PROTEINS OF SPERM NUCLEI EXAMINED BY AUTORADIOGRAPHY AT FERTILIZATION AND SUBSEQUENT NUCLEAR DIVISIONS IN LILIUM¹

DALE M. STEFFENSEN

Department of Botany, University of Illinois, Urbana

Received May 5, 1965

IT is often assumed that DNA is the only determinant received from the male parent. The possibility remains, however, that the male-contributed chromosomal, nuclear and cytoplasmic proteins and cellular organelles could have significant influence on early embryonic development. An attempt was made, therefore, to investigate the proteins of sperm and their later fate upon nuclear fusion and division in the embryo. In plants, the contribution of a second sperm nucleus can be followed during division of the endosperm in the embryo sac. This study with *Lilium* was initiated in order to examine the mode of assortment and stability of labeled paternal chromosome proteins during these divisions.

MATERIALS AND METHODS

Varieties: The Easter lily, *Lilium longiflorum*, was used in the experiments. The varieties Nellie White, Ace, Croft. and Georgii, were obtained as bulbs from commercial sources and cultivated in the greenhouse until the buds and flowers were ready for use.

Stages and embryology: With slight differences in varieties, the period of DNA and histone synthesis prior to the first microspore division, occurs in buds between 50 and 60 mm in length. A second synthesis of the generative nucleus takes place in buds that are between 70 and 80 mm in length (HOTTA and STERN 1961). This generative nucleus divides in the pollen tube at about 24 hours after pollination, giving two sperm nuclei (Table 1). The developmental stages of the embryo sac in *Lilium* are well known and were reviewed by MAHESHWARI (1950). The various stages and their time of occurrence, which are relevant to this study are given in Table 1.

Labeling of pollen in bud culture: It has proved possible to culture buds of lily to maturity *in vitro*. The perianths of buds 50 to 60 mm long were removed, being careful not to damage the anthers, filaments or style. Buds are sterilized before dissection in 10% "Clorox." By leaving on approximately 1 or 2 mm of pedicel, the anthers and the pollen so contained, will develop to maturity and produce viable pollen. The buds were placed in Hoagland's solution with 2% sucrose but without sulfate, using 0.5 to 1.0 ml of medium per vial or test tube for a single bud. These were cultured at 15°C as described by HOTTA and STERN (1963). Different levels of carrier free S³⁵, as sulfate, were then added in 0.1 ml the next day to obtain maximum counts per pollen grain without significant loss of pollen germination. (See next paragraph concerning addition of White's medium.) The results presented here are based primarily on bud cultures containing between 80 to 100 μ c of S³⁵ per ml of final solution. This level of radioactivity yielded pollen with at least 50% viability, ranging from 5 to 19 counts per pollen grain per minute, as determined by a gas flow counter (Nuclear-Chicago). The viability was always pretested by germination in stigmatic fluid on slides before pollinations were made. In one experiment S³⁵-methionine and S³⁵-cystine were used, giving similar results to those obtained with S³⁵-sulfate.

¹ Research supported by grant NSF-GB-1327 from the National Science Foundation.

TABLE 1

The time course of fertilization and divisions in the Lilium embryo sac at approximately 20°C

Stages	Time of occurrence (days after pollination)
Division of generative nucleus giving two sperm nuclei	1
Pollen tubes enter capsule	4
Sperm nuclei enter embryo sac, followed by fusion of polar nuclei with one sperm giving primary endosperm nucleus; 2nd sperm stays appressed to egg	5-6
First division of endosperm nucleus, 2-nucleus stage	7
Second division of endosperm, 4-nucleus stage	9-10
Fusion of egg and second sperm to form zygote	11-12
Third division of endosperm, 8-nucleus stage	13
First division of zygote	14-15

Attempts were made to label pollen with H^3 -arginine. This pollen was viable but nonradioactive, indicating little if any incorporation of arginine.

The "sulfur pool" in developing pollen was determined in some culture sets by counting the supernatant after cold perchloric acid extraction. Also a radiation survey probe was used to measure the time of incorporation into whole anthers. Both types of results showed that significant amounts of radioactivity appeared about 3 days after addition of isotope, and after one week most of the sulfur³⁵ to be taken up was already absorbed. This latter time in culture (7 to 10 days) coincides with the chromosome synthesis period of the generative nucleus. In order to assure continued growth and to obtain viable pollen, White's complete media containing non-radioactive sulfate was added at about 7 to 9 days after cultures were begun. Occasionally, additional White's media was added again to replenish water loss. The anthers dehisced at about 17 to 19 days from the start of bud culture.

