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Autoradiographic studies of DNA and histone synthesis in successive differentiation stages of pollen grain in *Hyacinthus orientalis* L.

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Abstract

DNA and histone synthesis in five consecutive morphological stages of *Hyacinthus orientalis* L. pollen grain differentiation were studied autoradiographically. DNA synthesis was found to occur in both the generative and the vegetative cell. DNA replication in the generative cell took place when the generative cell was still adhered to the pollen grain wall but already devoid of callose wall. DNA synthesis in the generative cell slightly preceded that in the vegetative cell. Histones were synthesized in phase S of the generative and vegetative cell. In the generative cell histone synthesis also continued at a lower level after completion of DNA replication. In the developmental stages under study the nuclei of the generative cells were decidedly richer in lysine histones than vegetative cell nuclei.

INTRODUCTION

The pollen grain, being made up of two sister cells — generative cell and vegetative cell, constitutes a good model for study of cell differentiation mechanisms. The first detectable symptom of differentiation is the polarity of the microspore cytoplasm (Sanger and Jackson 1971) followed by its asymmetric division resulting in the formation of a small parietally situated generative cell and a much larger vegetative cell. These two cells are separated from each other by a callose wall during initial development (Górska-Brylass 1967), which is in no doubt of great significance in the differentiation of pollen cells (Charzyńska and Pannenko 1976).

One of the commonly described differences between the generative and the vegetative cell lies in the degree of DNA replication in the pollen cell nuclei. This difference, however, does not occur in all species. There are species in which the DNA is completely doubled in the nuclei of both pollen cells (D'Amato et al. 1965, Corsi and Renzoni 1972), while in others DNA replication in the vegetative nucleus is incomplete (Moses and Taylor 1955, Rodkiewicz 1960), or does not take place at all (Swift 1950, Bryan 1951, Pasteels and Lison 1951, Taylor 1953, Woodard 1958, Howell and Hecht 1971, Charzyńska and Maleszka 1978, Thiebaud and Ruch 1978). In the same species two classes of vegetative nuclei with a DNA level of 2C and 1C at the same time may also occur (Pipkin and Larson 1973). It seems therefore that the differences in DNA replication in the vegetative nucleus are a specific characteristic of the species. Consequently, this factor should be eliminated as a possible essential cause of differentiation of two pollen cells.

Differences in the RNA template activity bring about the different development of the pollen cells. In the vegetative cell the level of RNA and protein synthesis is known to be higher than in the generative cell (Sauter and Marquardt 1967a, 1967b, Sauter 1969b, Jalouzot 1969).

Differences have also been found in the character of nuclear proteins of either pollen cell, those in the vegetative nucleus being more acidic (Bryan 1951). The amount of acidic (Pipkin and Larson 1973) and basic proteins (Sauter 1969a, Sheridan 1973, Pipkin and Larson 1973, Thiebaud and Ruch 1978) is different in the nuclei of the generative and vegetative cells.

Sauter's (1969a) cytochemical studies, Sheridan's (1973) biochemical and Thiebaud and Ruch's (1978) cytophotometric studies have shown that the vegetative nucleus is poorer in lysine histone and richer in arginine histon then the generative nucleus. The above results concern a rather indefinite stage of differentiation of the generative cell, since the maturity degree of the pollen grains was generally determined by the length of preceding anthesis.

An attempt has been made at relating the information on the time of DNA replication and the character and time of histone protein synthesis to the consecutive stages of morphological differentiation of the pollen grain.

Autoradiopraphic studies were carried out on the maturing pollen grain: in the stage of the parietally situated generative cell, consecutive phases of detachment of generative cell from the pollen grain wall, up to the stage of the spindle-shaped generative cell situated inside the vegetative cell.

MATERIAL AND METHODS

Pollen grains of *Hyacinthus orientalis* L. were studied in five developmental stages (Fig. 1). DNA and histone was studied autoradiographically using ^{3}H-thymidine (15 000 mCi/mM, 50 μCi/ml), 4,5-^{3}H--lysine (5 000 mCi/mM, 50 μCi/ml) and ^{3}H-arginine (5 000 mCi/mM, 50 μCi/ml). The anthers were incubated for 2 hours in radioactive precursors dissolved in 2% sucrose. Applicated incubation time was shorther than the duration of distinguished developmental stages in the anthers under natural conditions. After incubation the material was fixed for 24 hours in 10% formalin in phosphate buffer ph 7.0 at +4°C.

