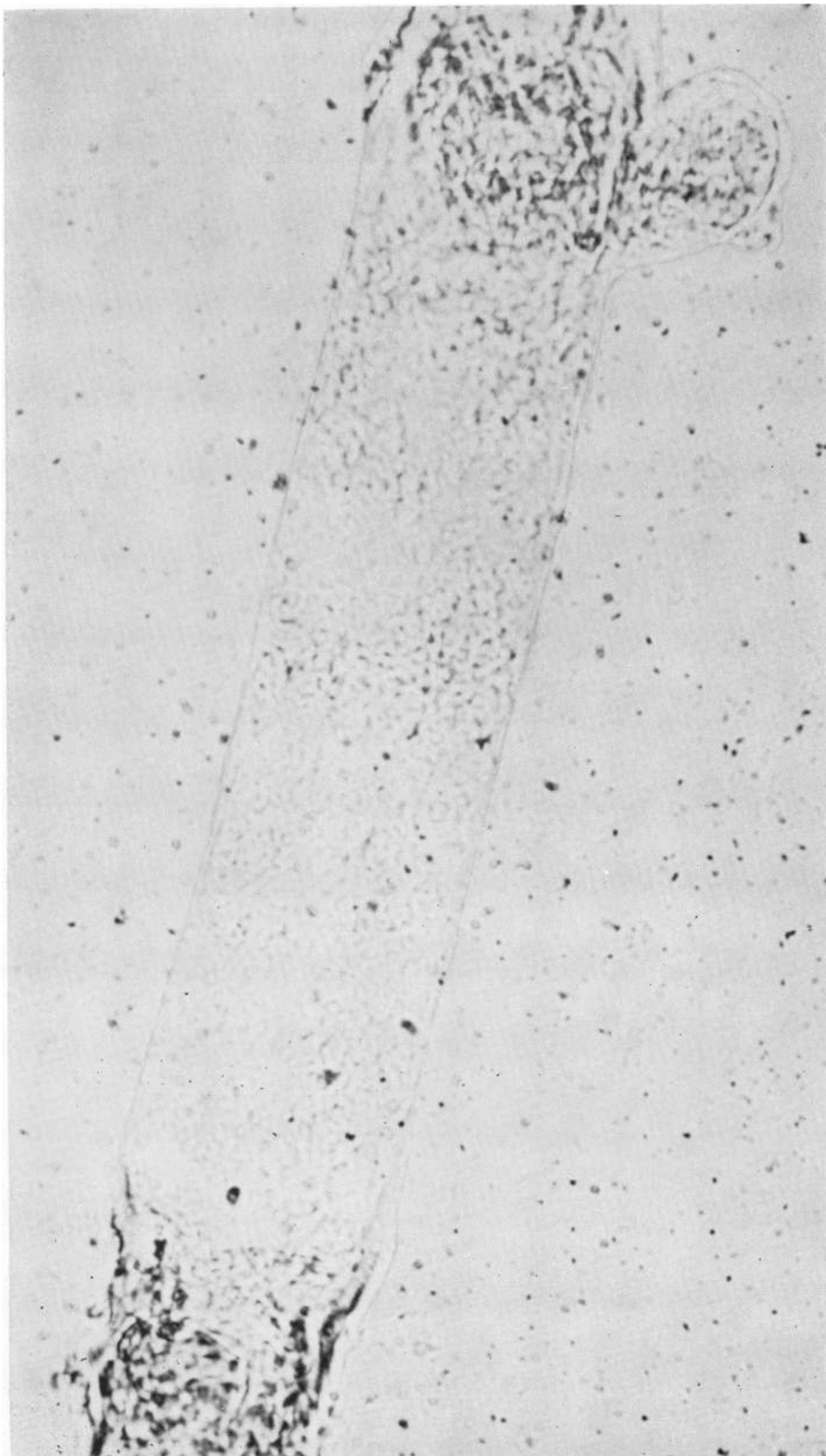

FIG. 7. Autoradiography of caulonema cell No. 6 and bud initial after 10 hours of ^{14}C-benzyladenine treatment followed by a 3 minute rinse in water. In this particular experiment a drop of ^{14}C-benzyladenine (0.1 mM) was placed on the agar surface under the cellophane disc.

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