DEVELOPMENT OF AN AUTORADIOGRAPHIC TECHNIQUE FOR USE IN BOTANICAL INVESTIGATIONS

By

ORAN D. STEFFEY

Bachelor of Arts
Phillips University
Enid, Oklahoma
1948

Master of Science
Oklahoma Agricultural and Mechanical College
Stillwater, Oklahoma
1950

Submitted to the faculty of the Graduate School of
the Oklahoma Agricultural and Mechanical College
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
August, 1953

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Thesis Approved:

Thesis Adviser

Dean of the Graduate School

PREFACE

In the spring of 1950 work was begun on research involving the attenuation of the effects of x-rays and other irradiations upon sorghum seedlings. During the progress of this research it was felt that the autoradiographic technique might be used in tracing tagged elements intracellularly and intercellularly. It was hoped that the observations made on the autograms might be correlated with the effects of irradiation and attenuation. With this in mind the author set out to develop a technique which could be used with plant material. This thesis is the result of this research.

This work has been made possible through Atomic Energy Project No. A T (11-1) -71 No. 2 which was obtained by Dr. R. M. Chatters under the auspices of the U. S. Atomic Energy Commission and the Research Foundation of the Oklahoma Agricultural and Mechanical College.

I wish to take this opportunity to express my thanks to Dr. R. M. Chatters, my adviser, for having made possible the research and having given much help in the work; to Dr. W. W. Hansen, head of the Department of Botany and Plant Pathology, who made available the equipment of the department for my use; to Professor O. C. Schultz for serving on my committee and giving advice; to Dr. J. S. Brooks, of the Agronomy Department, for giving of his time and serving on my committee.

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INTRODUCTION

Autoradiography, or radioautography as found in some literature, is a method for detecting radioactive substances in type specimens such as minerals or plant and animal tissues. The basis of this technique is the ability of radioactive substances to affect silver bromide crystals of photographic emulsions in much the same manner as does light. From a biological standpoint an autoradiograph is obtained by placing a plant or animal tissue section, containing a radioactive element, in contact with a photographic emulsion, allowing for sufficient exposure time, and developing the emulsion in the same manner as in ordinary photography. Thus the resultant autoradiograph will consist of black silver grains corresponding to the areas of localization of the radioactive element in the tissue.

Historically the development of autoradiography may be traced back to the introduction of silver halide emulsions as a medium for recording optical images. Not long after this several investigators placed various materials and objects in contact with photographic emulsions attempting to produce an image without action of light. In 1896 Henri Becquerel (3) discovered that a developable image could be produced with uranium sulfate. As a result of Becquerel's experiments, the photographic emulsion became a useful tool in investigations of radioactivity in different mineral and biological specimens. Shortly after Becquerel's discovery,

London (16), in 1904, placed a frog in a hermetically sealed bottle with a solution of radium. After death the animal was placed on a photographic plate, and, after proper exposure and development of the plate, an image was obtained. This is thought to be the first published autoradiograph. Probably the first time autoradiography was used to a great extent in detection of radioisotopes in tissue was made by Lacassagne and Lattes (14), in 1924. They used polonium for the demonstration of localization within tissues, and since then the usefulness of this technique has been greatly expanded.

A Review of Autoradiographic Techniques

One will find throughout the literature a great amount of material dealing with autoradiography, most of which is on animal tissues. There has been only a small amount of work devoted to plant tissues. Techniques vary greatly, therefore it seems desirable to review the most important of these methods and cite a few of the workers who have employed them.

1. The Contact Method:

This method consists of placing a specimen containing a radioactive element upon a photographic emulsion and holding the two in close contact during the exposure time. Various techniques are employed. For example, many workers place the specimen in direct contact with the photographic emulsion while others separate the emulsion and specimen with thin layers of various materials. Another technique

is to mount the specimen on a microscope slide, coat the specimen with celloidin and place the slide with specimen side down in contact with the emulsion.

Employing x-ray film, the contact method was used by Arnon et al (2) in 1940 to produce autoradiographs of P^{32} in tomato fruits. In studying the development of the thyroid gland with I^{131}, Gorbman and Evans (12) also used x-ray film. X-ray and panatomic films were employed by Harrison et al (13) to show the distribution of sulfur in wheat. X-ray film was used by Mullins (20) while studying radiophosphate in the cells of Chara and Nitella, and also by Armstrong et al (1) in the study of radioactive carbon in rat teeth and kidney. In 1950, Buie et al (9) used a nuclear track emulsion for studying the distribution and concentration of radioisotopes in bone.

The contact method has the advantage of simplicity and is quite adaptable to quick routine work when one wishes to gain knowledge of the general areas of localization of a radioactive element within tissues. Poor resolution and difficulty in comparing the specimen and the autograph under the microscope are disadvantages of this procedure.

When speaking of resolution, reference is to lateral and/or vertical resolution. Lateral resolution refers to the distance between discrete particles necessary to form separate and distinct images. Vertical resolution has been used to indicate depth of field; it refers to the vertical distance through which a microscope objective will form acceptable sharp images.

2. The Mounted Method:

In this method, the section is mounted directly on the photographic emulsion. Different types of techniques are employed in mounting the section on the emulsion and a number of various kinds of emulsions on plates are available. Eastman Kodak NTB experimental plates were used by Boyd et al (8) to make autoradiographs of single blood cells which contained C^{14}. These blood cells were smeared directly upon the emulsion. Endicott and Yagoda (10) floated tissue onto Eastman Alpha Particle plates in the quantitative microscopic study of tissue and cellular distribution of alpha emitting radioactive elements. Evans (11) has described the mounting of tissues directly onto lantern slides and other photographic emulsions. A method of autoradiography wherein the biological specimen was placed directly onto a photographic plate was described by Low-Beer (17) in 1950.

Whereas the advantages of the mounted method are simplicity and good resolution there are certain disadvantages which have been encountered: for example, (1) failure of the developer to penetrate the tissue uniformly may leave some areas undeveloped which have actually been exposed. Also, (2) the gelatin of the emulsion has the tendency to absorb stain with some tissue dyes thus obscuring the autograph to some degree. When there is stain in the emulsion, it is also difficult to produce photomicrographs of the autograph since many times there is insufficient contrast in the tissue to record it on the film. (3) Blackening may occur due to chemical action of tissue or to such

solutions as toluene or xylene.

3. The Coating Method:

Here, the tissue section is covered with either a fluid emulsion or a stripping emulsion. A number of different emulsions both in type and form are available for use.

In using fluid emulsion, it is melted and then a small quantity is applied to celloidin-covered sections with an eyedropper. It is then smoothed out with a camel's hair brush. Emulsion from Eastman Kodak medium contrast lantern slides was used by Belanger and Leblond (5) to coat histological sections. In studying localization of radioiodine in stained sections of thyroid gland, Leblond, Percival and Gross (15) in 1948 also employed Kodak medium contrast lantern slide emulsion. Another type of emulsion which may be used in the fluid technique is Ilford G-5. This was employed by Blundell and Rotblat (6) in 1951 for coating biological specimens in track autoradiography.

When stripping film is used, the sections are covered with a strip of film which is peeled from a base and transferred to the slide which bears celloidin-covered sections. Ilford half tone stripping plates, Kodak NTB_1 stripping film and NTB_2 stripping plates were employed by Bogoroch (7) in 1951 for studies of strip-coating technique. Mac Donald et al (19) in 1949 described a technique for coating slides with stripping film.

The advantage of the coating method is found in the great number of slides which may be processed at a time, especially with liquid emulsion. The thickness may be

fairly well controlled with liquid emulsion and various dilutions are possible. One fault of the coating technique may be found in the need for sections to be stained before coating. If one is working with a radioactive element which may be displaced from the tissues by the staining procedure, the preparation must be autographed unstained. However, non-interfering staining methods are possible and may be used in some cases.

4. Inverting Method:

In the inverting method, the section and emulsion are completely inverted. Usually Kodak matrix emulsion is employed to coat the celloidin-covered section which is unstained. After exposure and development, the slide is placed under water and the emulsion-tissue complex is lifted from the slide with a razor blade. The complex is then inverted and fixed to a clean slide so that the tissue section is on top of the emulsion. The tissue then may be stained and a cover slip applied. The inverted autoradiographic method was described by Belanger (4) in 1950. Mac Donald et al (18) in 1948 published an autoradiographic technique using stripping film to produce inverted autoradiographs.

The advantage of this method lies in the fact that the tissue sections may be stained after the autograph has been made and at the same time the celloidin protects the emulsion from the stains used. Probably a disadvantage is the time needed for manipulation which lengthens the process and limits the number of slides produced.

MATERIALS AND METHODS

Day Lily (Hemerocallis spp.) and Snapdragon (Antirrhinum spp.) were used for experimental plants. The plants were grown in outdoor flower beds on the Oklahoma A & M College campus. Flower bearing stems were cut off 6 to 12 inches in length, placed in wet toweling and brought to the laboratory where they were placed in tap water. The stems were trimmed to 5 to 7 cm. in length and placed in a solution containing 1000 lambdas of radioactive sulfur[1] (S^{35}) in 100 ml. of tap water. The activity of the solution was found to be about 1,584 cpm. per lambda (1/1000 ml.). Plant parts were allowed to remain in the radioactive solution from 24 to 72 hours after which they were removed for dissection. The flower of the Day Lily was dissected and ovary, style, anther and portions of the filament, petal and stem were killed and fixed in a solution of 100% ethyl alcohol saturated with picric acid. The plant parts were allowed to remain in the killing solution not less than 2½ hours. They were then placed in 100% normal butyl alcohol for about 2 hours, infiltrated with paraffin[2] by the butyl alcohol technique and embedded in a paraffin-beeswax mixture (120 gms. paraffin and 15 gms. beeswax). The tissue

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[1]The S^{35} was in the form of SO_4 in weak HCL.

[2]A commercial paraffin, "Texwax," manufactured by the Texas Co. was used.

was cut 12 to 15 microns on a rotary microtome. The Snapdragon was treated in the same manner, except no part of the petal was taken.

The autoradiographic procedure employed is a modification of the coating method which was developed for use with plant material by the writer. Ansco Radioautographic Emulsion A was used for coating the tissue. The following steps which have been found to be necessary in this procedure are taken up in order:

Preparation of Slides for Coating

1. Microscope slides were marked, cleaned in chrome-sulfuric acid cleaning solution[1] and dipped in warm water containing a synthetic detergent (alkyl aryl sulfonate). They were then rinsed thoroughly in hot water and placed in distilled water.

2. Slides were dipped in a subbing solution[2] and placed in glass staining racks to dry.

3. Paraffin ribbons containing plant material were floated in 4% formalin and heated to smooth the film after which the excess fluid was removed. The slides were then placed in glass staining racks to dry overnight.

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[1] Water . . . . . 300 ml.
Potassium dichromate. . . . . 60 gm.
Sulfuric acid (conc.) . . . . . 460 ml.

[2] Gelatin . . . . . 5.0 gm.
Chrom Alum. . . . . 0.5 gm.
Phenol (anhydrous). . . . . 10.0 gm.
Water (distilled) to make . . . . . 1000 ml.

After drying, the slides were processed as follows:

Xylene I	5 min.
Xylene II	3 min.
Xylene III	3 min.
100% Ethyl Alcohol I	1 min.
100% Ethyl Alcohol II	1 min.
100% Ethyl Alcohol III	1 min.
Stain (either 1% Safranin O in 95% ethyl alcohol or 1% Fast Green FCF in 100% ethyl alcohol)	3 to 15 min.
100% Ethyl Alcohol, to destain	1 min.
100% Ethyl Alcohol	1 min.
Celloidin (1%)	1 min.
Drain	10 to 15 sec.
70% Ethyl Alcohol, to harden	1 min.

After 70% ethyl alcohol, the slides were placed in glass staining racks and allowed to dry thoroughly.