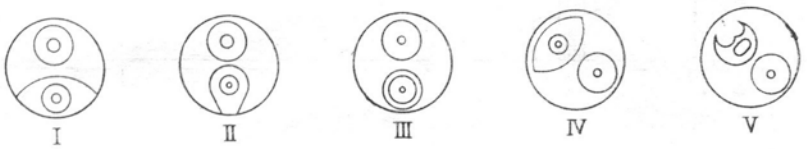


Fig. 1. Developmental stages of pollen grain in *Hyacinthus orientalis* L. Stage I — lens-shaped generative cell devoid of callose wall; stage II — balloon--shaped generative cell nearly completely detached from the pollen grain wall; stage III — spherical generative cell with no contact with the pollen grain wall; stage IV — lemon-shaped generative cell — the cytoplasm forms angles at two opposite poles of the nucleus; stage V — spindle-shaped generative cell (mature pollen grain)

Anthers embedded in paraffin were sliced 4 μm thick on an MPS-2 microtome. Histones were extracted after Spelsberg and Hnilica (1971) from part of the preparation incubated in ^{3}H-lysine or ^{3}H--arginine. The preparations were covered with Ilford L-4 emulsion. The exposure time for ^{3}H-arginine was 70 days, for ^{3}H-lysine 63 days. The autoradiograms were stained with methyl green and pyronine (UNNA) after Brachet.

For determination of callose wall one part of the autoradiograms, after counting the traces was investigated in 0.05% aniline blue (Eschrich and Currier 1964) in fluorescence microscope (Fluoval - Carl Zeiss Jena).

Traces of over 30 pollen grains were counted with reference to cytoplasm, nucleus and nucleolus in both pollen cells. The intensity of the labelling was determined on the basis of the total traces proceeding from all succesive sections of the same pollen grain. The results were statistically analysed.

RESULTS

^{3}H-THYMIDINE INCORPORATION

Traces of ^{3}H-thymidine incorporation into pollen grain were found over the nucleus of the generative and of the vegetative cell (Photos 1, 2, Fig. 2).