Crossing: The rationale of the experimental procedure was to label the protein of the male parent and to use its pollen to fertilize a nonradioactive female. The Easter lily is self incompatible, and therefore it was necessary to use males from a variety different than the female parent. The varieties, Nellie White or Ace, were labeled in bud culture and used as male parents, while Croft and Georgii were usually the nonradioactive female parent.

Fixation and histological technique: Lily ovaries were fixed in formalin, 70% ethanol and glacial acetic acid (5:90:5, v:v:v) at various times after pollination. In latter experiments glutaraldehyde was substituted for formalin in the same proportion since it has often proved to be a better fixative in electron microscopy. In two additional sets of fixations, the freeze substitution method in methanol was used (WOODS and POLLISTER 1955). There was no discernable difference in protein retention of tissues fixed in these three ways. This finding is similar to that of DE (1961). After several changes in 70% ethanol, the tissue was dehydrated in a tertiary butanol series, imbedded in paraffin and sectioned. After development of autoradiographs, most of the sectioned material was processed by the Azure B method for staining nucleic acids at pH 3.6. Some tissue was tested for histone by hydrolyzing the DNA with hot trichloroacetic acid before autoradiography, followed by fast green staining at pH 8.2 after development of the emulsion. The details for the last two procedures are given by CONN, DARROW and EMMEL (1960).

Autoradiography: Most autoradiographs were prepared by the dipping method using Ilford G.5 Nuclear Emulsion, diluted 1 to 1 with water (w:w) and melted either at 45° or 50°C, depending on the thickness desired. In enzymatic hydrolysis experiments it proved necessary to use the maize protein, zein, as the adhesive to retain the tissue and emulsion on the slides. Zein is an ideal adhesive for enzymatic experiments since it is low in basic and aromatic amino acids, and is also water insoluble. Acid cleaned slides were coated with a 0.5% zein in 70% ethyl alcohol (w:v).

Enzymatic procedures: The enzymatic degradations of protein in the sections were carried out by the use of trypsin, chymotrypsin and pepsin, obtained from Worthington Biochemical Corp., Freehold, N. J. The separate reaction mixtures of trypsin and chymotrypsin were made with potassium phosphate buffer at pH 6.8, containing 0.5 mg/ml or one or the other enzyme. Pepsin was prepared at concentrations of 2 mg/ml in 0.02 N HCl. All enzymatic reactions were carried on for 3 hours at 37°C.

RESULTS

Distribution of radioactivity in the embryo sac and endosperm after fertilization with S^{35} labeled pollen: As might be expected, most of the S^{35} radioactivity was concentrated at the micropylar end of the embryo sac where the pollen tube enters one of the synergids. The radioactivity persisted there for at least two weeks after fertilization. The first question asked was: What happened to the sperm protein in the endosperm nucleus? Is this protein lost immediately or does it persist and stay bound to the paternal chromosomes after a number of divisions?

Twelve chromosomes are brought in by the male parent and 48 chromosomes are contributed by the female to the endosperm of *L. longiflorum*. Before the first division of the $5n$ endosperm nucleus there are always regions of high radioactivity, "hot spots," and regions exhibiting background radiation levels. There are usually five nucleoli in the endosperm nucleus, each attached to a nucleolar organizer chromosome. Almost always one of the five nucleolar organizer regions shows labeling, while the other four are unlabeled. After the first division of the primary endosperm nucleus, the resulting daughter nuclei are still quite radioactive, again with heavily labeled regions or hot spots.

At the four-nucleus stage, a quantitative study was attempted, as given in Table 2. A thicker nuclear emulsion was used in these particular autoradiographs in order to detect electron tracks from the S^{35} beta particles. Track counting is a reasonably accurate method since the tracks have a vector and much of the radioactivity background comes from the K^{40} in the glass slide below, most of which is out of range. All of the four nuclei were labeled, as seen in Table 2, but the radioactivity was unequally distributed.

After three divisions of the endosperm (Table 3) there is some indication that the data fall into classes, with a few nuclei having two or three times the number

TABLE 2

Distribution of sulfur³⁵ tracks over endosperm nuclei at the 4-nucleus stage as determined from autoradiographs of serial sections, radioactivity arises from labeled protein contributed by the male parent

Embryo sac No.	Number of tracks* over each of four endosperm nuclei			
	1	2	3	4
1	29	25	12	29
2	35‡	17	28	†

* A track is defined as a cluster or row of three or more silver grains.

† Too near sperm and synergids, therefore not scored.

‡ One nucleolar region with seven tracks.