Coating Slides with Emulsion

1. A coplin jar was placed in a 600 ml. beaker and both were filled with water. A 50 ml. beaker was fitted into the mouth of the coplin jar and held in place by two wire hooks secured to the walls of the jar by a rubber band wrapped about them, (Fig. 1). This device was placed on a slide warming table in the darkroom and the water was brought to a temperature of about 37° C.

2. Using a Wratten Series 1 Safelight with a 15 watt bulb at a distance of about 3 feet, the desired amount of Ansco Radioautographic Emulsion A was placed in the 50 ml. beaker and allowed to melt.

Fig. 1. Apparatus used for melting the emulsion prior to coating slides.

Fig. 2. Dark box used for storing slides during exposure time.

3. After the emulsion was completely melted, slides were coated by placing by eyedropper one drop of the emulsion to about every 2.5 sq. cm. of area on the slide. This was smoothed out with a small camel's hair brush which was kept in the emulsion along with the dropper. Before coating, the slides were preheated to about 40° C on a level glass plate heated at one end by the slide warming table.

4. After coating, the slides were returned to the level glass plate and momentarily warmed to allow the emulsion to further spread evenly. After this they were moved to a cool, level glass plate and cooled until reset. Resetting was aided by a small electric fan.

5. All slides were placed in dark plastic slide boxes which contained a small amount of anhydrous calcium chloride wrapped in cheese cloth which served as a dessicant (Fig. 2). The top and bottom of the boxes were taped together with adhesive tape to keep out external moisture. During the exposure time, they were stored in a refrigerator near the freezing compartment and in such a position so that all the slides would rest horizontally in the boxes.

Developing, Clearing, and Washing Slides

After exposure, the slides were placed in glass staining racks and held in a horizontal position by a rubber band placed around each rack of slides. Each rack of slides was taken through the solutions which were contained in pyrex baking dishes (6 3/4 in. x 3 3/4 in. x 2 1/4 in.). The temperature of the solutions was about 18° C to 20° C.

A Wratten Series 1 Safelight with a 15 watt bulb was used at a distance of about 2 feet. The developing schedule used is as follows:

D-72	1 to 2 min.
Acid stop bath (1½ oz. of a 28% solution of glacial acetic acid per 32 oz. of water)	1 min.
Kodak Acid Fixer	10 to 15 min.
Wash in running water	1 hr.

Dehydration

After the slides were washed they were carried horizontally through the following solutions contained in petri dishes:

95% Ethyl Alcohol	3 min.
100% Ethyl Alcohol	3 min.
100% Ethyl Alcohol and Xylene (50-50)	3 min.
Xylene I	3 min.
Xylene II	2 min.
Xylene III	1 min.

At this point in the schedule, the slides were placed in coplin jars containing xylene and allowed to clear for at least 1 hour after which they were mounted in balsam.

Most of the slides were studied with a Spencer Phase Microscope using a 20x, B minus L objective and a 15x widefield eyepiece. A few of the slides were studied with a Bausch and Lomb research microscope at magnifications of 150x and 675x using widefield eyepieces. Photomicrographs were taken of autoradiographic and histological slides,

using both the phase and standard research microscopes with a Leitz Micam photographic attachment.

Exposure time was determined by preparing a number of slides and developing a few at different times until a correct exposure was obtained. One experiment was run to gain a general idea of exposure time in relation to energy contained in sections.

OBSERVATIONS AND RESULTS

Day Lily and Snapdragon were found to be quite suitable for the experimental work. The parts of Day Lily flower are quite simple in structure and were easily dissected out. The floral parts of Snapdragon were not particularly difficult to remove, but since some of the organs are zygomorphic it was found that great care was often necessary when orienting the paraffin block on the microtome prior to cutting the tissue. The radioactive sulfur was taken up by the plants in a most satisfactory manner. Since sulfur 35 has a half life of 87.1 days, plants containing the isotope could be used at some later date for experimentation.

Since it was desirable to reduce leaching of the isotope, it was found that 100% ethyl alcohol saturated with picric acid served quite satisfactorily as a killing and fixing solution. This solution not only acted rapidly, but it was possible to go directly into 100% normal butyl alcohol and then into paraffin for infiltration. Thus the whole process of killing, fixing, and infiltration of the tissue was shortened. A mixture of paraffin and beeswax was found best in which to embed the tissue since temperatures were quite high in the laboratory at times making microtoming difficult when paraffin alone was used. Thicknesses from 12 to 15 microns were found best for the various types of tissues used since not only autoradiographs of the tissues

were of interest, but also observations at cellular levels were desired. If sections were microtomed less than 10 microns thick, many of the cells were, for the purpose of this research, of little value.

Thorough cleaning of the slides was found to be very important. Any trace of grease or oil made it impossible to obtain the desired results. Cleaning the slides in concentrated chrome-sulfuric acid cleaning solution, washing in warm water containing a synthetic detergent (alkyl aryl sulfonate) followed by rinsing in hot water removed any grease or oils. Placing the slides in distilled water seemed to aid in reducing surface tension when going into the subbing solution and also guarded against a great amount of impurities which might have been found in tap water. In earlier experiments the celloidin was found to become loosened from the slide and consequently there was a tendency for the celloidin-emulsion complex to be lost completely or for buckling to occur. To overcome this difficulty the slides were dipped in a subbing solution before the tissue was fixed to the slides. The tissue was floated on the slides in 4% formalin and the subbing solution acted as an adhesive. After the sections were flattened by heating the slide and the formalin was drained off, the sections were further affixed to the slide by gently rolling the forefinger over them. It was observed that by using this technique along with allowing the slides to dry overnight under dustfree conditions, the possibility of the tissue coming loose was reduced.

All paraffin had to be removed from both tissue and slide, otherwise the celloidin did not adhere completely and in the final preparation the tissue could not be properly studied. All staining of the tissue was done in ethyl alcohol since it was felt that staining in an aqueous solution would remove a great amount of the isotope. While some tissues retained stain with difficulty, others did not. A 1% celloidin solution was found best in which to dip the slides since concentrations greater than this caused areas of uneven thickness. It was also found that it was best to make up a new solution of celloidin for each new batch of slides. After dipping the slides in the celloidin solution it was necessary that they be drained sufficiently before they were placed in 70% ethyl alcohol or uneven areas were formed. About one minute in 70% ethyl alcohol was found sufficient to harden the celloidin. After hardening, the slides were allowed to dry overnight. If sufficient time was not given for the celloidin to dry, it tended to loosen in the developing and washing process.

In coating the slides with emulsion it was found that certain conditions had to prevail throughout the process. A temperature of between 37° C and 40° C was found best for melting the emulsion. If the temperature was allowed to rise above 40° C there was a great likelihood that background or fog would be built up in the emulsion. It may be said that background or fog is the presence in the emulsion of black granules due to factors other than irradiation. The emulsion was transferred from its container to the melting

apparatus with a clean stainless steel teaspoon. The emulsion was not broken up or stirred while melting since this might provoke the development of background and fogging. Sufficient time was allowed for the emulsion to completely melt so that there would be no contained bubbles or lumps when it was applied to the slides. The slides were pre-heated to about 40° C before coating so that the emulsion would flow more evenly over the surface. It was found that about one drop of emulsion to every 2.5 sq. cm. of slide surface was the most desirable amount with which to coat the slides. If too much emulsion was used, the thickness became so great that poor resolution was obtained in the finished slide. There was also more chance for bubbles and irregularities to form in the emulsion. Care was essential when emulsion was expelled from the dropper onto the slide else there was danger of the introduction of bubbles. The emulsion was smoothed out with a small camel's hair brush using long, even strokes. The strokes were kept at a minimum so that less background would be produced. Any bubbles formed were carefully worked out. It was found that the emulsion reset quite well if the slides were laid out on a large glass plate. A small electric fan was placed so that it would blow fairly evenly upon the plate and the air would sweep the slides. It was observed that if the emulsion was not thoroughly reset it would swell, bubbles would form in it during developing and washing, and the whole would tend to loosen and come off the slide. About 15 to 20 minutes was required for the emulsion to reset. During the entire

coating procedure a Kodak Wratten Series 1 safelamp was used at a distance of about 3 feet. It was felt best to work at this distance since there was less possibility of the emulsion fogging.

Dark plastic slide boxes were found best in which to store the slides during exposure. These boxes were easily cleaned and did not contain wood fibers which might have become entrapped in the emulsion. The plastic boxes had the advantage of not absorbing moisture and were easily stored since they were smaller in size than the wooden boxes. It was found desirable to wrap some calcium chloride in a small piece of cheese cloth and place it in one end of the slide box to serve as a dessicant. This was separated from the other slides by a blank slide. It was observed that by taping the top to the bottom of the slide box, the external moisture which might enter was kept at a minimum.

It was felt best to store the slides in a refrigerator near the freezing compartment since the background was kept lower under cold conditions. The temperature of the air surrounding the slides during exposure was about 0° C. The boxes were placed so that the slides would rest horizontally and there would be less chance for any creeping of the emulsion.

During the developing process it was found best to maintain the solutions at a temperature of between 18° C and 20° C. At higher temperatures, background greatly increased and under extremely high temperatures the emulsion swelled

or melted and came loose. Throughout developing, fixing, washing and dehydrating, the slides were all kept horizontal since there was great possibility for creeping or flowing of the emulsion even a few microns which might have rendered the finished autographs useless. It was found essential to have all chemicals used in the developing and clearing process completely in solution. Crystals were found to be deposited in some slides if the chemicals used were not properly mixed or were allowed to crystallize from solution (Fig. 38). While it was felt that the slides should be fixed in acid fixer for as short a time as possible in order that the latent image would not be reduced, it was found that if slides were not fixed long enough a white milkiness would form in the emulsion sometime after the coverslip was added (Fig. 33). As with most other emulsions, a general rule of fixing a minimum time of twice as long as it takes to clear the emulsion should be followed. It was found that washing the slides in water about 20° C for at least 1 hour was best. All the acid fixer needed to be removed otherwise there was a possibility of the latent image eventually being reduced. Washing was done in a container where there was constant turnover of water flowing from a tap fitted with a rubber tube. The temperature of the washing water was not allowed to rise because it was found that the emulsion would swell and come loose.

After washing, enough time was needed for dehydration of the emulsion since any water left caused the final

autoradiographic preparation to appear hazy under the microscope and poor resolution was the result. It was necessary for all slides to be kept horizontal again throughout dehydration to reduce creeping or flowing of the emulsion. After dehydration, it was apparently safe to place the slides in coplin jars of xylene to clear. While it was felt that clearing in xylene for at least one hour was essential, allowing the slides to remain overnight was best.

One experiment was run to gain information on exposure time in relation to energy emitted by the isotope contained in tissue sections. The data are shown in Table 1. All slides were processed at the end of a 7 day exposure period and showed latent image formation proportional to the energy contained in tissue sections of the respective size as indicated in the table. This does not show the minimum energy necessary to produce an autograph, nor does it set any strict limits in exposure time.

Autoradiographic Results

In Day Lily, the isotope was taken up through the vascular tissues and distributed throughout the plant. In the ovary, each vascular strand carried great quantities of sulfur 35. While in the parenchyma tissues the isotope often appeared to be uniformly distributed within individual cells, it was observed that the S^{35} exposed emulsion granules were concentrated at the periphery of the cells (Figs. 3, 5). In the ovary wall there was great uptake in the vascular tissues and a heavy concentration in the

Table 1.

Energy emitted by the isotope contained in tissue and its relationship to exposure time.