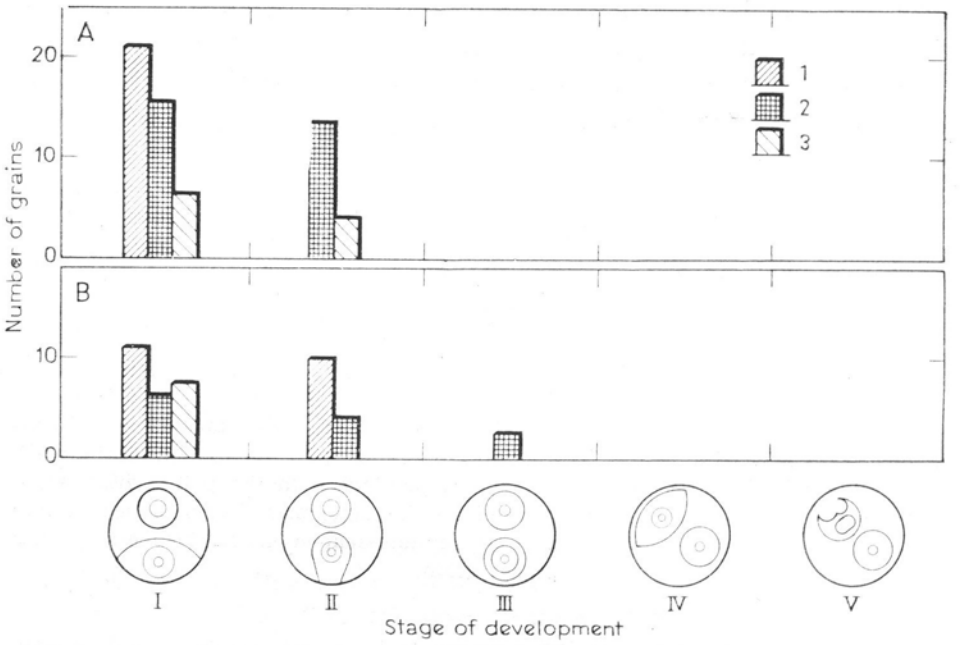
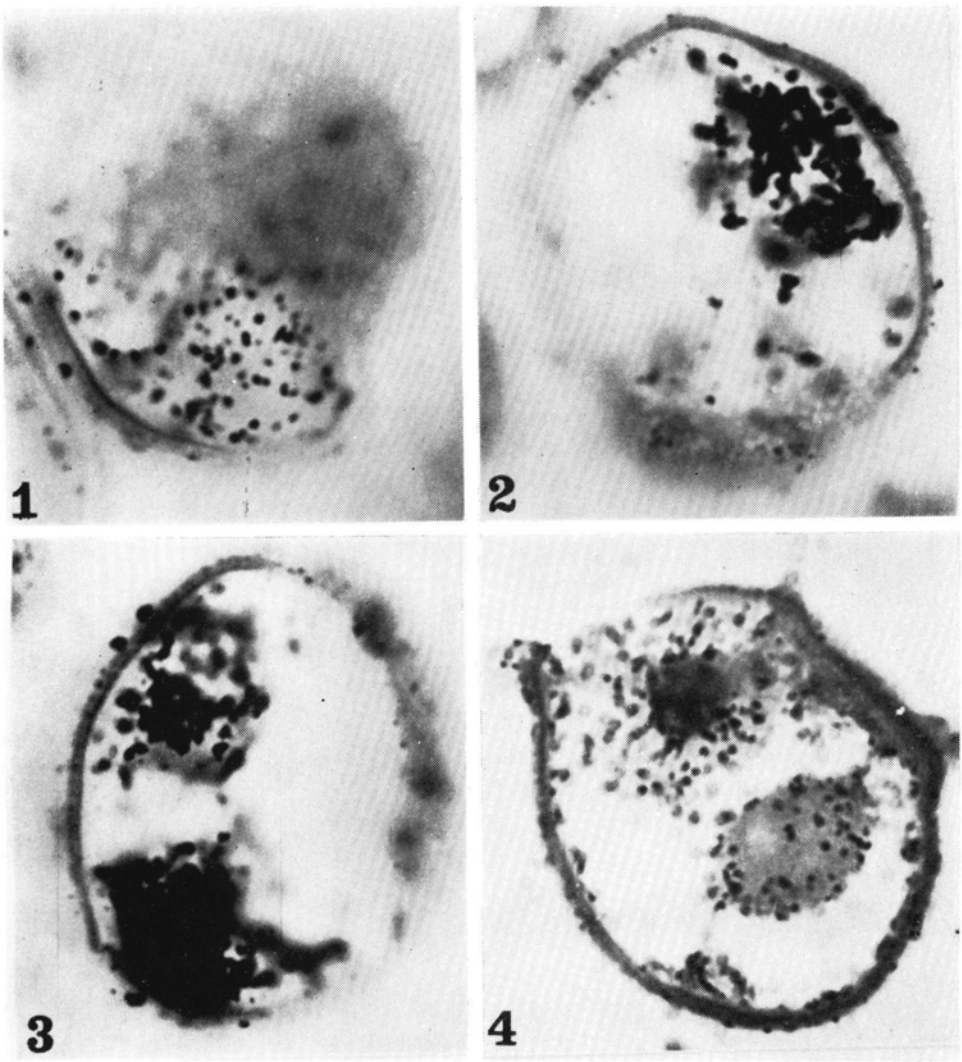


Fig. 2. Intensity of DNA and histone synthesis in the generative and vegetative cell nucleus of developing pollen grain of *Hyacinthus orientalis* L.
A — generative cell nucleus; B — vegetative cell nucleus; 1 — DNA; 2 — lysine rich histone; 3 — arginine rich histone

Traces of ^{3}H-thymidine incorporation, over the generative cell nucleus, were observed in stage I, in the parietally localized generative cell after the disappearance of the callose wall (Photo 1). About 30% of generative cells in this morphological stage of pollen grain development showed ^{3}H-thymidine incorporation (phase S). Labelling analysis of pollen grains situated in the particular anther loculus has excluded different ^{3}H-thymidine accessibility to pollen grains as the possible cause of this phenomenon. The time of DNA synthesis is then decidedly shorter than the duration of the parietal stage of generative cell.