TABLE 3

Distribution of sulfur³⁵ after three divisions of the endosperm nuclei. The data were obtained by analyzing the number of electron tracks emitted from each nucleus in the embryo sac

Embryo sac No.	Number of tracks* over each of eight endosperm nuclei							
	1	2	3	4	5	6	7	8
1	10†	6	14	8	5	10	4	—
2	12	17	9	15	8	11	13†	10†

* A track defined as a cluster or row of three or more silver grains.

† Near micropylar end, values probably too high.

‡ Seven of the ten tracks originated at and tangentially from one nucleolus.

of tracks of other nuclei. Such a distribution might be expected if 12 chromosomes were distributed at random to eight nuclei.

The data from Tables 2 and 3 indicate at best, that a single chromosome should produce about seven or so electron tracks at the fourth or fifth division. The only conceivable way one can be certain of distinguishing this level of radioactivity from background is to have the silver grains and tracks occur in clusters or "stars." All factors have been maximized to insure detection of any labeled protein (i.e., highly sensitive emulsion, long exposure and maximum isotope incorporation up to killing levels). The embryo sacs were sectioned so that each nucleus occurred in two or three adjacent sections. A labeled region of a nucleus almost always exhibited labeling in the same location in the adjacent section, thus providing a check against errors due to the occasional radiation from background. For the considerations below, a labeled nucleus was defined as a silver grain density from 5 to 15 times background levels.

If the sulfur labeled proteins stay with the "old strands" of the original 12 male chromosomes, then after one more division, the 16-nucleus stage, some nuclei should be unlabeled. At the 32-nucleus stage these differences between labeled and unlabeled nuclei should be even more apparent. Some attempts were made, therefore, to score the entire embryo sacs in serial sections at these 16- and 32-nucleus stages. In this material there were always some "hot" nuclei and some "cold" nuclei. The radioactive nuclei appeared as stars, where silver grains and tracks were localized in one part of the nucleus. In the 32-nucleus stages, at least half of the endosperm nuclei were either unlabeled or no higher than background. It does seem evident then that some chromosome protein coming in with sperm can persist at least through five divisions of the endosperm. However, the evidence so far is only indicative that the original 12 male chromosomes retained some of the protein synthesized in the pollen, suggesting that the labeled protein must go in one direction when DNA reduplicates and is not dispersed.

Enzymatic degradation: A number of slides were prepared from the same lily capsule, which had been fixed 8 days after pollination. At this stage fusion of sperm and egg has not yet occurred. However, the endosperm nucleus has undergone one division. Separate sets of slides containing serial sections of this material were subjected to four separate treatments: trypsin, chymotrypsin, pepsin, and a

phosphate buffer control, all incubated at 37° for 3 hours. These enzymatic hydrolyses were made in an attempt to characterize the chromosome protein: trypsin cleaves the peptid chain only at sites with basic amino acids, chymotrypsin breaks the chain only at sites with aromatic amino acids, while pepsin is least specific and cleaves at a number of locations. From the autoradiographs examined, it appeared that little if any labeled protein was lost by the buffer control treatments alone. As might be expected, pepsin hydrolysis removed all of the radioactivity from the embryo sac, synergids and pollen tube. The radioactive protein was completely removed from the endosperm nuclei by trypsin. However, the label remained near the micropylar end and the synergids (Figures 1 and 2). On the other hand, chymotrypsin removed little if any of the labeled protein either from the endosperm nucleus and the sperm nucleus appressed to the egg, but it did remove the slight amount of "cytoplasmic label" throughout the embryo sac and most of the label from the synergids, micropyle and pollen tube (Figures 3 and 4). These characteristic digestion patterns by trypsin and resistance to chymotrypsin are typical of a histone or protamine, which are high in arginine and lysine or both and low in aromatic amino acids. It is concluded, therefore, that the protein followed in the endosperm nuclei is a basic protein and probably a histone.

Preliminary study of the egg and embryo: Investigation of the fate of the sperm proteins in the zygote has proved more difficult than that of the endosperm owing to the closely adjacent labeled areas of the pollen tube and synergids. When the pollen tube first enters the embryo sac and the endosperm starts to develop, the male pronucleus is intensely radioactive, so much so that the surrounding region is obscured with overlying silver grains. In slightly oblique sections, however, the unfertilized egg can be observed, free of contaminating radioactivity. In this case the egg is definitely nonradioactive, as would be expected before the two gametes fuse. The evidence so far indicates that the zygote formed from the fusion of a labeled sperm and unlabeled egg is nonradioactive, in the one cell, two cell, and later stages. There seems little doubt that the major proportion of the protein from the male was replaced, destroyed or left behind before fusion. Entirely new chromosome proteins appears to be made by the male chromosome complement in the initial stages before zygote formation.