Snapdragon Tissue	No. Sect. per slide	cpm. per slide	Average cpm. per section	Vol. of section in cu. mm.	Latent Image Resultant
Stem X sect.	24	246	10	0.045	Well Formed
Stem L sect.	12	137	12	0.045	Well Formed
Ovary X sect.	10	222	19	0.105	Well Formed
Ovary L sect.	10	535	54	0.195	Well Formed
Style X sect.	12	0	0	0.092	Not Formed
Anther X sect.	17	240	14	0.034	Well Formed

epidermal portion and in the tissue lining the locule (Fig. 9). In the ovule even though there was some isotope in peripheral areas, there was generally only a small amount of the radiochemical taken up by this organ. General distribution was indicated in most of the parenchyma tissues, greater uptake by the three vascular strands and localization in the cells surrounding the lacuna of the style (Fig. 11). The stamen filament showed isotope uptake through the two vascular strands and uniform distribution in the parenchyma tissue (Figs. 13, 15). There was also a great concentration in the epidermal tissue of the filament, particularly in the uppermost portion lying in the anther sacs (Fig. 15). The tissue in the anther sacs took up the isotope and a large amount was present in the pollen grains (Figs. 17, 19). Along with uptake in the pollen grains, there was no doubt adsorption of the isotope on the exine of the grains. While in the petal a large amount of isotope was present in the vascular tissues and epidermis there were smaller but yet evenly distributed quantities in the parenchyma tissues (Fig. 21).

In the stem of Snapdragon there was a heavy uptake of isotope in all the vascular tissues (Figs. 23, 25). The isotope was found in the cortex of the stem and the epidermis showed heavy localization especially in the epidermal glandular hairs (Figs. 29, 31). The parenchyma tissues of the pith indicated that the isotope was distributed in the peripheral region along the cell walls (Figs. 25, 27).

Uptake of the isotope by the ovary wall, ovules, vascular tissue of the placenta, filament, anther, and style was similar to that observed in Day Lily.

Since the experimental plants were placed with their cut stems in the radioactive sulfur, a great amount of the isotope was in a transient state at the time samples were collected for study.

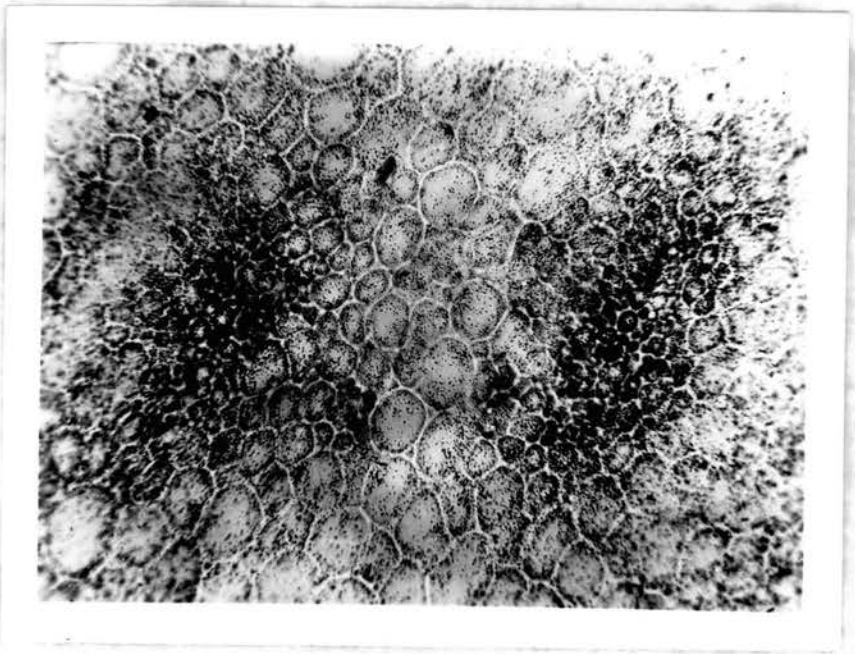

Fig. 3. Autoradiograph of a cross section from the lower portion of Day Lily ovary. Phase 300x.

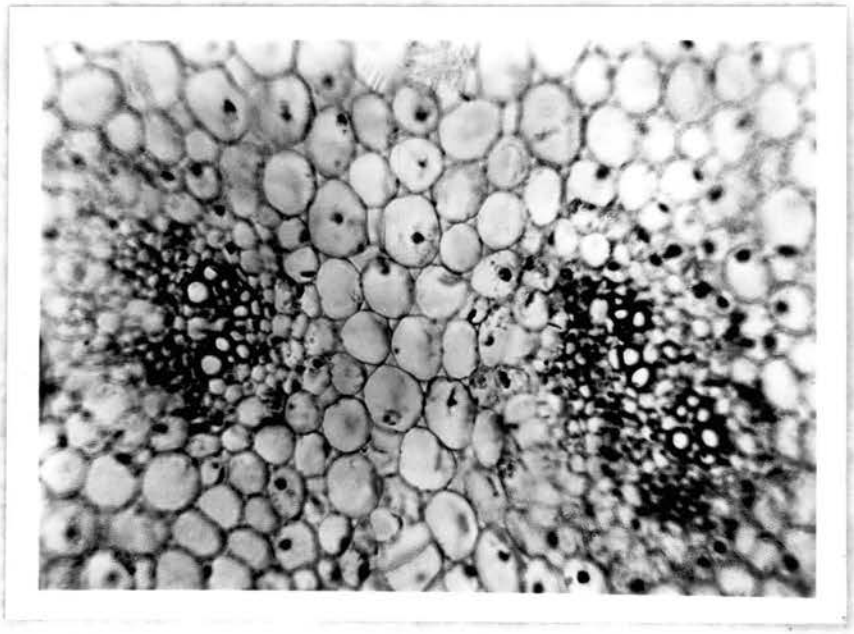

Fig. 4. Histological cross section from the lower portion of Day Lily ovary. Phase 300x.

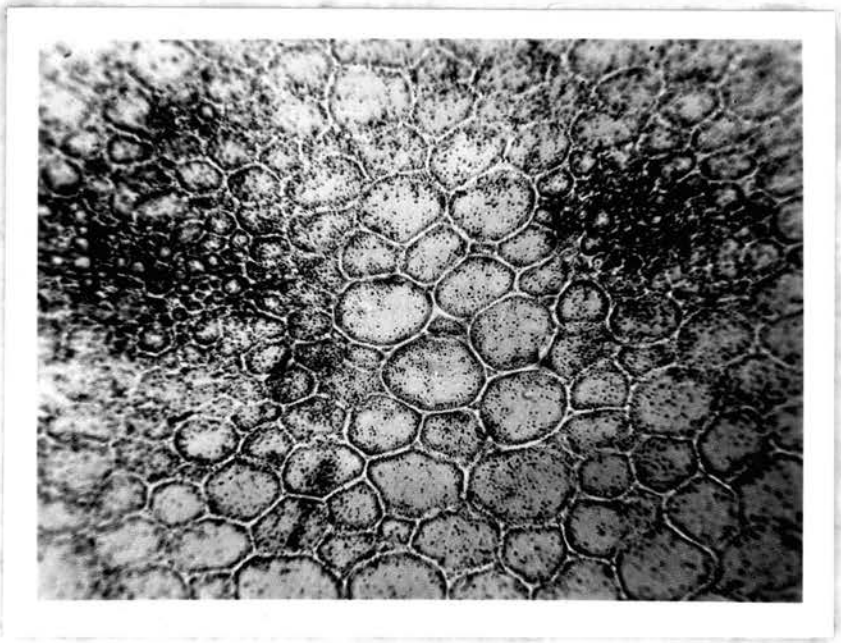

Fig. 5. Autoradiograph of a cross section from the lower portion of Day Lily ovary. Phase 300x.

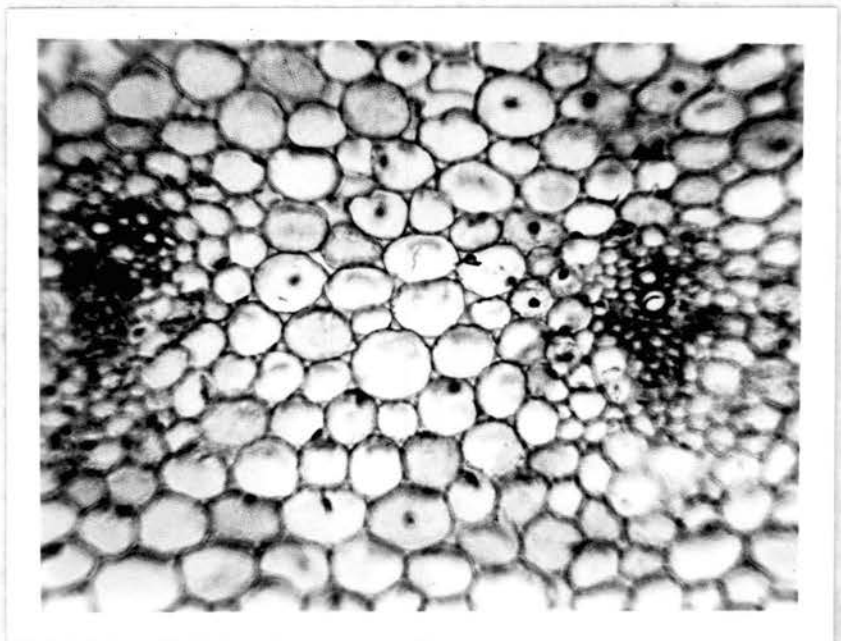

Fig. 6. Histological cross section from the lower portion of Day Lily ovary. Phase 300x.

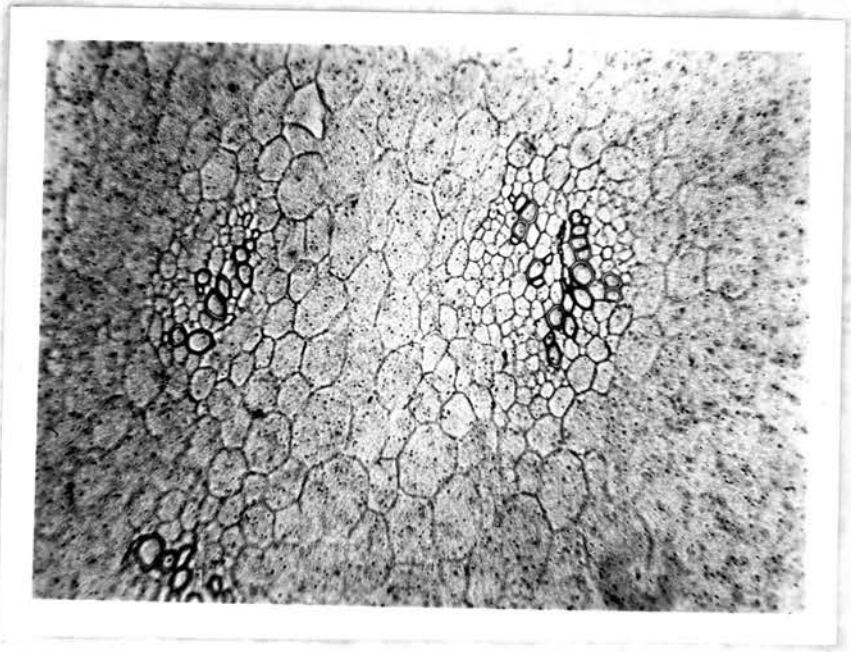

Fig. 7. Control cross section from the lower portion of Day Lily ovary. Phase 300x.

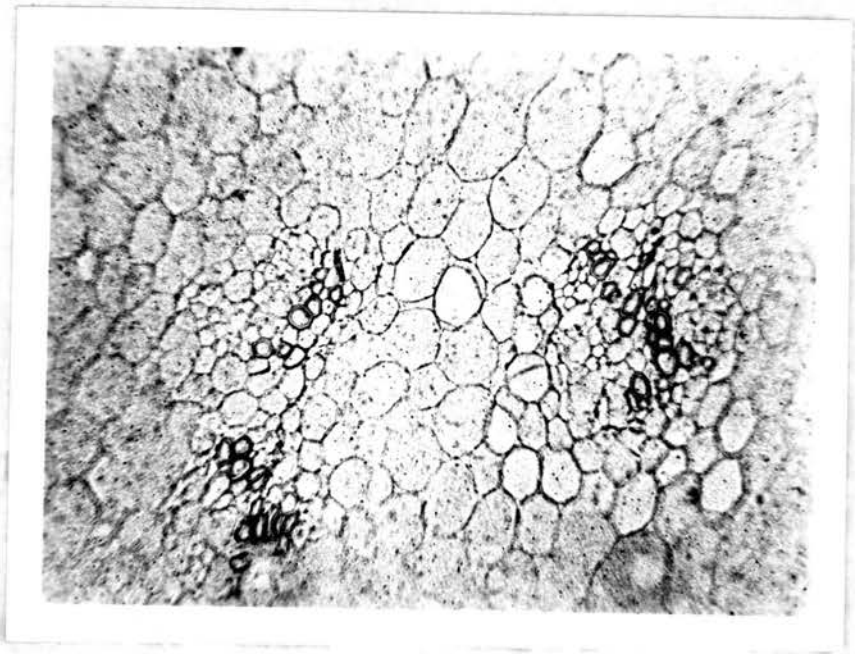

Fig. 8. Control cross section from the lower portion of Day Lily ovary. Phase 300x.