Radioactive thymidine incorporation into the vegetative cell nucleus was observed in stages I and II of pollen grain development (Photo 2). The above results indicate that DNA replication in the vegetative cell

Plate I



DNA and histone synthesis during maturation of the pollen grain of *Hyacinthus orientalis* L. × 1200. Photos 1 and 2. ^{3}H-thymidine incorporation into pollen grain (stage I): Photo 1. DNA synthesis in generative nucleus; Photo 2. DNA synthesis in vegetative nucleus. Photos 3 and 4. ^{3}H-lysine incorporation into pollen grain: Photo 3. Parietally situated generative cell, strong labelling of generative nucleus visible (stage I); Photo 4. Decrease in labelling intensity in lemon-shaped generative cell (stage IV)

continued with the same intensity throughout two morphological stages (I and II), whereas phase S in the generative cell was shorter — this coincides with one developmental stage of the pollen grain. Consequently, simultaneous labelling of equal intensity was not observed in both pollen nuclei.

^{3}H-LYSINE INCORPORATION

The generative cell

Maximum intensity of ^{3}H-lysine incorporation in the nucleus was found in stages I and II of pollen grain development (Photos 3, 4). In further developmental stages there was a decline in the number of traces of radioactive aminoacid incorporated into the nucleus. The mature pollen grain was devoid of any ^{3}H-lysine traces over the nucleus of the spindle-shaped generative cell.

In stages I and II considerable differences were observed in the number of traces over nuclei in the same morphological stage. About 30% of the nuclei were very strongly labelled (ca 40 traces per nucleus),

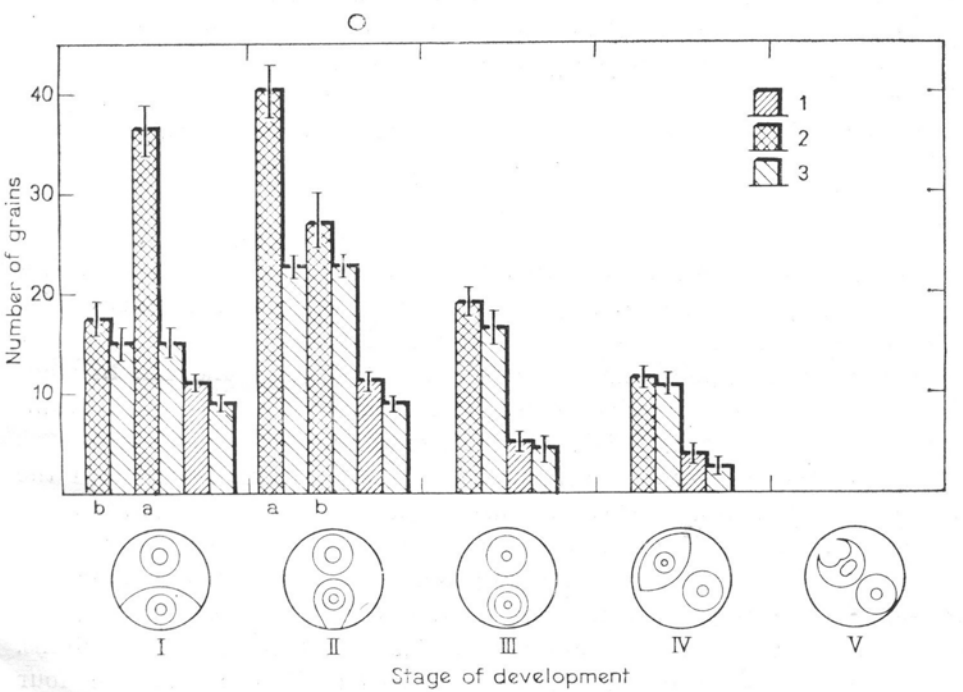


Fig. 3. Incorporation of ^{3}H-lysine into generative cell of developing pollen grain of *Hyacinthus orientalis* L.
1 — cytoplasm; 2 — nucleus; 3 — number of silver grains after lysine histone extraction

while over the remaining nuclei the number of traces was markedly smaller (ca 20 traces per nucleus). Therefore, two categories of nuclei had been distinguished, ^{3}H-lysine synthesis intensity was considered separately in each category (Fig. 3).

After extraction of lysine histone the two categories of nuclei in stages I and II, distinguished according to labelling intensity, were not observed. The number of traces over the nuclei in these two stages approximated the level characterizing nuclei of categories Ib and IIb (Fig. 3). A significant difference in the number of traces before and after extraction was found in nuclei of categories Ia and IIa. This result indicates that the categories of nuclei strongly labelled with ^{3}H-lysine (Ia and IIa) are those in which lysine histone, other histone and basic proteins appear, while categories Ib and IIb include nuclei in which other histon and basic proteins appear.

In a small number of generative cells the nucleolus showed little labelling. The small dimensions of the nucleolus (1-2.5 μm) may cause considerable errors and the results are doubtful.

