Culture of anthers of henbane (Hyoscyamus niger, annual variety) at an appropriate stage of development in a simple mineral salt medium provokes division in the embryogenic pathway in a small proportion of the enclosed pollen grains, resulting in the formation of embryoids (4, 11). However, in other plants the origin of embryoids has been traced to the large vegetative cell of the pollen grain (1, 6-8, 10, 14), whereas in henbane a large number of embryoids originate exclusively by the division of the small generative cell. Here, the vegetative cell either does not divide or undergoes but a few divisions and forms a short suspensor-like structure, although it is not determined whether the latter is functionally analogous to the suspensor of zygotic embryos. In a small number of pollen grains, division products of both generative and vegetative cells contribute to embryoid formation, but segmentation of the vegetative cell alone in the embryogenic pathway is seldom observed (12). These results, as well as the rapidity with which embryogenic divisions are initiated in the pollen grains, support the proposition that embryoid induction in henbane is a function of the physiological state of the cell nucleus and that the nucleus of the generative cell, which is primed for DNA synthesis in preparation to forming gametes, enters a state of continued mitotic activity with relative ease. Here, I report a simple, light-microscope autoradiographic method in which thin sections of plastic-embedded anthers are used to follow the pattern of DNA synthesis in embryogenic pollen grains. The results of this study show that dedifferentiation of the pollen grain into an embryoid is accompanied by autoradiographically detectable DNA synthesis in the nucleus of the generative cell, while the vegetative cell nucleus undergoes no DNA synthesis after it is cut off, or synthesizes DNA only during a limited number of cell cycles.

MATERIALS AND METHODS

Culture of Anthers

Plants of H. niger were raised from seeds sown in soil in 10-cm diameter pots and maintained with regular nutrient feeding in a growth chamber at 25°C under an 18-h photoperiod provided by fluorescent and incandescent lamps. Flower buds 6.0-6.5 mm long were sterilized in Clorox (Procter & Gamble Inc., Cincinnati, Ohio) for 5 minutes and washed several times in sterile water. Anthers were aseptically removed from flower buds and cultured in 1-oz French square bottles containing 10 ml of Bourgin and Nitsch's (3) solidified mineral salt medium (basal medium). Cultures were maintained at 26°C in a culture room provided with fluorescent light 12 h daily. Four anthers were planted from each flower bud, and the fifth was used to determine the stage of pollen development by acetocarmine staining. At intervals of 24 h after culture, anthers from one bottle were examined in acetocarmine to follow the stages of embryogenesis.

Autoradiography

For autoradiography of [3H]thymidine incorporation, immediately after excision and at intervals of 24 h in culture thereafter (up to 96 h), anthers containing pollen grains in the uninucleate stage of development were transferred to 2.5 ml of the basal medium supplemented with 5.0 μCi/ml of [methyl-3H]thymidine (25.0 Ci/mmol), serving as a precursor of DNA synthesis. Anthers were exposed to the isotope for 24 h, after which they were collected and fixed in 10% acrolein for 24 h at 5°C. The fixed tissues were processed for microtomy and embedded in glycol methacrylate according to the method of Feder and O'Brien (5). Sections cut at 5-7 μm thickness on a rotary microtome with a steel knife were overlaid with a thin layer of Kodak NTB1 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) and then exposed to X-ray film.
exposed in the dark for 2 wk. The slides were developed in Kodak D-19 developer, fixed, washed, and stained in azure B before mounting in Euparal (GBI Laboratories Ltd., Manchester, England). To confirm that the radioactivity associated with the nucleus was actually going into DNA, a few slides were washed in DNase (0.1 mg/ml in 0.003 M MgSO₄, pH 6.5, 4 h at 30°C) before the emulsion was applied; this treatment eliminated most of the silver grains from the autoradiograms. For each sampling, anthers from at least four flower buds were exposed to the radioactive label, although, for the sake of simplicity, only a general picture of the labeling patterns observed is given here. Fig. 1a is a representative of the type of preparations obtained in this study by the method described. Time scales indicated in the text and in the figure legends are inclusive of the labeling period.

RESULTS
Acetocarmine staining of fresh material and azure B staining of sectioned material proved ideal to differentiate between the generative and vegetative cells of embryogenic pollen grains. The nucleus of the generative cell was granular and stained intensely with both stains, whereas the vegetative nucleus was diffuse and stained lightly. When wall formation occurred in the embryogenic pollen grain, separating a large vegetative cell from a small generative cell, the cytoplasm of the latter stained intensely with both acetocarmine and azure B. During later stages of embryogenesis, daughter cells cut off from the vegetative and generative cells retained the staining characteristics of the parent cells through successive generations, which made it possible to trace their origin to particular cell types. The histochemical differences between vegetative and generative cell products were noticeable even in heart-shaped embryoids constituted of more than 200 cells.