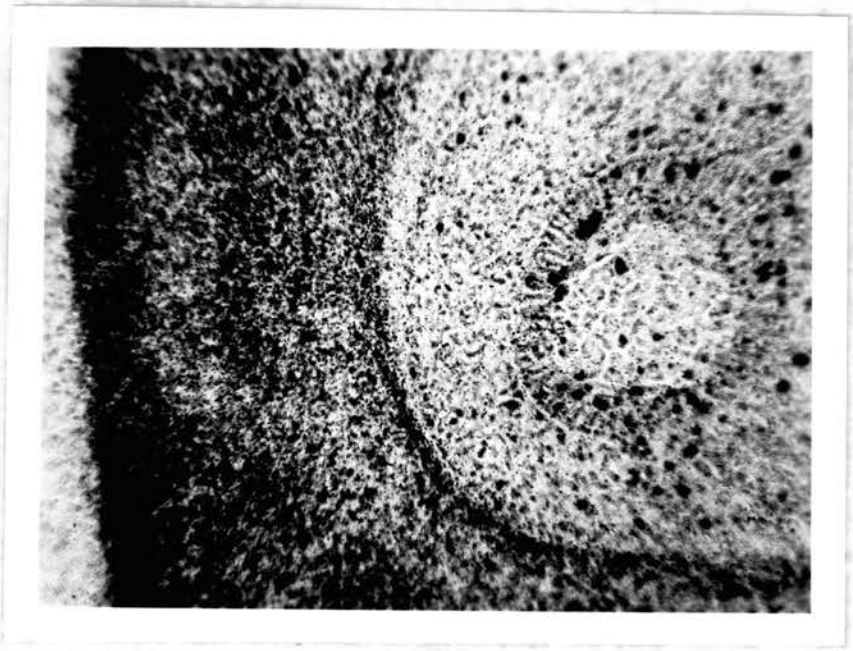

Fig. 9. Autoradiograph of the cross section of Day Lily ovary and ovule. Phase 300x.

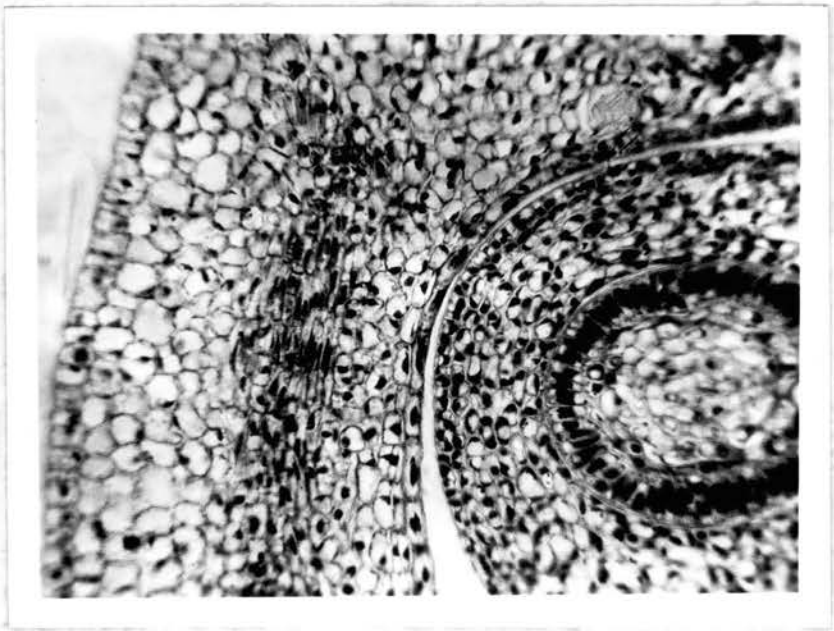

Fig. 10. Histological cross section of Day Lily ovary and ovule. Phase 300x.

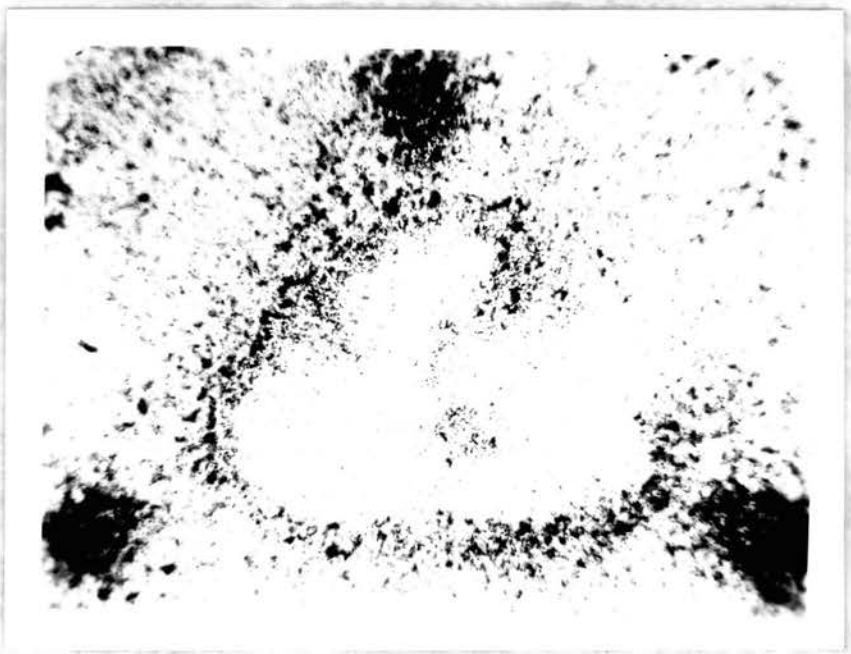

Fig. 11. Autoradiograph of the cross section of Day Lily style. Phase 300x.

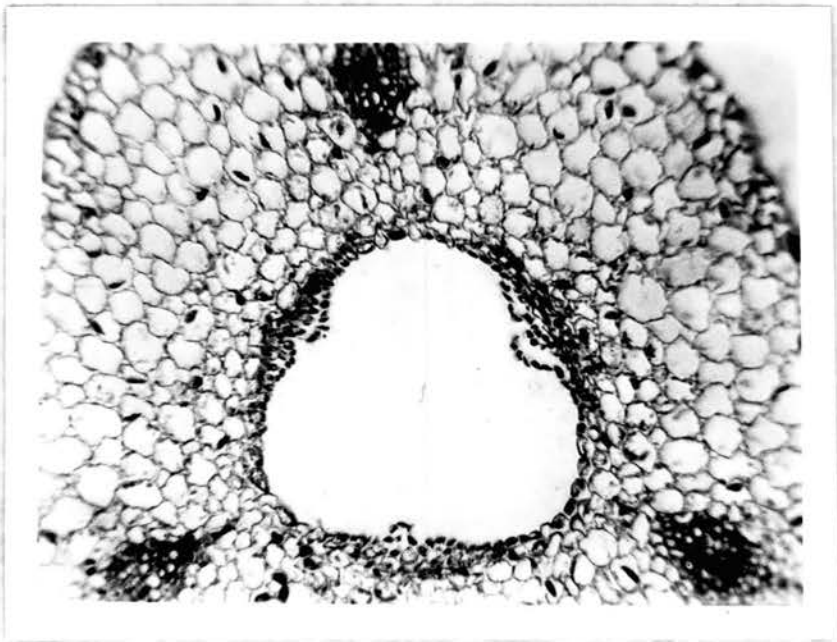

Fig. 12. Histological cross section of Day Lily style. Phase 300x.

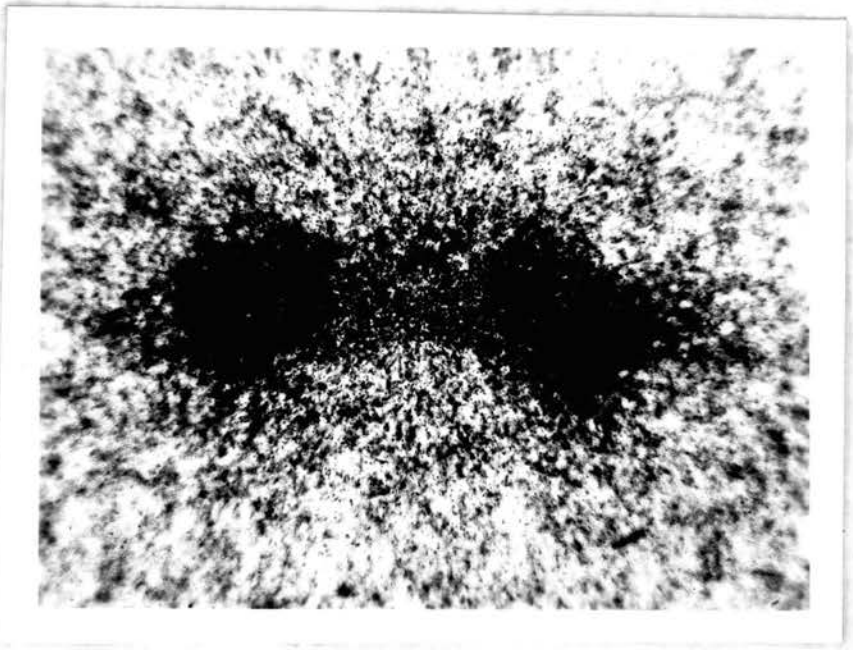

Fig. 13. Autoradiograph of the cross section of Day Lily filament. Phase 300x.

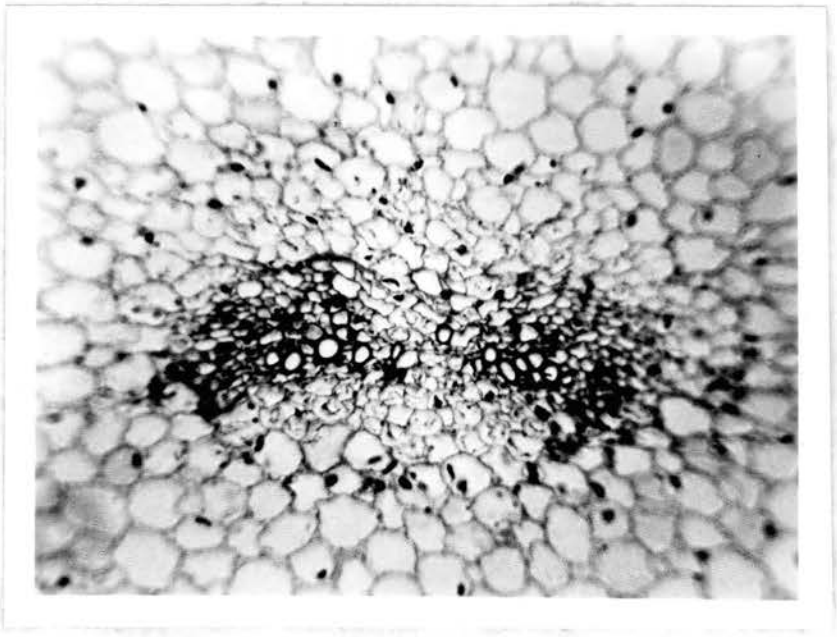

Fig. 14. Histological cross section of Day Lily filament. Phase 300x.