^{3}H-lysine incorporation into the cytoplasm of the generative cell occurred in the first four developmental stages of the pollen grain (Fig. 3). The largest number of traces was observed in stage II.

After lysine histone extraction a very slight difference was found in stage II. In the remining stages no significant difference in the number of ^{3}H-lysine traces was found before and after extraction, which would point to synthesis of other histones and basic proteins.

The vegetative cell

The vegetative cell incorporated ^{3}H-lysine into the nucleus, nucleolus and cytoplasm during four pollen grain developmental stages. The vegetative cell nucleus showed the strongest labelling in stage II (Fig 4). Somewhat weaker labelling was observed in stages I and III. In stage IV the number of ^{3}H-lysine traces decreased. In mature pollen grains there were no traces of ^{3}H-lysine incorporation.

Lysine histone extraction resulted in a significant decrease in the number of ^{3}H-lysine traces in stages I and II, and a slight one in stage III. In stage IV the number of traces over vegetative nuclei did not significantly change. This result indicates that the lysine histone appears in the vegetative nucleus in stages I, II and III. In stage IV lysine is incorporated only into other histone and other basic proteins.

The vegetative cell nucleolus incorporated ^{3}H-lysine in the four initial pollen grain development stages. Lysine histone extraction did not change the number of traces of precursor. This would indicate that in this case ^{3}H-lysine is incorporated into the basic proteins of the nucleolus.

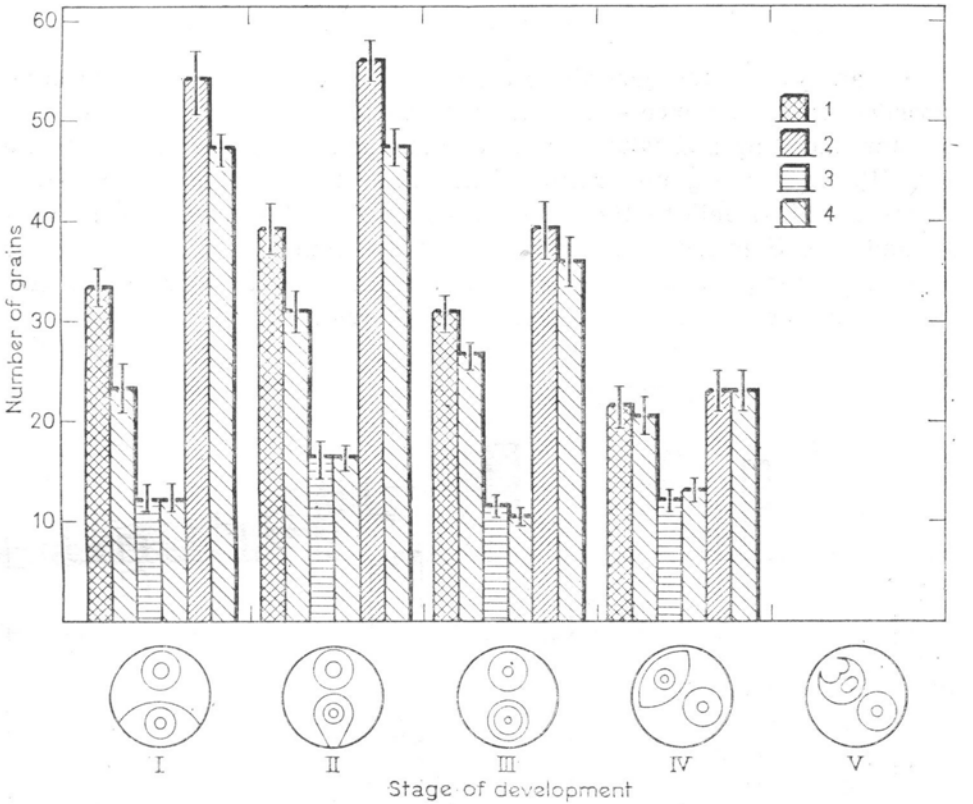


Fig. 4. Incorporation of ^{3}H-lysine into vegetative cell in developing pollen grain of *Hyacinthus orientalis* L.
1 — cytoplasm; 2 — nucleus; 3 — nucleolus; 4 — number of silver grains after lysine histone extraction

^{3}H-lysine is incorporated into the cytoplasm of vegetative cells in four developmental stages. The peak of ^{3}H-lysine incorporation occurred in stages I and II. In later stages a drop was observed in the number of ^{3}H-lysine traces. Mature pollen grains did not show any incorporation.

