Autoradiographs of Pollen Tube Nuclei with Calcium-45*

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PLATES 158 AND 159

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ABSTRACT

Autoradiography with Ca^{45} has been used to obtain information about the relation between calcium and chromosomes. Labelled pollen from the Easter lily, *Lilium longiflorum*, was allowed to develop into pollen tubes between 5 and 6 cm. long in the styles of non-radioactive flowers. All of the nuclei, namely the tube nucleus and the two sperm nuclei, retain Ca^{45} after this period of growth and development.

Since the two sperm nuclei have formed during this interval by the mitotic division of the generative nucleus and growth of the tube has occurred under the influence of the tube nucleus, it is inferred that the calcium was bound in a stable nuclear component, the chromosomes.

INTRODUCTION

Although the nutritional requirements of plants for divalent metals are quite well known, the physiological basis for the requirement is poorly understood in many instances. The controversy over the relation between chromosome stability and divalent cations is a case in point.

Scott (13) used the microincineration technique to follow the behavior of inorganic salts during mitosis. The spodograms showed a progressive increase of inorganic salts in the chromatin so that by metaphase the chromosomes appeared as aggregates of white ash. According to Scott the bulk of this ash is composed of water-soluble salts, probably of calcium and magnesium. Spodograms of the salivary chromosomes of *Drosophila* have been described by Barigozzi (2) who found the ash concentrated in the chromosomal bands. At the biochemical level, analyses and solubility studies of nucleoprotein by Allgén (1), Luck *et al.* (8), and Frick (6) have revealed bound calcium and magnesium. Cytochemical and cytogenetic studies have indicated that chromosome instability is associated with a deficiency in calcium or magnesium (14, 15); it was inferred by Mazia (10) and Steffensen (16) that these cations play a direct role in stabilizing the structure of the chromosome. Conversely, the results of cytological studies of the effect of cation removal with EDTA[1] and enzymic treatments have led Kaufmann and McDonald (7) to the view that the chromosome abnormalities which are associated with metal deficiencies are the result of disturbed metabolism. Physicochemical studies with nucleohistones have influenced Davison and Butler (4) to deny the possibility that metal ions play a significant role in chromosome structure.

Autoradiographic studies with tracers are especially suited for obtaining information on the uptake and exchange of substances. Our experiments with Ca^{45} are based on a simple assumption: if Ca^{45} associates with nuclear structures but exchanges readily with non-isotopic calcium under physiological conditions, then the cation is not a part of the stable components of chromatin; conversely, if the Ca^{45} is incorporated and remains bound during metabolic activity and mitosis, then it is part of the stable components of the chromatin. This report describes the results which are obtained when pollen grains from Easter lilies labelled with Ca^{45} are allowed to grow and develop into

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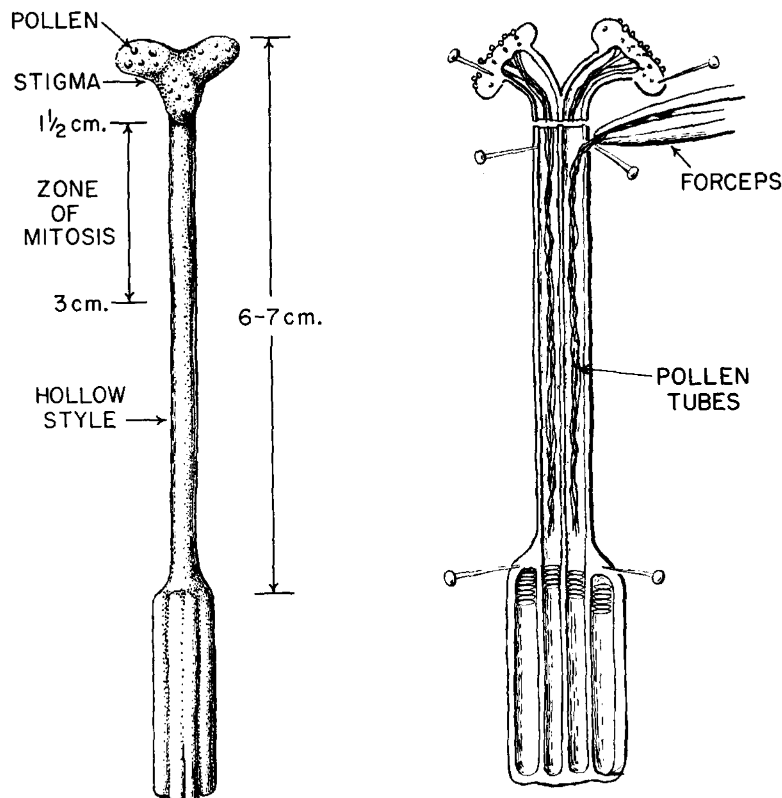
[1] Abbreviations employed: ethylene diamine tetra-acetic acid (EDTA), deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and adenosine triphosphate (ATP).