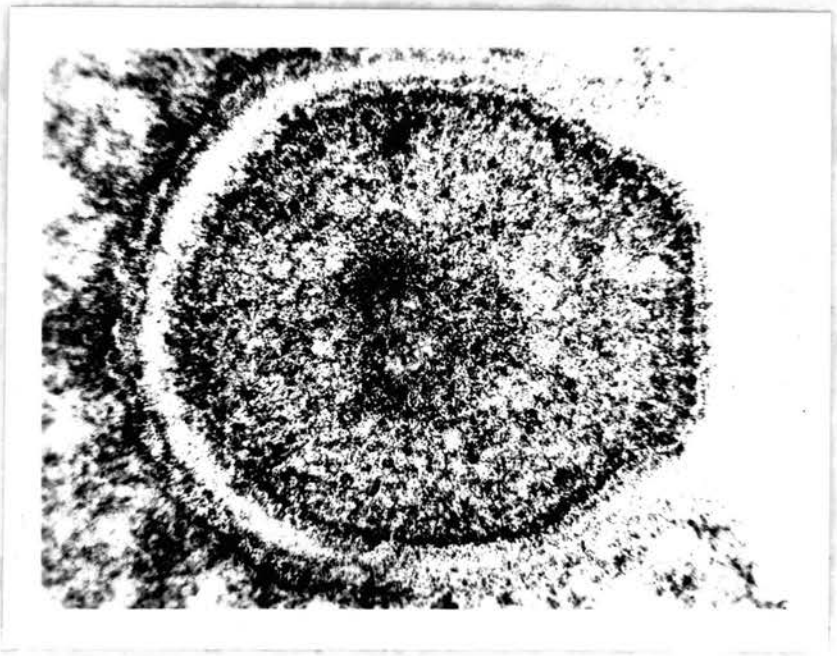

Fig. 15. Autoradiograph of the cross section of Day Lily anther and filament. Phase 300x.

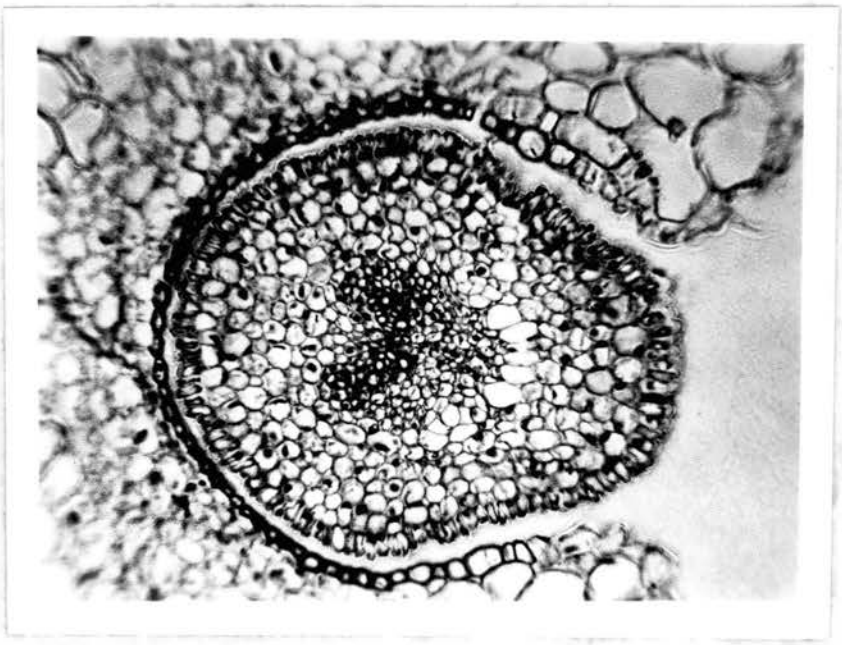

Fig. 16. Histological cross section of Day Lily anther and filament. Phase 300x.

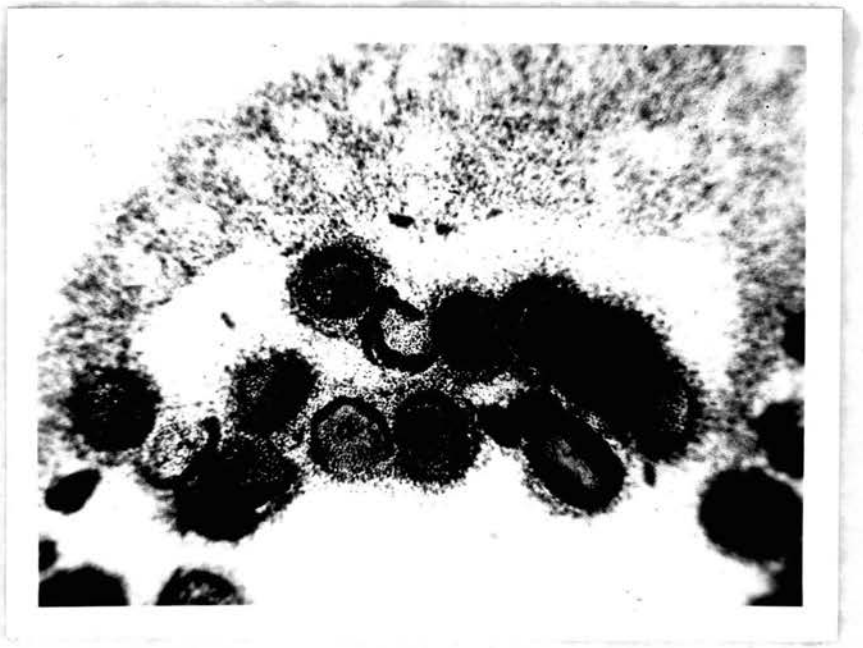

Fig. 17. Autoradiograph of the cross section of Day Lily anther showing pollen grains. Phase 300x.

Fig. 18. Histological cross section of Day Lily anther showing pollen grains. Phase 300x.

Fig. 19. Autoradiograph of the cross section of Day Lily anther showing pollen grains. Phase 300x.

Fig. 20. Histological cross section of Day Lily anther showing pollen grains. Phase 300x.

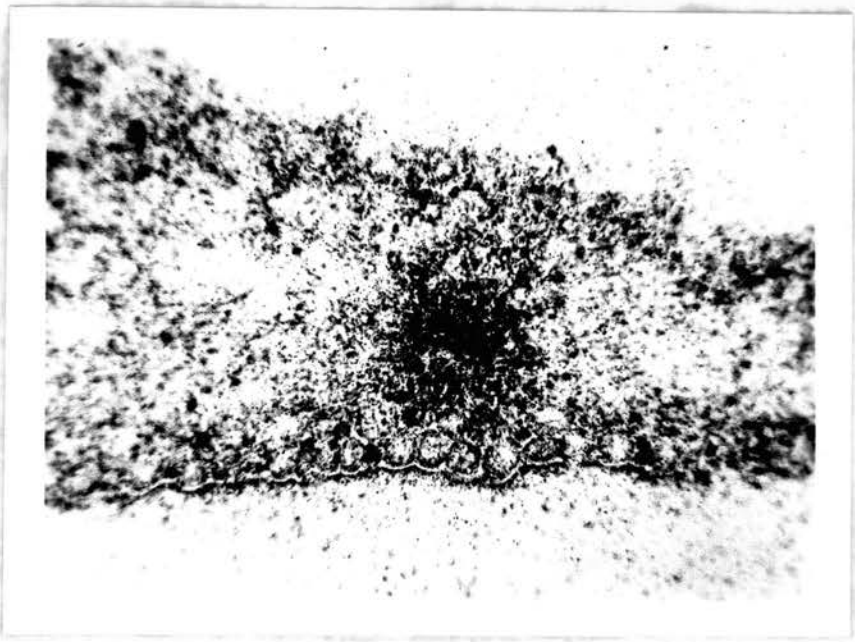

Fig. 21. Autoradiograph of the cross section of Day Lily petal. Phase 300x.

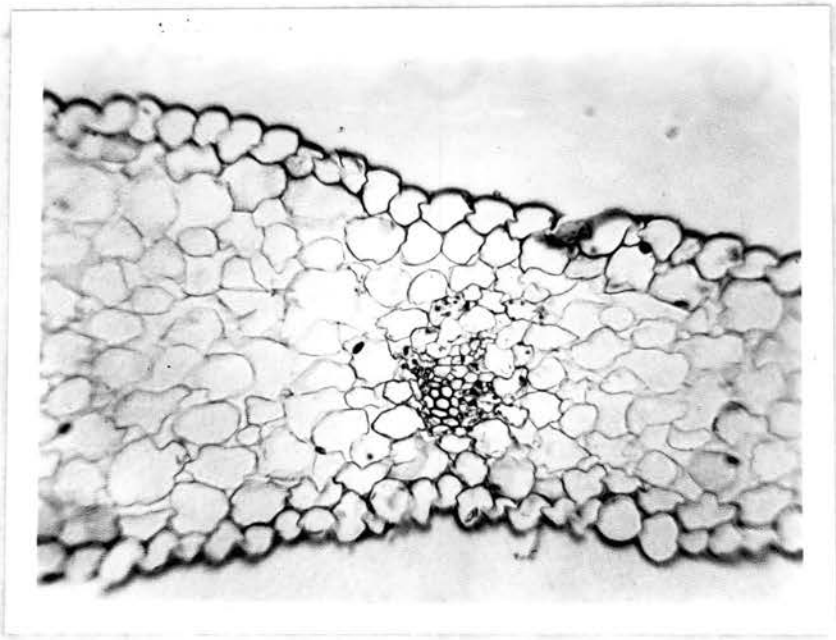

Fig. 22. Histological cross section of Day Lily petal. Phase 300x.

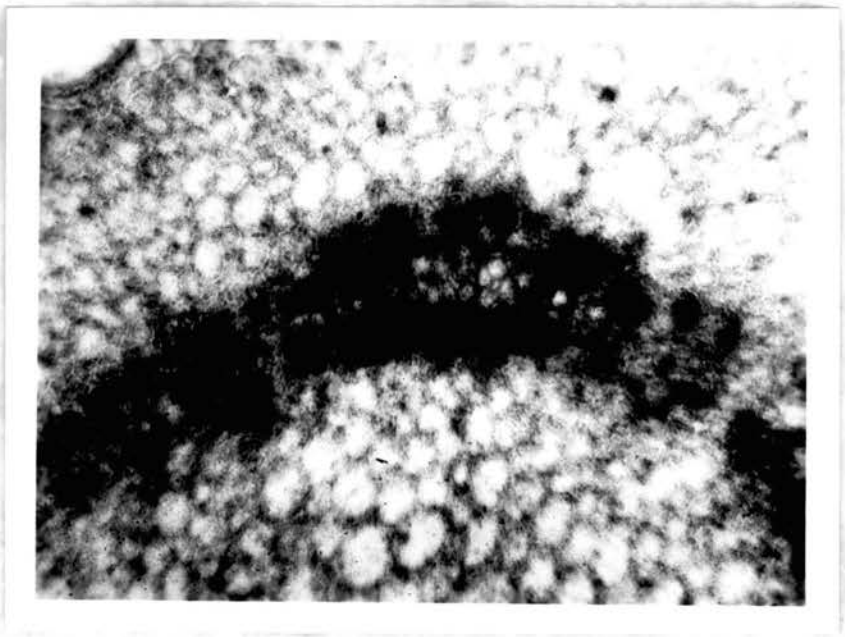

Fig. 23. Autoradiograph of the cross section of Snapdragon stem. Phase 300x.