Lysine histone extraction induced a significant decrease in the number of ^{3}H-lysine traces over the cytoplasm of the vegetative cell in stage I and II. In stages III and IV there was no significant difference in the number of traces after extraction. The results suggest that in stages I and II histones are synthesized along with other basic proteins, whereas in stages III and IV only other proteins are synthesized.

^{3}H-ARGININE INCORPORATION

^{3}H-arginine incorporation was found in both pollen cells in the first four developmental stages. In mature pollen grains no labelling was found.

The generative cell

An analysis of the generative cells labelled with radioactive arginine revealed, the occurrence of two distinct categories of nuclei: with a small amount labelling and with a large amount of labelling (Fig. 6Ia, Ib, IIa and IIb). After arginine histone extraction the number of traces in stages I and II fell to the level characteristic of nuclei of categories Ib and IIb. Significant differences in trace number before and after extraction were found in nuclei of categories Ia and IIa. This suggests the occurrence of arginine histone in these nuclei.

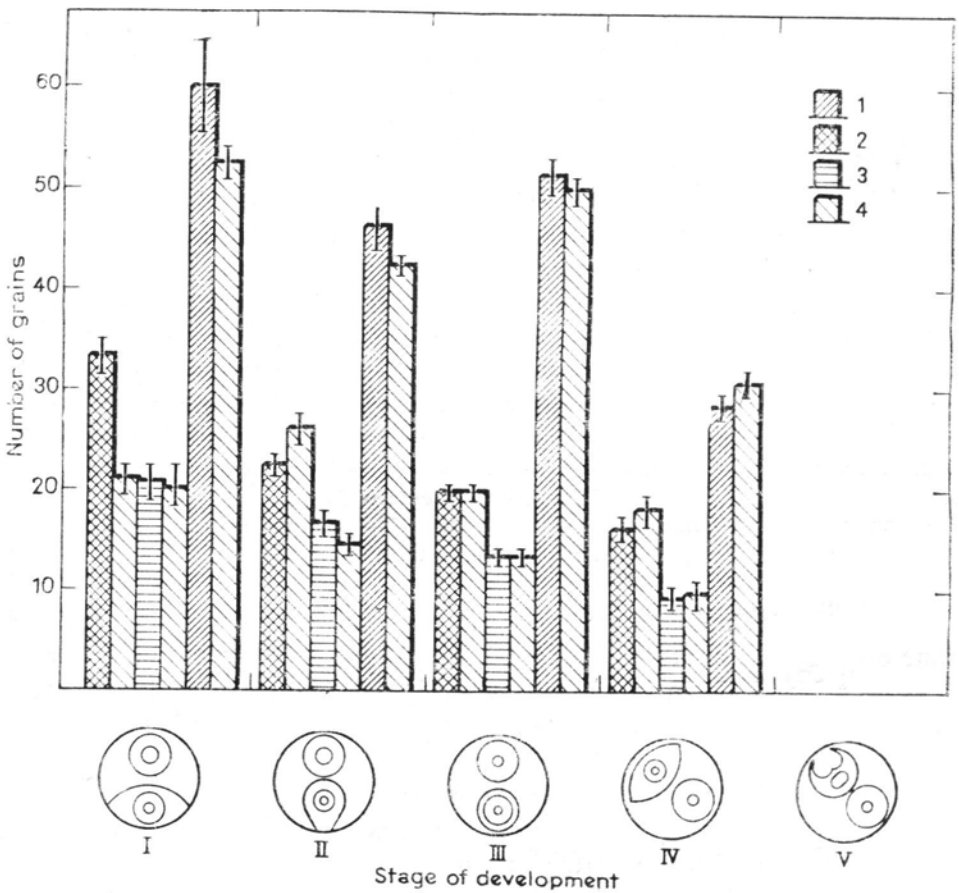


Fig. 5. Incorporation of ^{3}H-arginine into vegetative cell in developing pollen grain of *Hyacinthus orientalis* L.
1 — cytoplasm; 2 — nucleus; 3 — nucleolus; 4 — number of silver grains after arginine histone extraction

^{3}H-arginine was incorporated into the generative cell cytoplasm in the first four stages of pollen grain development. A significant difference

in the trace number before and after arginine histone extraction was found in stages I and II. This result indicates that histone synthesis takes place in the generative cell cytoplasm during this period of pollen grain development.