Pool and exposure times: As in many labeling experiments, one of our principal concerns has been the fate of the endogenous pool of sulfur³⁵ radioactivity in pollen and pollen tubes growing down the style. The observations on this matter are as follows. When autoradiographs were made of tubes from labeled pollen, obtained either by growing pollen *in vitro* or by removing tubes from the style 24 hours after pollination, the proper exposure time was only a few hours. If exposed 24 hours or longer, then the emulsion was black from over exposure. When the same pollen source grew 4 to 5 days in the style, about 10 to 11 cm in length, the proper exposure time is about 2 to 3 weeks. Apparently most of the "sulfur pool" was used and the protein left behind in the wall and in the vacuoles, as were the other sulfur compounds. The exposure time for autoradiographs of embryo sacs after fertilization took at least 2 to 3 months, more than 200 times

longer than the time required for germinated pollen tubes. It is safe to state that the original amino acid pool is depleted and the remaining label in the embryo sac is stable protein. Also the incoming radioactive protein is not reutilized in any

detectable amount, since the egg and zygote are unlabeled even though surrounded by radioactive protein.

DISCUSSION

The failure to detect a radioactive protein contribution by the male in the zygote of *Lilium* agree with the cytochemical findings in *Helix* by BLOCH and HEW (1960) and in *Drosophila* by DAS, KAUFMAN, and GAY (1964). These animal data indicate a complete shift of incoming proteins contributed by the sperm to an "embryonic or cleavage histone," proteins with more lysine than arginine. Apparently zygotes start with little if any proteins contributed by the male, again consistent with the usual absence of cytoplasmic and paternal effects contributed by sperm.

The possibility still exists, however, that some proteins contributed by the male may survive and persist in the zygote. The experiment presented here was not definitive in this regard. Hypothetically at least, certain genes or genotypes might control or "regulate" the conservation of certain chromosome bound proteins. If this mechanism were possible, then what cases might be cited as examples? The phenomenon of paramutations described by BRINK (1964) appears as a plausible example. In this thoroughly investigated case one fact is very clear: the genotype of the sporophyte controls the R^r mottling patterns exhibited immediately by the endosperm and in the embryo as detected in the next generation. Could this phenomenon be brought about by a protein bound to the paramutated allele, R^r ? If the maize endosperm behaves as *Lilium*, then the selfed colored allele, R^r , could be temporarily under the influence of a R^{st} protein made in the sporophyte. KERMICLE (cited by BRINK 1964) obtained hyperploid endosperm using B-chromosome translocations of the following types: $r^g r^g$ (♀)/ $R^r R^r$ (♂) and $R^r R^r$ (♀)/ $r^g r^g$ (♂) from reciprocal crosses. The dosage response was considerably different in these two supposedly identical constitutions. The R^r gametes contributed by the male did not produce the expected dosage of aleurone pigmentation, indicating that the sperm contribution might have been turned off temporarily.

A unique response in a strain of *Drosophila robusta* has been described by LEVITAN (1963) and LEVITAN and SCHILLER (1963) where chromosome aberrations are induced in the paternally derived complement. The chromosome aberrations are apparently produced in the zygote since no mosaics were detected

FIGURE 1.—An autoradiograph of a sectioned embryo sac, eight days after pollination with S^{35} -pollen that has been digested with trypsin, hot TCA hydrolyzed and stained with fast green at pH 8.2. Phase contrast. End (endosperm nucleus), Mic (micropyle), Ant (antipodals). $\times 435$.

FIGURE 2.—The same embryo sac digested with trypsin as in Figure 1 but photographed with dark field illumination to show the silver grains more distinctly. Note: the silver grains, showing as white dots (those out of focus are white halos), are in no higher number over the endosperm nucleus than the background levels over the nucellar cells. The micropylar end and synergids are strongly radioactive. $\times 435$.

FIGURE 3.—An autoradiograph, S^{35} labeled material as in Figure 1, except that this embryo sac was digested with chymotrypsin. Phase contrast Syn (synergid), End (endosperm nucleus), Ant (antipodal end). $\times 440$.

FIGURE 4.—The same embryo sac as in Figure 3, digested with chymotrypsin but with dark field illumination. Note the intense radioactivity persisting over the endosperm nucleus and the greatly reduced radioactivity at the micropylar end. $\times 440$.

(LEVITAN 1964). Could the basis of selection between male and female chromosomes possibly be determined by their respective proteins? These questions are put forth only to emphasize the fact that the chromosome is a nucleoprotein.