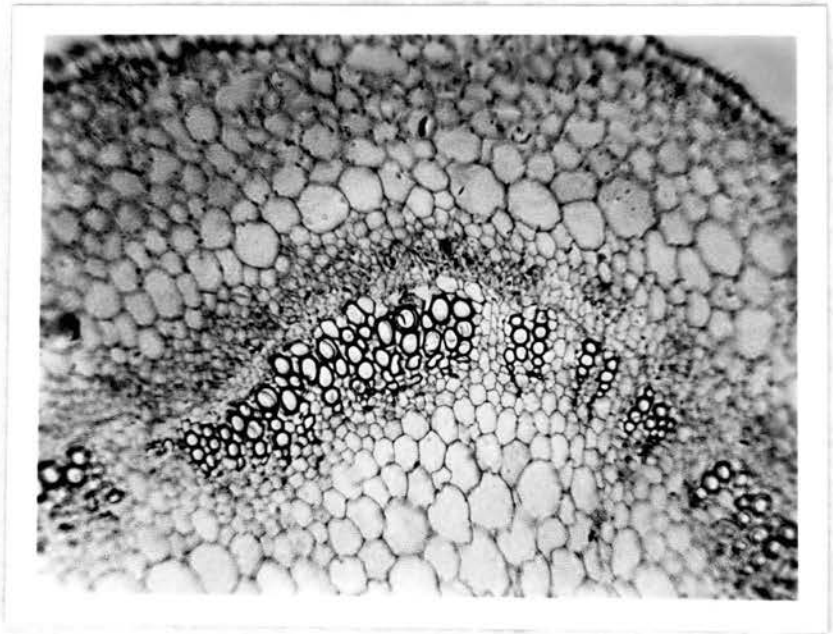

Fig. 24. Histological cross section of Snapdragon stem. Phase 300x.

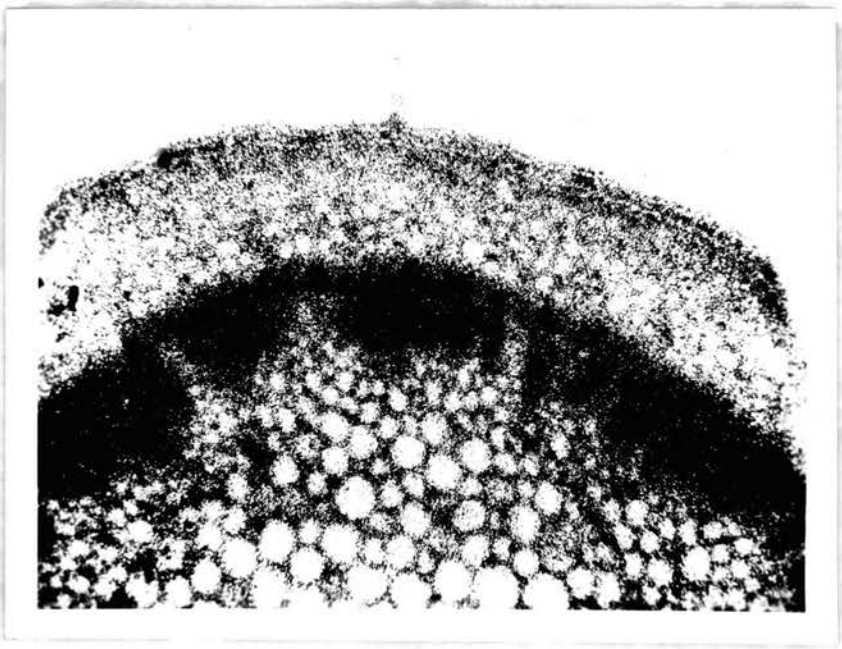

Fig. 25. Autoradiograph of the cross section of Snapdragon stem. x150.

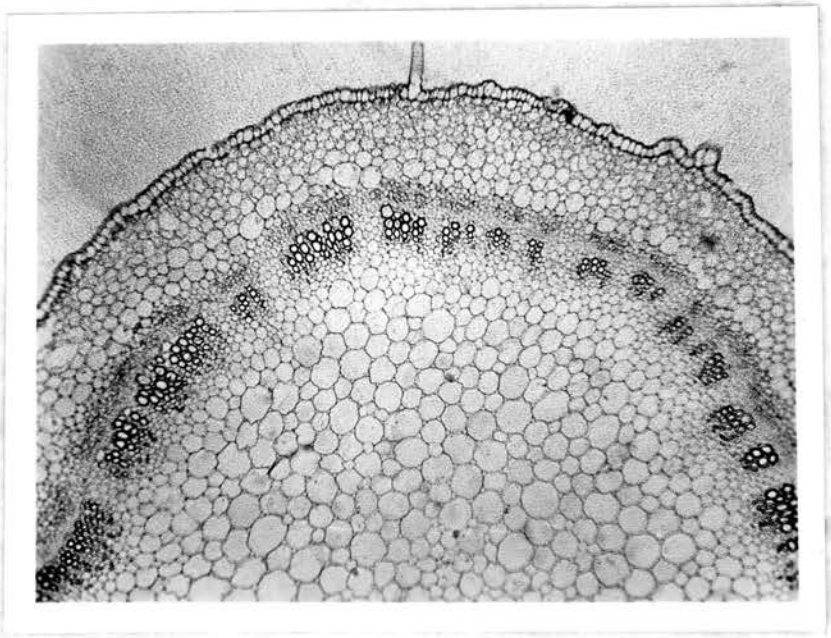

Fig. 26. Histological cross section of Snapdragon stem. x150.

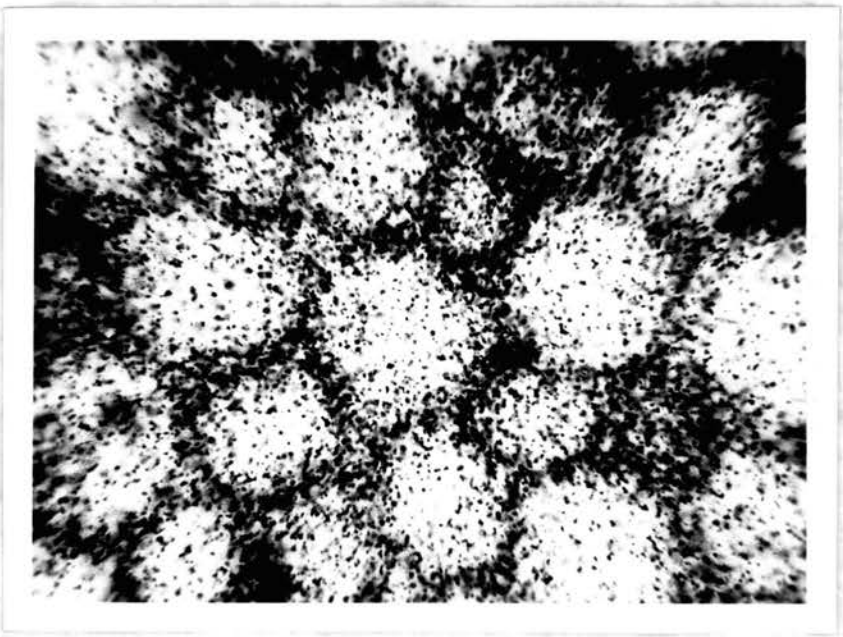

Fig. 27. Autoradiograph of the parenchyma cells of Snapdragon stem. x675.

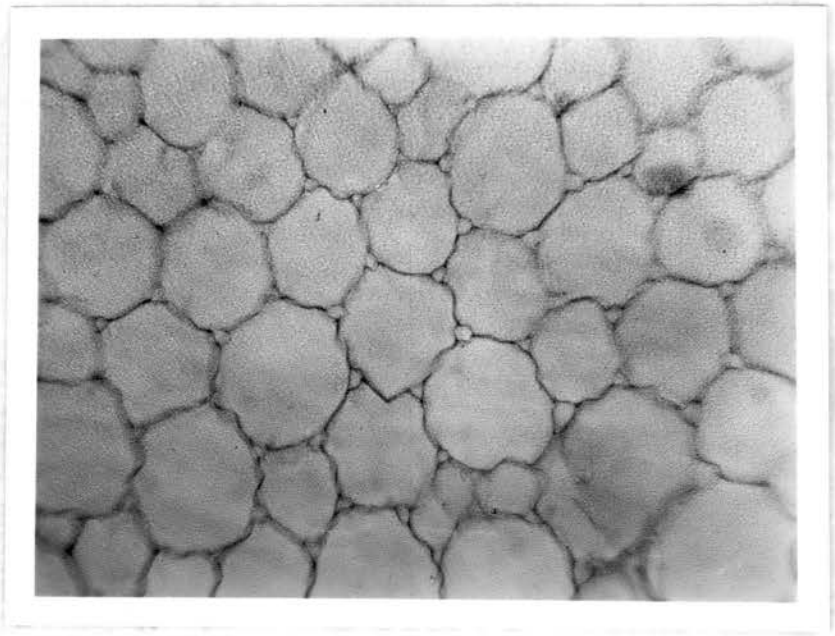

Fig. 28. Histological cross section of the parenchyma cells of Snapdragon stem. x675.

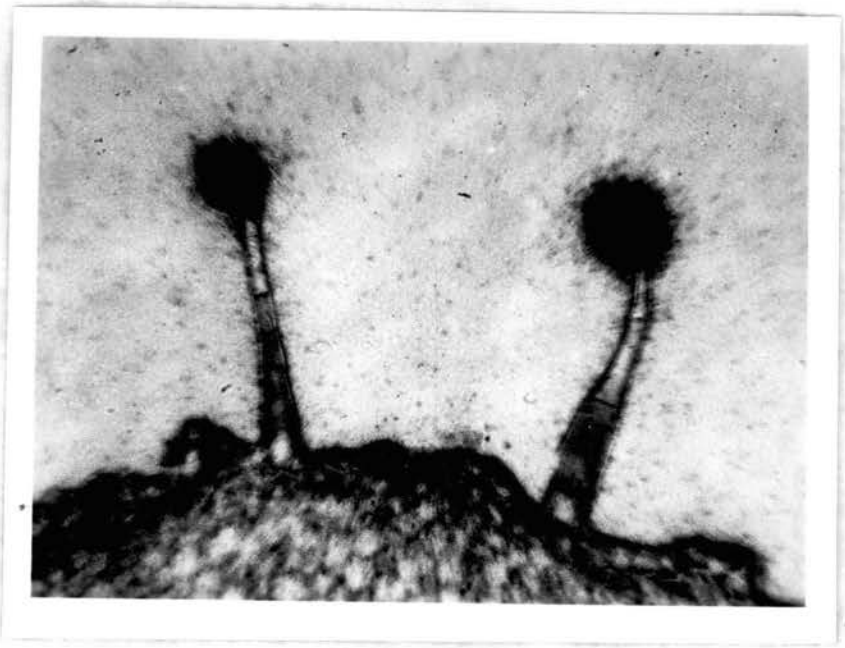

Fig. 29. Autoradiograph of the glandular hairs of Snapdragon stem. Phase 300x.

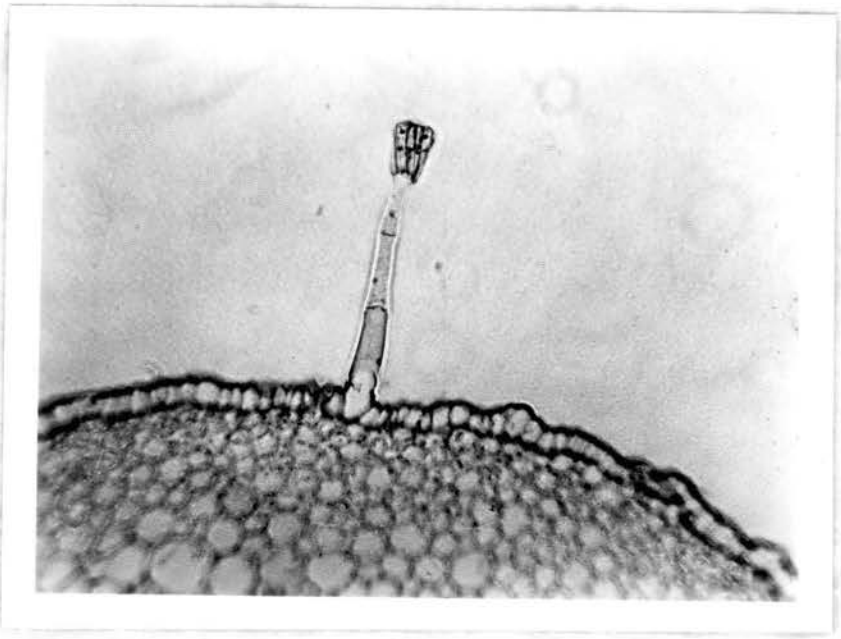

Fig. 30. Histological section of a glandular hair of Snapdragon stem. Phase 300x.