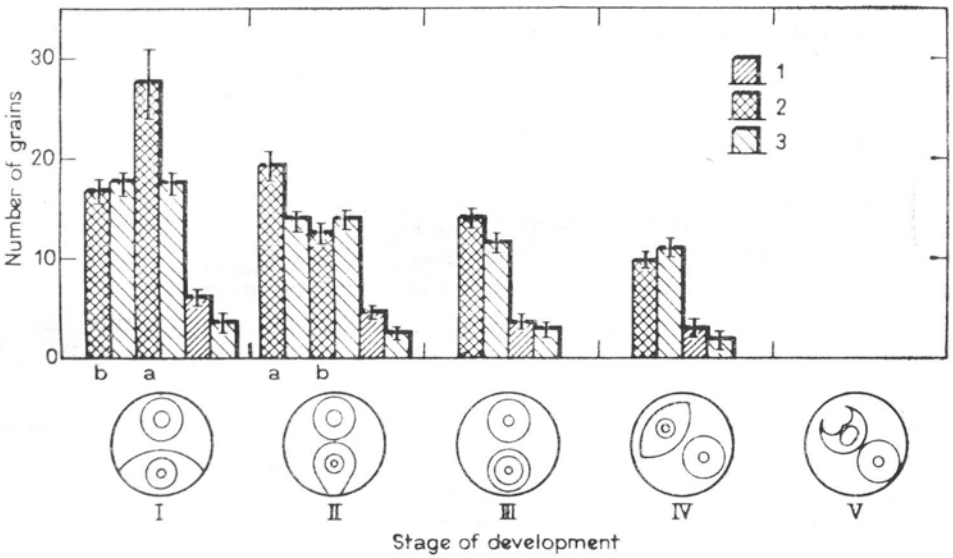


Fig. 6. Incorporation of ^{3}H-arginine into generative cell in developing pollen grain of *Hyacinthus orientalis* L.
1 — cytoplasm; 2 — nucleus; 3 — number of silver grains after arginine histone extraction

The vegetative cell

^{3}H-arginine traces were found over the nucleus, nucleolus and cytoplasm of the vegetative cell (Fig. 5). Arginine histone extraction did not induce any significant differences in the number of traces over the nucleolus, which suggests the occurrence of other basic proteins or other histones.

After arginine histone extraction a significant difference was found in the cytoplasm in stages I and II, which sugests that arginine histone are synthesized during these stages. In stages III and IV no significant differences were detected, which would point to the synthesis of other proteins in the vegetative cell cytoplasm.

A significant decrease in the number of traces over the vegetative nucleus after arginine histone extraction was observed in stage I. In stages III and IV no significant differences were revealed, and in stage

II there was even an increase in the number of traces after extraction. Considering this it can assumed that in stage I of pollen grain development ^{3}H-arginine is being incorporated into histones. However, the increase in the number of traces in one case may suggest that this increase occurred as a result of physico-chemical changes during extraction. Consequently, the absence of significant difference need not be regarded as evidence that arginine histone synthesis does not take place, but it is possible that it proceeds with less intensity.

DISCUSSION

DNA synthesis in the pollen grain of *Hyacinthus orientalis* L. was found in the nucleus of the generative and vegetative cell. DNA replication in the generative nucleus took place in one morphological stage of the pollen grain (stage I), whereas in the vegetative nucleus it proceeded over, the stages of parietally localized generative cell and the stage of the "balloon-shaped" generative cell.

The beginning of DNA replication in the vegetative nucleus was retarded in relation to that in the generative nucleus. This is evidenced by the fact that no simultaneous labelling of equal intensity was observed in both cell nuclei and by the intensive DNA synthesis in the vegetative nucleus which continued when the generative cell became balloon-shaped (stage II).

The analysis of ^{3}H-thymidine incorporation showed the connection between the DNA replication phase in the nucleus of the generative cell and its morphological traits. Intensive DNA synthesis took place in the parietally situated generative cell in *Tradescantia* (Charzyńska and Maleszka 1978) yet already devoid of callose wall (stage I). These and unpullished data concerning DNA synthesis in generative cells in *Paris quadrifolia, Convallaria majalis* (Górska-Brylass and Świerzowicz) and in *Muscri racemosum* (Bednarska and Deruszewska) suggest that in monocotyledons there is a more general regularity concerning the coincidence of this morphological stage with the DNA replication period in the generative cell.