TEXT-FIG. 1. The technique for removing pollen tubes from the style of lily flowers.

pollen tubes in the styles of normal, non-radioactive flowers.

Material and Methods

Commercial varieties of the Easter lily, *Lilium longiflorum*, were used. Croft and Ace varieties were maintained in the laboratory as the source of radioactive pollen. Easter lilies are generally self-incompatible and outcrosses were made. The varieties used for the non-radioactive female parent (Georgii, Floridii, Giganteum) were obtained in flower from commercial sources.

The presumptive pollen parents were grown in soil to a point (premeiosis) just prior to the appearance of flower buds. The soil was washed away and the plants were cultured in Hoagland's solution with the calcium omitted in order to lower the concentration of this cation in the plant. After 2 to 3 weeks, when the root system had developed sufficiently, individual plants were transferred to 2 liters of the labelled nutrient solution. Each water culture contained 10 to 20 mc. of $Ca^{45}Cl_2$ (specific activity 55 to 66 mc./gm.) in 2 liters of a modified Hoagland's solution with the pH adjusted between 5.5 and 6 with 0.2 molar NaOH. The $Ca^{45}Cl_2$ was substituted for the usual $Ca(NO_3)_2$ and the potassium concentration was halved in order to increase the rate of upward movement of calcium (9). The solution for the P^{32} experiments contained about 10 mc. of carrier-free $P^{32}O_4$ in 2 liters of solution. The degree of labelling (counts/pollen grain/minute) was estimated by counting pollen on planchets in a gas-flow counter (Nuclear-Chicago Corporation, Chicago, model 186) equipped with an end window of mylar film.

The non-radioactive flowers were pollinated and the pollen tubes were allowed to grow to a length of 5 to 6 cm. The procedure for removing the pollen tubes from the styles is illustrated in Text-fig. 1. The stigma was severed in order to prevent radioactive debris on the stigmatic surface from entering the stylar canal. Another clean blade was used to open the style, then the pollen tubes were gently stripped away with watchmaker's forceps. The subsequent treatment depended upon the autoradiographic procedure to be employed.

When the Ilford G.5 nuclear emulsion plates (1 x 3 inches, 50 microns thick) were used, the pollen tubes were fixed 1 to 2 minutes in 70 per cent ethanol saturated with calcium carbonate (pH about 7.5) on microslides which had been coated with silicon grease dissolved in petroleum ether. The ends of the fixed pollen tubes were dispersed in deionized water on the sili-

conized slide and the tubes were transferred to the surface of the emulsion (presoaked in the ethanol fixative) by gently pressing the two slides together. The preparations were transferred to 95 per cent ethanol for 1 to 2 minutes, then were air-dried and stored for exposure at 1°C. over silica gel in desiccators. After varying exposure times, the preparations were developed and fixed in accordance with the standard photographic procedures and the slides were stained by Bergeron's (3) procedure.