It would be premature to ascribe any role for the paternal protein in *Lilium*, which can be detected even after four or five divisions in endosperm nuclei. A number of proposed functions have been attributed to histones and nuclear proteins and some of the possibilities are as follows: (1) a structural protein called a "linker" between DNA molecules had been proposed (TAYLOR 1963); (2) STEDMAN and STEDMAN (1947) suggested a role for histones in regulation of gene function during development; (3) heterochromatic or "repressed" proteins, that turn off chromosome segments, are proposed by FRENSTER, ALLFREY and MIRSKY (1963); (4) there may be cell division and chromosome coiling proteins; (5) "feedback" proteins, like those moving from nucleus to cytoplasm and back again, are indicated in the experiments of GOLDSTEIN (1963); (6) protein is a candidate for the repressor substance in the JACOB-MONOD operon model; (7) histones are supposed to control RNA synthesis according to ALLFREY, LITTAU and MIRSKY (1963) and BONNER and HUANG (1963), with synthesis proceeding when histone is absent from the DNA and with no synthesis when histone is bound. By and large the evidence for most of these hypotheses is fragmentary and lacking in rigorous chemical and genetic substantiation. It seems fair to conclude that we are just beginning to understand the nature and potential roles for chromosome protein.

A few experiments have dealt more specifically with the question of turnover and distribution of chromosome proteins after more than one division of mitotic chromosomes after labeling. In one case using *Vicia faba*, PRENSKY and SMITH (1964) found heavy labeling at the first division in chromosomes pretagged with H^3 -arginine, yet at the second division, the chromosomes were almost unlabeled. The meaning of this experiment is in doubt since the acetic acid-ethanol fixative, hydrolysis in normal hydrochloric acid and the spreading with 45% acetic acid used by PRENSKY and SMITH are likely to have extracted some histones from the *Vicia* roots tips, particularly the lysine rich fraction (ROBERT H. GRAY, personal communication). MATTINGLY (1963) using H^3 -lysine to label *Vicia* protein, and employing a method of formalin fixation and trichloroacetic acid hydrolysis, found that chromatin had lost only one quarter of its activity in 48 hours. Using chromosomes of hamster cells grown in tissue culture, PRESCOTT (1964) followed four mitotic cycles after labeling with a mixture of six amino acids, and concluded that the assortment of radioactive proteins was dispersive and the proteins had almost completely turned over after the fourth division. Since there are at least ten or more types of histone (PHILLIPS 1962; BONNER and T'so 1964) and an undetermined number of other proteins, then any qualitative or quantitative study of chromosome protein turnover using autoradiography is inconclusive until supported by data concerning changes in specific activity for each of the proteins in question. A diversified approach to the biology and chemistry of histones will have to be pursued in order to understand their many complexities.

One serious difficulty in analyzing mitotic cells from root tips or from tissue

culture is that heavily labeled cells are often blocked in the G_1 period following the first division and never divide again, apparently owing to radiation effects. For example, when root tip cells of *Vicia faba* were heavily labeled with H^3 -thymidine, followed by a colchicine block until fixation, then no labeled $4n$ cells reached the second division even though they were examined up to seven days after labeling and the experiment was repeated three times (STEFFENSEN, unpublished). On the other hand, unlabeled $4n$ and $8n$ cells were observed at the 2nd and 3rd divisions, respectively. The mitotic rates of the unlabeled cells proceeded as expected. If radioactive amino acids had been used, rather than thymidine for DNA, then it might have been mistakenly concluded that the protein had turned over at the second division, where in fact the cells in question would have never been labeled. The advantage of the lily experiment, reported here, is that one follows the protein from single pollen grains and the amount of radioactivity and the number of divisions can be determined directly.

SUMMARY

Sulfur³⁵ labeled pollen was obtained by growing buds of *Lilium longiflorum* in sterile culture. This radioactive pollen was then used to pollinate nonradioactive female plants in order to follow the course and fate of the protein in sperm nuclei at fertilization and subsequent divisions in the embryo sac. The autoradiographic results with endosperm nuclei indicate that the male contributed protein can still be detected after the 3rd, 4th, and 5th divisions—the 8-, 16-, and 32-nucleus stages respectively.

Enzymatic degradation with trypsin, chymotrypsin and pepsin of sectioned lily ovaries was carried out before autoradiography. The presence and absence of radioactivity after enzyme digestion indicated that the protein is basic, and probably a histone.

Preliminary experiments with the zygote suggest that little if any of the protein contributed by sperm accompanies the male gamete when fusion with the egg occurs.

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