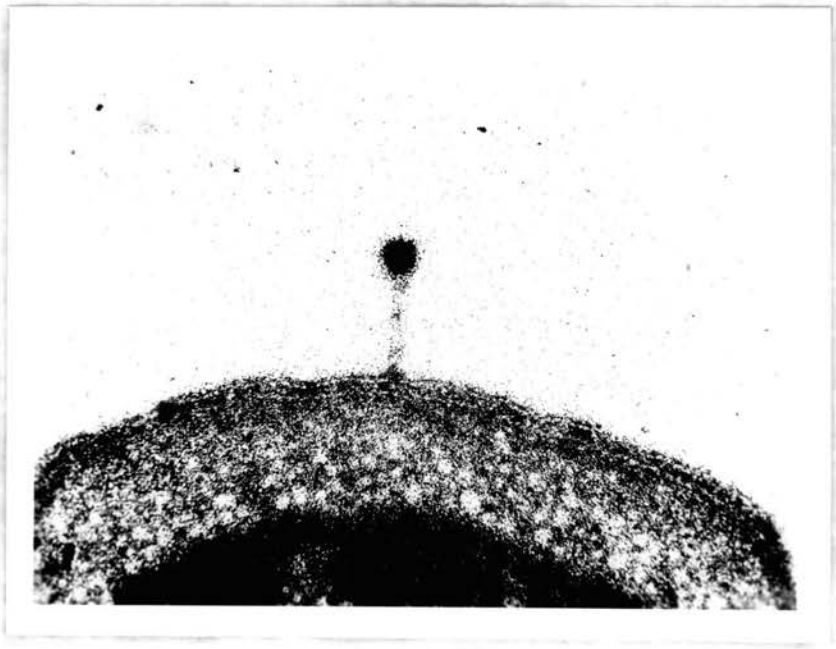

Fig. 31. Autoradiograph of the cross section of Snapdragon stem and glandular hair. x150.

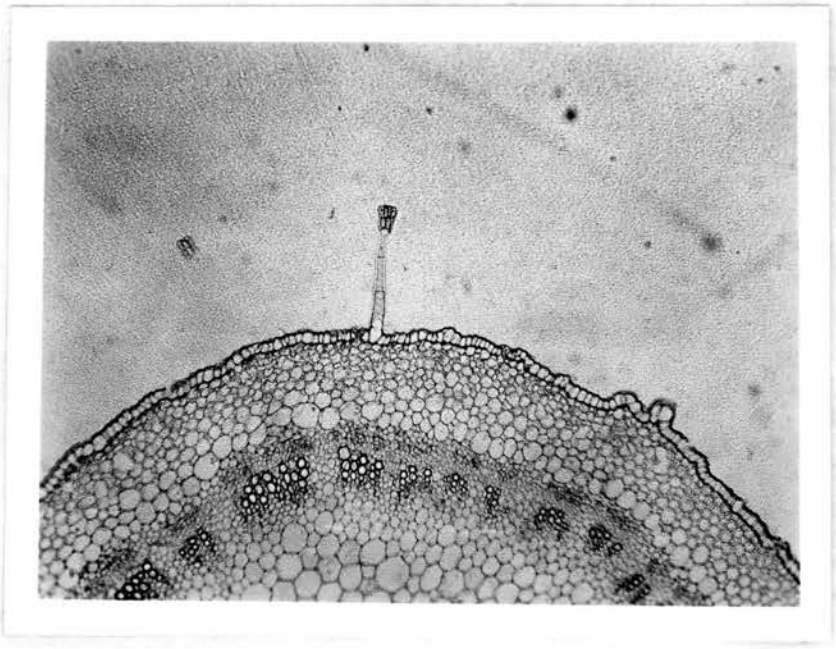

Fig. 32. Histiological cross section of Snapdragon stem and glandular hair. x150.

Difficulties encountered throughout the autoradiographic process are listed as follows:

1. Incompletely cleaned slides: It was observed that if the slides were not completely cleaned and rinsed, the subbing failed to adhere and consequently the tissue, celloidin and emulsion either loosened or came completely off the slide.

2. Slides improperly coated with celloidin: When slides were dipped in celloidin which was too thick, improperly drained or improperly hardened, areas of poor vertical resolution resulted. Improperly hardened celloidin caused the celloidin-emulsion complex to come loose during the washing and dehydrating process resulting in the formation of wavy surfaced slides.

3. Foreign materials: Dirt or other foreign solid substances entrapped in either the celloidin or emulsion masked the tissue and made study of the autoradiograph difficult. It was found that foreign substances could come from various sources and become incorporated in the film at a number of stages in the processing of the slides.

4. Background: Several possible sources of background in the emulsion were observed. Background might have been caused by high temperatures encountered during shipment of the emulsion, during coating, or development. Melting the emulsion at too high a temperature or stirring would likewise produce fogging.

5. Uneven coating of the emulsion: Uneven coating of the emulsion caused areas of poor horizontal and vertical

resolution. It was also observed that in the areas of greater thickness there was likelihood of insufficient clearing.

6. Milky emulsion: It was observed that if the emulsion was not cleared and fixed sufficiently, milky areas soon developed after the coverslip was applied (Fig. 33).

7. Checks and folding of the emulsion: Checks and folding of the emulsion was observed to be produced by swelling during development and washing. The swelling was the result of the emulsion failing to adhere to the celloidin or of improper hardening and drying of the celloidin. Tendency of the emulsion to take up moisture during exposure or failure to completely reset after coating, also resulted in swelling (Fig. 34). Checking of the emulsion in a regular pattern was observed but no complete account could be given. It was thought that it might have been produced by improper dehydration or other improper techniques which would cause shrinkage and the formation of checks.

8. Bubbles in the emulsion: It was observed that bubbles would be produced on the slide by using the emulsion from which all bubbles had not worked out during the melting process. Bubbles were also introduced if the dropper was not properly filled with emulsion and if it was not carefully placed on the slide and brushed out completely (Fig. 35).

9. Exposed silver granules and aggregates: There were observed in a number of slides large silver granules or aggregates forming one black mass (Fig. 36). It was believed

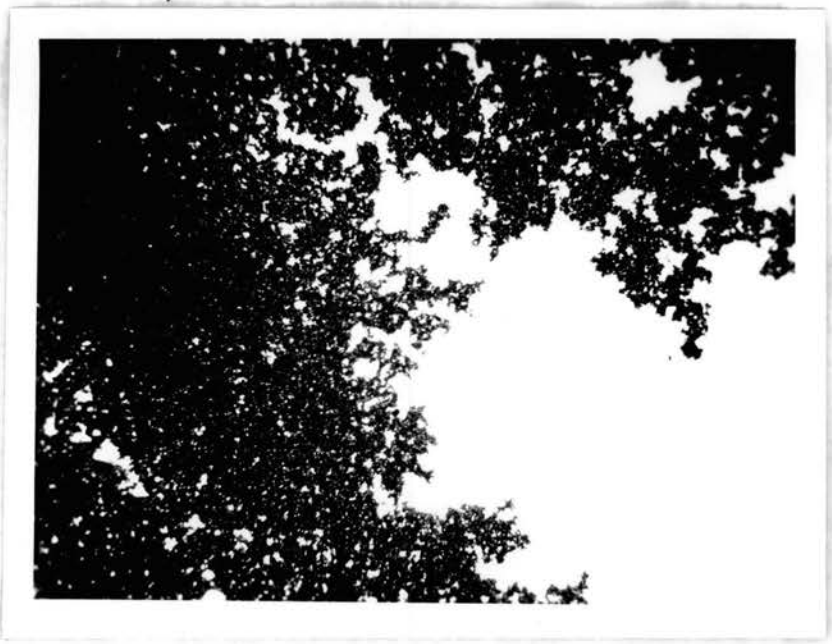

Fig. 33. Appearance under the microscope of white milky areas caused by improper fixing in Acid Fixer. x150.

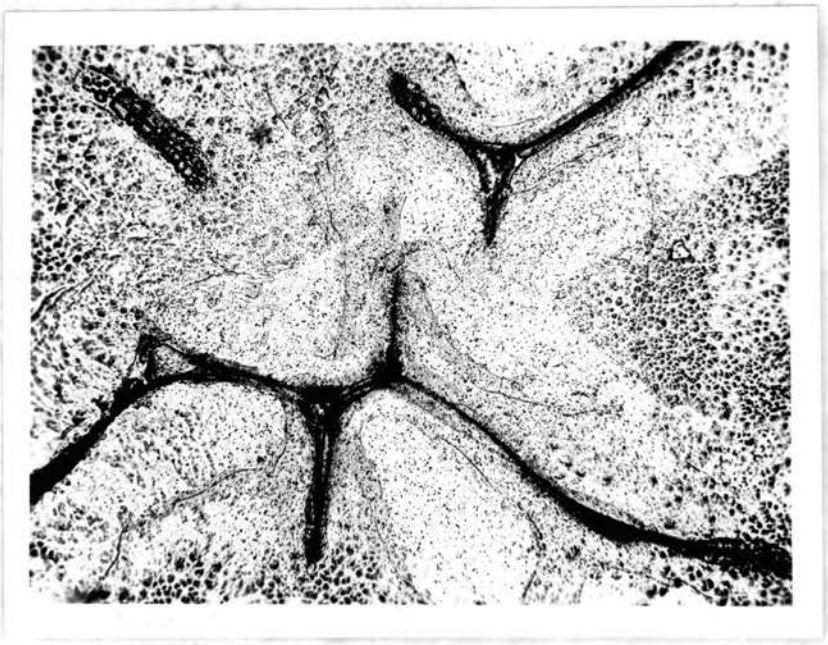

Fig. 34. Checks and folds resulting from swelled emulsion. x150.

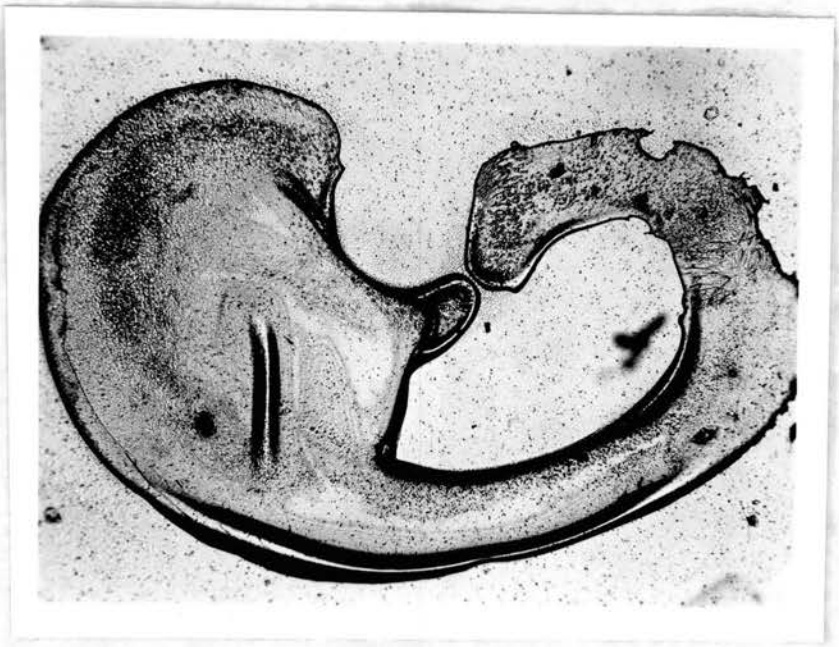

Fig. 35. A bubble formed in the emulsion during the coating process. x150.