Synthesis of basic proteins other than lysine and arginine histons went on during the first four developmental stages of pollen grain in both pollen cells. In mature pollen grains, i.e. with a spindle-shaped generative cell, there was no synthesis of basic proteins. The intensity of basic protein synthesis was much higher in the vegetative cell.

The use the autoradiographic method with simultaneous lysine or arginine histone extraction made it possible to define lysine and arginine histone among other histones and basic proteins synthesized and to pinpoint the time and place of their synthesis during the course of

pollen grain development (Fig. 2). In *Hyacinthus orientalis* L. histone synthesis was found in both pollen cells. Similar results were obtained by Sauter (1969a) in *Peonia tenuifolia* and by Sheridan (1973) in *Lilium*. In *Tradescantia* (Rasch and Woodard 1959). *Hippeastrum belladonna* (Pipkin and Larson 1973) and *Lilium* (Reznikova et al. 1978) histone synthesis has been observed only in the generative cell nucleus.

The fact that not all generative nuclei in the parietal stage incorporated ^{3}H-thymidine and the occurrence of two categories of nuclei differing significantly in intensity of ^{3}H-lysine labelling in stages I and II indicates that the metabolic stages of the processes in question do not entirely coincide with the morphological stages distinguished. It seems that stages I and II are longer than the metabolic stages related to DNA and histone synthesis (Fig. 7).

Fig. 7. DNA and histone synthesis into generative and vegetative cell of developing pollen grain of *Hyacinthus orientalis* L.

The results obtained with arginine and lysine histone extraction point out that the intensity of basic protein synthesis other than lysine and arginine histons in the generative nucleus is about the same throughout the stage, and the differences are due to differences in intensity of lysine and arginine histone synthesis. Histone synthesis in the generative cell does not end with the completion of DNA replication. In the stage related to phase S the intensity of histone synthesis reached its maximum, the synthesis however continued in the balloon-shaped generative cell stage, i.e. after completion of DNA replication. Histone synthesis after phase S in cells of the male generative line has been observed both in animals and in plants (Bloch and Brack 1964, Bloch and Teng 1969, Olszewska 1974). This may suggest that histone synthesis during and after phase S is a fairly general in the development of male gametes.

The application of lysine histone extraction revealed that generative nuclei are considerably richer in lysine histones than vegetative nuclei. This may account for the higher degree of chromatin condensation in the generative nucleus observed just after microspore division. Lysine

histones participate, as we know, in the chromatin condensation processes. The condensation process may cause nonspecific inactivation of genetic information in the generative cell, which results in differentiation.

The increase in the number of traces after arginine histone extraction found over the vegetative nucleus in stage II is difficult to explain. It should be noted in the generative nucleus the amount of arginine histone is markedly smaller than of lysine histone.

Arginine, and lysine, histone synthesis continued longer than phase S. The synthesis of this group of histone occurred both in the stage I and II of the generative cell.

The differentiation of the two pollen cells in *Hyacinthus orientalis* L. reflected by the described syntheses of DNA histone took place during in the stages preceding pollen grain maturity. Just before anthesis mature pollen grains characterized by a spindle-shaped generative cell did not synthesize DNA or histone protein.

The results presented concerning DNA and histone synthesis in *Hyacinthus orientalis* L. reflect the differentiation condition, which determines the following steps of the development and functional distinctness of the two pollen cells.

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Badania autoradiograficzne syntezy DNA i białek histonowych w kolejnych stadiach różnicowania się ziarna pyłkowego Hyacinthus orientalis L.

Streszczenie

Badano metodą autoradiograficzną syntezę DNA i histonów w pięciu kolejnych stadiach morfologicznych różnicowania ziarna pyłkowego *Hyacinthus orientalis* L. Synteza DNA odbywała się zarówno w komórce generatywnej jak i wegetatywnej. W jądrze komórki generatywnej replikacja DNA ma miejsce w okresie, kiedy komórka ta przylega do ściany ziarna pyłkowego, jednakże pozbawiona jest już ściany kalozowej. Synteza DNA w komórce generatywnej trwa krócej aniżeli w komórce wegetatywnej. Histony są syntetyzowane w fazie S komórki generatywnej i wegetatywnej. W komórce generatywnej po zakończeniu replikacji DNA synteza histonów zachodzi mniej intensywnie. Jądra komórek generatywnych w badanych stadiach rozwojowych są zdecydowanie bogatsze w histony lizynowe aniżeli jądra komórek wegetatywnych.