When the stripping emulsion technique was used, the pollen tubes were fixed in neutral 70 per cent ethanol, dispersed in deionized water on subbed slides, and flattened with a coverslip. When the preparations were frozen in liquid nitrogen it was possible to remove the coverslips with a razor blade without disturbing the pollen tubes on the slide. The pollen tubes were allowed to thaw in 95 per cent ethanol, then the standard stripping film techniques were employed with the AR.10 emulsion (Kodak Limited, England). The preparations were processed and stained in the manner described above.

RESULTS

An exposure time of 1 to 2 weeks sufficed to produce good autoradiographs on stripping film (AR. 10, Kodak Limited) of the pollen which had been labelled with enriched Ca^{45}. The silver grains in the emulsion followed a pattern over the thick regions of the cell wall indicating that most of the Ca^{45} was bound in that area. These pollen grains had an activity of 0.5 to 0.9 counts per grain per minute. With pollen tubes, however, stripping film proved to be unsatisfactory for differentiating the relative radioactivities of nucleus and cytoplasm. 6 to 7 months were required to ascertain that nuclei were 5 to 10 times more radioactive than the cytoplasm, although the original pollen grains had activities like those above. To establish whether the failure to obtain good autoradiography was due to insensitivity of the emulsion or to low level retention of Ca^{45} in nuclei, P^{32} a well known label for chromosomes was used in a similar way. The degree of radioactivity obtained with carrier-free P^{32} was about 0.7 count/minute/pollen grain. With P^{32} an exposure of 3 or 4 weeks, or more than a half-life, was needed to obtain convincing autoradiographs of the nuclei in the pollen tubes. Since the half-life of Ca^{45} is 160 days and carrier-free calcium was not available in sufficient quantity, it was decided that thicker and more sensitive emulsions were better suited for studies on Ca^{45} retention. Of the several emulsions used (Kodak No-Screen, Kodak Limited AR.50, Ilford liquid (G.5, etc.) the Ilford nuclear research plate G.5 (50 microns thick) proved the most satisfactory. Figs. 1 to 4 illustrate typical results obtained with the pollen tubes after an exposure of 2 to 4 weeks. The pollen of the Croft variety was labelled when the buds were 15 to 20 mm. long; according to Ogur *et al.* (12) and Taylor (17) at this bud length the chromosomes in the microsporocytes are between the pachytene and tetrad stages. The activity of this pollen was between 0.5 and 0.9 count/minute/grain. These pollen tubes had grown 4 days in the non-radioactive styles and had reached a total length of 5 to 6 cm.

At this time each tube contains three nuclei, the tube nucleus and two haploid sperm nuclei. The tube nucleus, so called because it is generally regarded as involved in the growth, is located in the distal end of the tube. During the growth of the tube the generative nucleus, initially in prophase, undergoes a mitotic division which produces the two sperm nuclei. The two sperm nuclei are always proximal to the tube nucleus as the tube grows.

Since all of these nuclei retain Ca^{45} activity, it seems clear that the calcium is bound in a stable nuclear component, namely, the chromosomes.

DISCUSSION

The results indicate that Ca^{45} can form rather stable linkages within the chromosome, probably with the nucleoprotein. The nature of such linkages is still an open question. Although there are several possibilities, a number of studies suggest that divalent metals are bound preferentially by the phosphate groups of the DNA. The relative stability of complexes of calcium with polyphosphate and nucleotides has been discussed by Van Wazer and Callis (19) in a recent review. Divalent metals such as magnesium, calcium, manganese, or zinc are required for the formation of two- and three-stranded polyribonucleotides according to Felsenfeld and Rich (5); sodium is only $1/100$ as effective. Zubay (22) has shown that formaldehyde titration of DNA decreases the ratio of magnesium to phosphate from 0.7 to 0.39 indicating that in addition to phosphate the negative sites of guanine and cytosine also bind magnesium.