At the time of culture the anther contained a homogeneous population of thick-walled, furrowed pollen grains harboring a centrally placed nucleus which stained moderately with acetocarmine or azure B. The cytoplasm of the grain was granular, free of starch grains, and stained to the same extent as the nucleus. During the next 24-48 h of development of the anther in situ on the flower bud, vacuolation of the cytoplasm, appearance of starch grains, and nuclear division followed in succession in the entire pollen population. However, autoradiograms of the anther exposed immediately after excision to [³H]thymidine (i.e., a total of 24 h after culture) revealed the existence of four major types of pollen grains with respect to their ability to synthesize DNA: (a) completely vacuolate, unlabeled, uninucleate pollen grains; (b) completely vacuolate, unlabeled binucleate pollen grains; (c) nonvacuolate or slightly vacuolate, enlarged binucleate pollen grains, in which the generative nucleus incorporated [³H]thymidine, and (d) same type as c but not detectably labeled by the tritiated precursor. While type a pollen grains constituted up to 90% of the total, the percentages of the other types varied from anther to anther. It is reasonable to conclude that DNA synthesis for the first pollen mitosis must have occurred in the binucleate pollen grains before the anther was cultured; otherwise, some label would have been noted in both nuclei of these grains after 24 h in the isotope. Despite the absence of radioactive label in the nuclei of type a grains, their subsequent fate to be described below makes it difficult to determine from autoradiographic data alone whether or not DNA synthesis was completed in them before culture.

The early incorporation of [³H]thymidine into the generative nucleus identified type c pollen as potentially embryogenic, compared to types a and b grains which gradually disintegrated. Disintegration was accompanied by collapse of the vacuole, loss of cytoplasm and agglutination of the nuclear material. The fate of type d grains was difficult to follow, but available evidence shows that, while most of them became vacuolate and thus nonembryogenic, DNA synthesis and embryogenesis were initiated in others. The two principal DNA synthetic patterns observed in the embryogenic pollen grains are: (i) [³H]thymidine incorporation occurs only in cells originating from the generative nucleus, and (ii) [³H]thymidine incorporation occurs in cells formed from both generative and vegetative nuclei. Autoradiograms of [³H]thymidine incorporation into the nucleus of the generative cell and its division products at three different stages of embryogenesis are shown in Figs. 1b, 2a, and b. DNA synthesis and mitosis in the generative nucleus occurred so fast that by 96 h after culture globular to heart-shaped embryoids were found. In many embryogenic pollen grains, incorporation of [³H]thymidine into the generative nucleus during the first 24 h of culture was also accompanied by the deposition of starch granules and cell enlargement. Giant pollen grains thus formed became nonembryogenic due to failure of mitosis in the generative nucleus or failure of the daughter nuclei formed to enter the cell division cycle. The potentially embryogenic nature
FIGURE 1 Autoradiograms of embryogenic pollen grains. (a) 120 h after culture: section of an anther containing embryogenic pollen grains incorporating [3H]thymidine (heavy arrows), starch-containing pollen grains (light arrows) and vacuolate nonembryogenic pollen grains (asterisk). × 90. (b) Two-nucleate embryogenic pollen grain, 24 h after culture, showing heavy [3H]thymidine incorporation into the generative nucleus; the vegetative nucleus (arrow) is unlabeled. Scale bars are 20 μm. b × 1,000.

of the giant pollen grains was evident from the fact that DNA synthesis and division in the embryogenic pathway were resumed in occasional grains after a delay of 24−48 h (Fig. 2c, d). A feature of embryoids originating exclusively from the generative cell was the complete absence of DNA synthesis in the vegetative cell, which was delineated as a single-celled suspensor-like structure.
In other embryogenic pollen grains, following division of the generative nucleus, the vegetative nucleus also began to synthesize DNA (Fig. 2e). Nuclear division and wall formation occurred, resulting in a four-celled embryoid all cells of which incorporated \([^3H]\)thymidine heavily (Fig. 2f). During further development of the embryoid, DNA synthesis, mitosis and cytokinesis were restricted to cells cut off from the generative nucleus which formed the embryoid proper. On the other hand, nuclei of the two vegetative cells which constituted the suspensor-like part continued to synthesize DNA unaccompanied by division and wall formation (Fig. 2g and h). It seems likely, although not proved by the data presented, that nuclei of the vegetative cells might have undergone endoduplication and attained the cytological characteristics of the suspensor cells of zygotic embryos of some plants (9). In a few instances where the vegetative nucleus formed a multicellular suspensor-like structure, labeling of nuclear DNA in their cells was much less extensive than in cells cut off from the generative nucleus (Fig. 2i). In a small proportion of the embryogenic pollen grains, wall formation delimiting the generative cell from the vegetative cell was followed by rapid DNA synthesis in the latter to form a group of cells before active DNA synthesis commenced in the generative cell (Fig. 2j and k). After 96 h in culture, embryoids were observed similar to those illustrated in Fig. 2l and m. Here, DNA synthesis had ceased in the vegetative cells constituting the suspensor-like structure, and the organogenetic part of the embryoid formed from the generative cell was actively synthesizing DNA. In occasional embryoids which lacked a suspensor-like structure, cells cut off from the vegetative nucleus contributed partly to the formation of embryoid proper; however, in contrast to the heavy incorporation of \([^3H]\)thymidine into the generative cells, DNA synthesis in the vegetative cells was relatively low (Fig. 2n). Embryogenesis solely from the vegetative cell was not observed in the sampling of anthers used in this study.

DISCUSSION

The present results are of interest for several reasons. Although pollen embryogenesis by anther culture has been achieved in several plants (13, 15), no one has studied the timing and pattern of DNA synthesis in the embryogenic pollen grains. This is important since a knowledge of the molecular events responsible for reprogramming of the developmental pattern of the pollen grains would facilitate formulation of a general theory to account for pollen embryogenesis. Furthermore, the results provide additional and apparently conclusive evidence for the totipotency of the generative cell and its involvement in embryogenesis in henbane. Despite the fact that the organogenetic part

![Figure 2](image-url) DNA synthetic patterns in embryogenic pollen grains. (a) 48 h after culture; wall formation separating a densely staining generative cell from a lightly staining vegetative cell. Nucleus of the generative cell is labeled. (b) A globular embryoid showing labeled cells cut off from the generative nucleus; 120 h after culture. Arrows in a and b refer to the unlabeled nucleus of the vegetative cell. (c) 48 h after culture; \([^3H]\)thymidine incorporation into the generative nuclei (gn) of a starch-filled pollen grain; vegetative nucleus (arrow) is unlabeled. (d) 96 h after culture. Labeled cells originating from the generative nucleus of a starch-filled pollen grain. Arrow indicates the undivided vegetative cell. (e) 48 h after culture; a three-nucleate pollen grain showing two labeled generative nuclei (gn) and one labeled vegetative nucleus (vn). (f) 72 h after culture; labeled generative and vegetative cells of a four-celled embryoid. (g and h) Both 120 h after culture; labeled vegetative cells forming embryo and labeled vegetative cells forming a suspensor-like structure. (i) 96 h after culture; embryo with several labeled cells cut off from the generative nucleus and a multicellular suspensor-like structure formed from the vegetative nucleus. Cells of the latter are lightly labeled. (j) 48 h after culture; \([^3H]\)thymidine incorporation into an early stage embryoid constituted of two vegetative cells and one generative cell; (k) 96 h after culture; later stage of an embryoid similar to that shown in j, with several labeled vegetative cells and two or three labeled generative cells. (l and m) Both 96 h after culture; termination of DNA synthesis in the vegetative cells which form the suspensor-like part; active DNA synthesis in the generative cells which form the embryoid proper. Arrows point to nuclei of vegetative cells. (n) 96 h after culture; DNA synthesis in an embryoid formed by division products of both vegetative and generative nuclei. Arrow points to a separation line observed in such embryoids which demarcates the two groups of cells. Scale bar in A (applying to all parts except I, M, and N) = 20 μm. a–h, j, k × 1,000; i, m, n × 600.

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of the embryoid is formed mainly from the generative nucleus, that both generative and vegetative nuclei are capable of DNA synthesis is supported by the data. The absence of an ideal situation involving continued DNA synthesis, followed by mitosis and cytokinesis in the vegetative nucleus and disintegration of the generative nucleus, may explain the absence or the rare occurrence in henbane of embryoids of exclusive origin from the vegetative nucleus. In contrast, in plants such as tobacco (1, 14) and Datura (7) where embryoids originate exclusively by the division of the vegetative cell, DNA synthesis, mitosis, and cytokinesis are presumably more efficient in this cell than in the generative cell, which promptly disintegrates. Pollen grains in cultured anthers of tobacco undergo a protracted induction period of 12-14 days after culture before division in the embryogenic pathway is initiated in the vegetative cell. Since the vegetative cell does not divide in the normal ontogeny of the pollen grain, organization of a new molecular program designed to return the vegetative cell nucleus to a mitotic state has been suggested as one of the events of the induction period (2).

SUMMARY

Continued DNA synthesis in the generative cell nucleus, followed by mitosis and cytokinesis, results in the formation of pollen embryoids in cultured anthers of H. niger. In contrast, the nucleus of the vegetative cell undergoes no DNA synthesis after it is cut off, or synthesizes DNA only during a limited number of cell cycles. DNA synthetic patterns in the generative and vegetative cell nuclei confirm the ontogeny of embryoids described in this plant.

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