The amount of metal bound to the protein of the chromosome is unknown but some analogous systems suggest possibilities. Zittle *et al.* (21) have studied the aggregation of casein, orthophosphate, and calcium. The binding of phosphate to casein above pH 6 is accompanied by a large increase in calcium binding. This phenomenon seems to depend upon the ionization of the secondary

phosphate hydrogen. Waugh (20) has proposed a model for the orthophosphate-calcium-casein complex. In this model calcium forms cross-linkages between phosphates, the phosphates being attached to the casein. The studies of Ts'o (18) with microsomes led to the conclusion that 80 per cent of the divalent cations were bound to RNA and 20 per cent were bound to the protein; furthermore, metal removal with EDTA led to a fragmentation of the microsome. The DNA protein used by Frick (6) in reconstruction experiments contains about 35 per cent DNA and is calculated to have about one calcium for each eleven nucleotides.

The evidence is strongly suggestive that divalent cations particularly calcium and magnesium are bound in chromosomes to negative sites which probably are on the DNA. The energy of such bonds could be of the order of 4 to 4½ kcal./mole which is the value obtained by Nanninga (11) for magnesium and calcium complexes with ATP.

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EXPLANATION OF PLATES

PLATE 158

FIG. 1. Autoradiograph of tube nucleus in *Lilium* labelled with Ca^{45}. The end of pollen tube is located at *B*.

FIG. 2. Autoradiograph of the sperm nucleus. Non-radioactive cytoplasm (*CYT*) of another pollen tube is in contact with the emulsion and crosses under the sperm nucleus at *A*.

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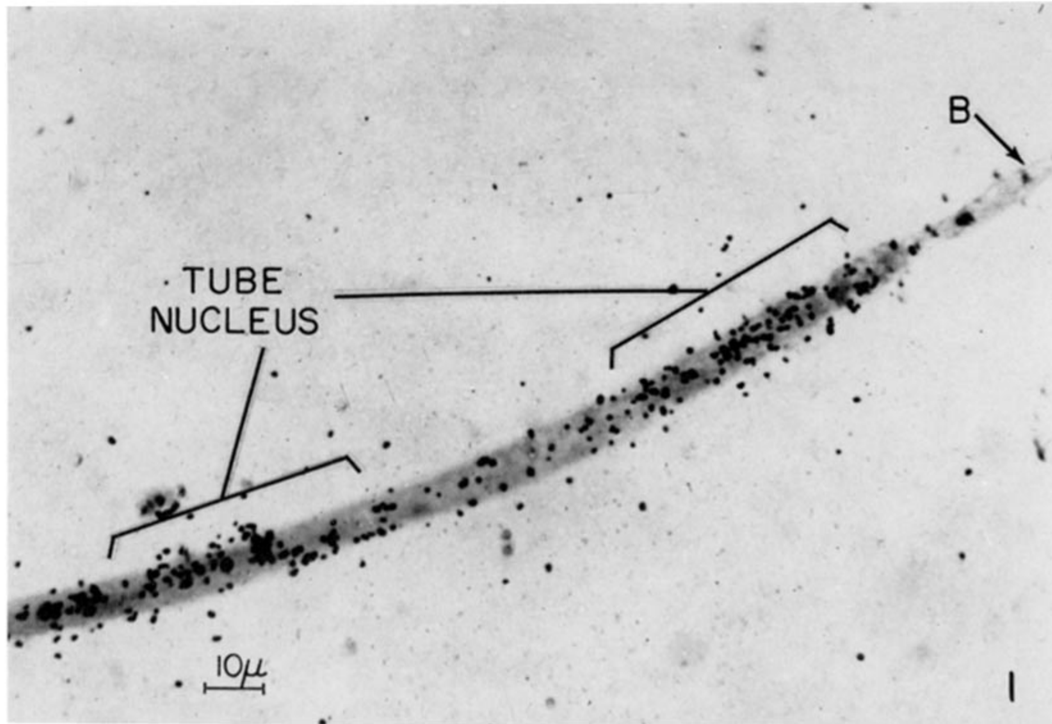
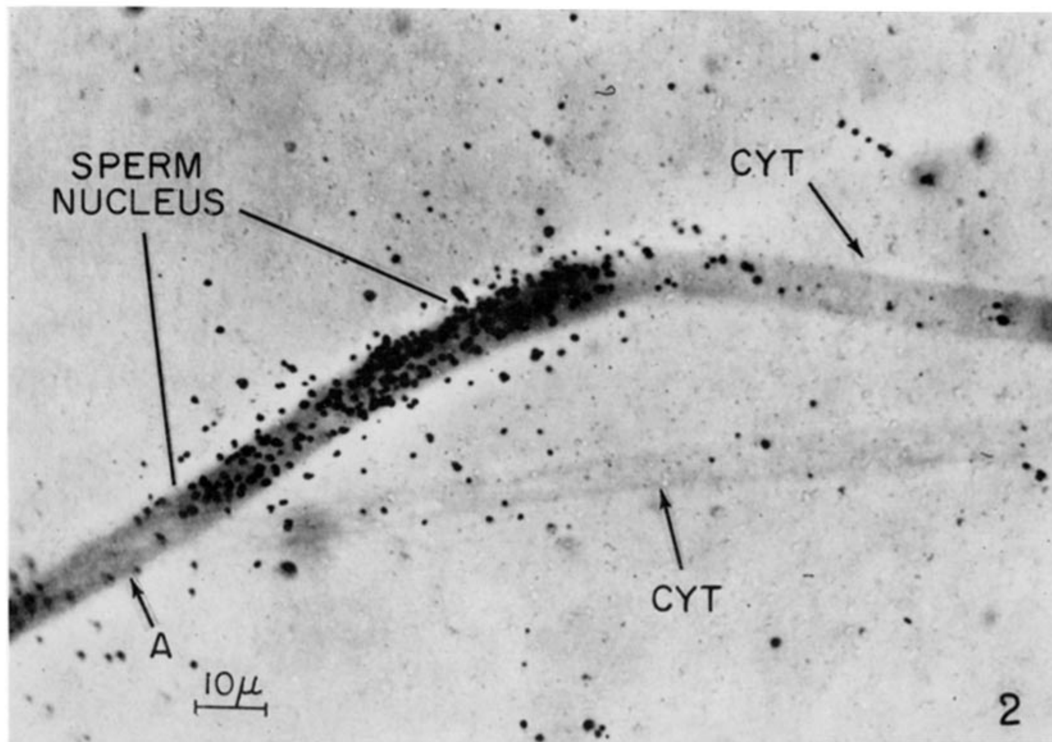


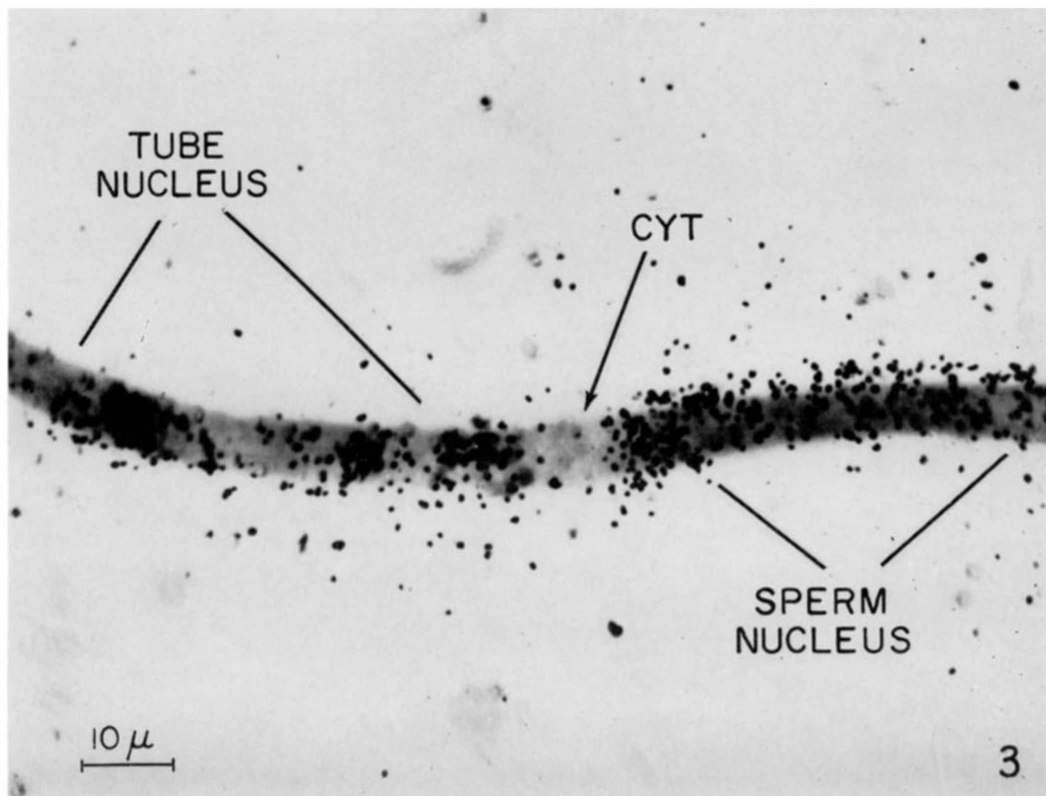


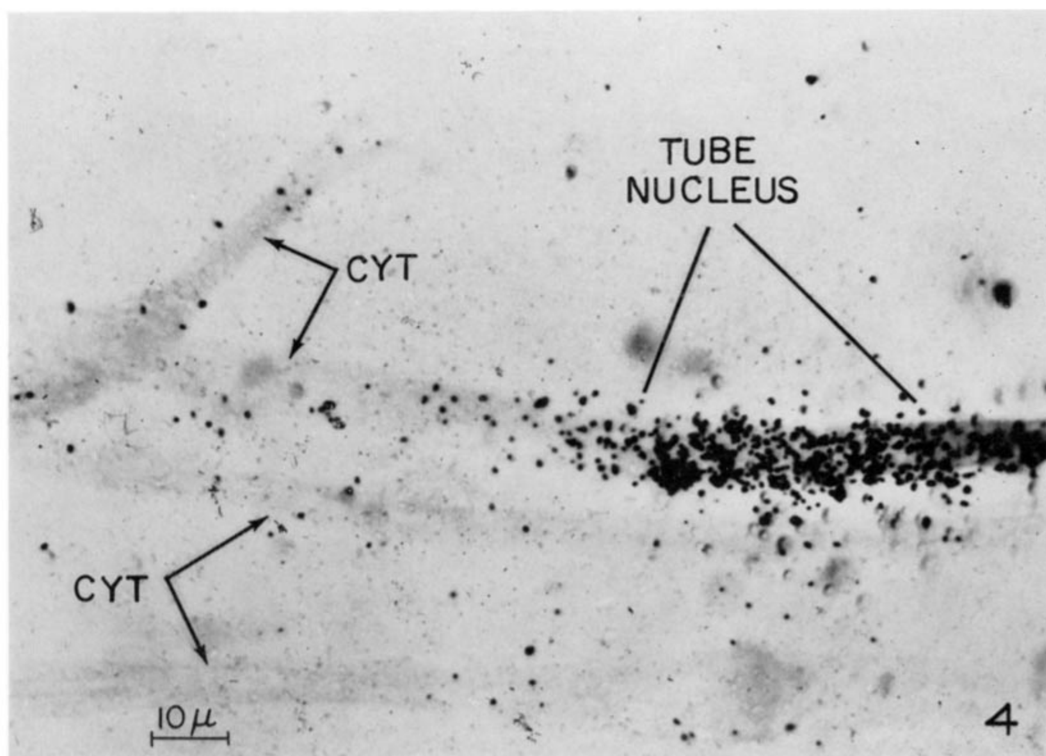
(Steffensen and Bergeron: Autoradiography with calcium-45)

PLATE 159

FIG. 3. A tube nucleus and a sperm nucleus labelled with Ca^{45}.

FIG. 4. A labelled tube nucleus. Note the unlabelled cytoplasm in the pollen tubes.





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