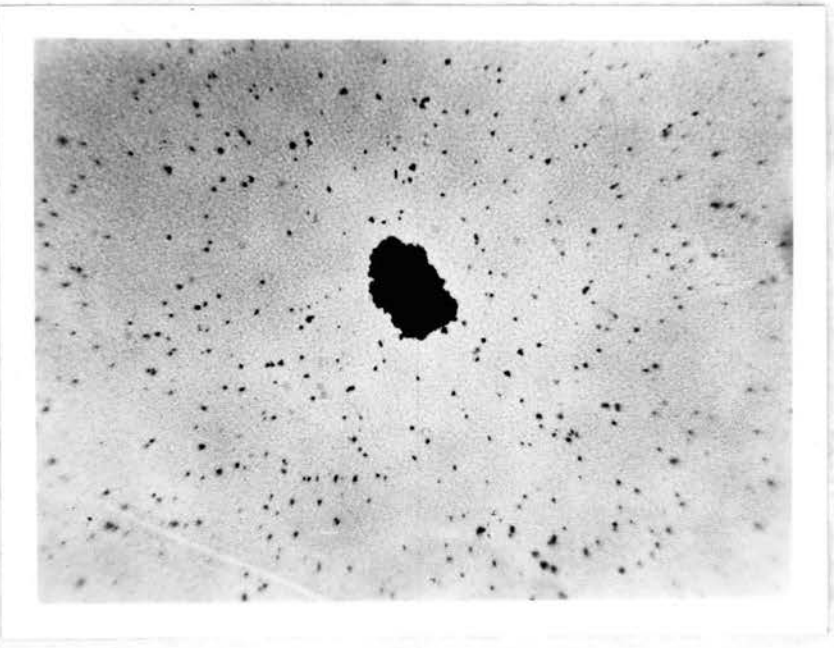

Fig. 36. Silver aggregation or developmental center. x675.

that these might be caused by exposed emulsion left upon or in the hairs of the coating brush and which had not been completely washed out. Another theory might be that for some unknown reason there were developmental centers involving a number of silver granules and as a result a build up of black silver aggregates.

10. Lumpy emulsion: Lumpy areas were due to insufficiently melted emulsion at the time the slides were coated.

11. Tissue poorly fixed to slide: When tissue was not fixed to the slide properly or when certain portions of the tissue did not adhere completely, areas nearly optically transluscent appeared (Fig. 37).

12. Crystals present in the emulsion: Crystals were observed near the surface of the emulsion and were attributed to the developing and clearing solutions in which the chemicals had not been completely dissolved or which had recrystallized from solution upon standing (Fig. 38).

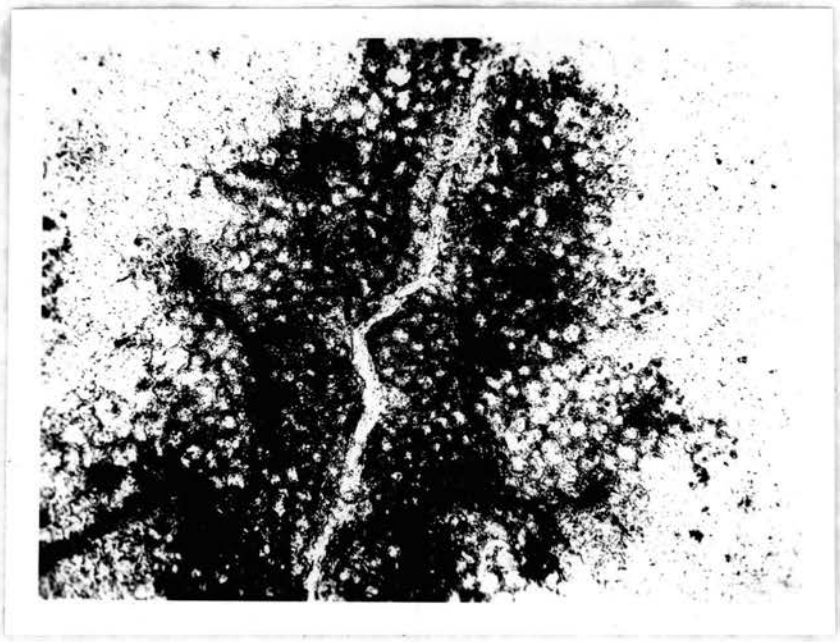

Fig. 37. Tissue poorly attached to slide. x150.

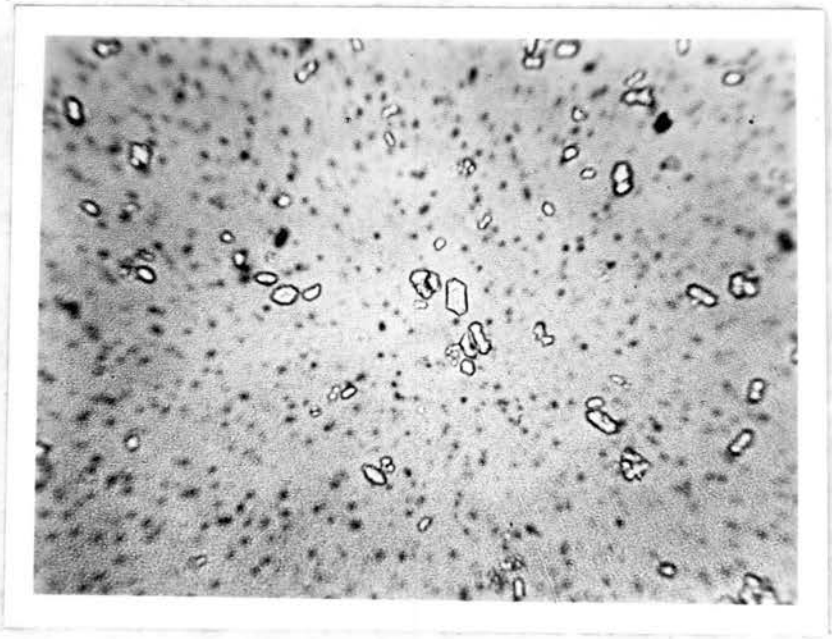

Fig. 38. Crystals in the emulsion caused by improperly mixed developing and clearing solutions. x675.

SUMMARY

Day Lily (Hemerocallis spp.) and Snapdragon (Antirrhinum spp.) were selected as experimental plants in a study of the application of the autoradiographic technique to plant research. Stems bearing flowers were cut and placed in tap water containing radioactive sulfur (S^{35}). Autoradiographs were obtained from sections of ovary, style, anther and portions of the filament, petal and stem. A modification of the coating method of the autoradiographic procedure was developed and was found most satisfactory for use in this research. It was found that Ansco Radioautographic Emulsion A was readily adaptable to plant research. Resolution was found to be quite satisfactory under low power if the slides were processed correctly and in many cases tissue could be studied under high power. Large grain size proved to be a disadvantage for the study of small detailed structures. The possibility of processing a great number of slides at a time was an advantage.

It was felt that the autoradiographic technique described in this thesis has a possible application to a great many branches of plant research.

LITERATURE CITED

1. Armstrong, W. D., J. Schubert, and A. Lindenbaum. 1948. Distribution of radioactive carbon administered as carbonate in body and excreta of mature rat. Proc. Exp. Biol. and Med., 68: 233-240.

2. Arnon, D. I., P. R. Stout, and F. Sipos. 1940. Radioactive phosphorus as an indicator of phosphorus absorption of tomato fruits at various stages of development. Am. J. Botany, 27: 791-798.

3. Becquerel, H. 1896. Sur les radiations émises par phosphorescence. Compt. rend. Acad. d. sc., 122: 420, 501, 698.

4. Belanger, L. F. 1950 Method for routine detection of radiophosphates and other radioactive compounds in tissue: inverted autograph. Anat. Rec., 107: 149-160.

5. Belanger, L. F., and C. P. Leblond. 1946. A method for locating radioactive elements in tissues by covering histological sections with a photographic emulsion. Endocronology, 39: 8-13.

6. Blundell, Mary, and J. Rotblat. 1951. Use of liquid nuclear research emulsion in track autoradiography. Nature, 167: 645-646.

7. Bogoroch, Rita. 1951. Detection of radio-elements in histological slides by coating with stripping emulsion - The "strip-coating" technique. Stain Technol., 26: 43-50.

8. Boyd, G. A., C. W. Casarett, and A. I. Williams. 1950. Making autoradiographs of individual blood cells. Stain Technol., 25: 13-16.

9. Buie, D. H. Jr., F. E. Hoecker, and P. G. Roofe. 1950. A constant registry autoradiographic technique. Proc. Soc. Exp. Biol. and Med., 75: 747-749.

10. Endicott, K. M., and H. Yagoda. 1947. Microscopic historadiographic technic for locating and quantitating radioactive elements in tissues. Proc. Soc. Exp. Biol. and Med., 64: 170-172.

11. Evans, T. C. 1947. Radioautographs in which the tissue is mounted directly on the photographic plate. Proc. Soc. Exp. Biol. and Med., 64: 313-315.

12. Gorbman, A., and H. L. Evans. 1941. Correlation of histological differentation with beginning of function of developing thyroid gland of frog. Proc. Soc. Exp. Biol. and Med., 47: 103-106.

13. Harrison, B. F., M. D. Thomas, and G. R. Hill. 1944. Radioautographs showing the distribution of sulfur in wheat. Plant Physiol., 19: 245-257.

14. Lacassagne, A., and J. S. Lattes. 1924. Méthod auto-histo-radiographic pour la détection dan les organes du polonium injecté. Compt. rend. Acad. d. sc., 178: 488-490.

15. Leblond, C. P., W. L. Percival, and J. Gross. 1948. Autographic localization of radio-iodine in stained sections of thyroid gland by coating with photographic emulsion. Proc. Exp. Biol. and Med., 67: 74-76.

16. London, E. S. 1904. Études sur la valeur physiologique et pathologique de l'émanation du radium. Arch. d'électric med., 12: 363-372.

17. Low-Beer, B. V. A. 1950. The clinical use of radioactive isotopes. Springfield, Ill.: Chas C. Thomas.

18. Mac Donald, A. M., Jack Cobb, and A. K. Solomon. 1948. Radioautographic technique with C^{14}. Science, 107: 550-552.

19. Mac Donald, A. M., Jack Cobb, A. K. Solomon, and D. Steinberg. 1949. Stripping film technique for radioautography. Proc. Soc. Exp. Biol. and Med., 72: 117-121.

20. Mullins, L. J. 1947. The localization of radiophosphates in cells. Proc. Soc. Exp. Biol. and Med., 64: 296-297.

VITA

Oran Dean Steffey
candidate for the degree of
Doctor of Philosophy

Thesis: DEVELOPMENT OF AN AUTORADIOGRAPHIC TECHNIQUE FOR USE IN BOTANICAL INVESTIGATIONS

Major: Botany

Biographical:

Born: The writer was born at Billings, Oklahoma, May 19, 1921, the son of Onis O. and B. Leona Steffey.

Undergraduate Study: He attended grade school in Marshall, Oklahoma, junior high school in Enid, Oklahoma, and graduated from the Enid High School in 1941. In the fall of 1941 he matriculated at Phillips University, Enid, Oklahoma, from which he received the Bachelor of Arts degree, with a major in Zoology and a minor in Botany, in May, 1948.

Graduate Study: In September, 1948 he entered the Graduate School of Oklahoma Agricultural and Mechanical College from which he received the Master of Science degree, with a major in Botany, in 1950. Requirements for the Doctor of Philosophy degree were completed in May, 1953.

Experiences: The writer was employed by the Mid-western Procurement District for the USAAF from 1943 till 1945. During 1947 and 1948 he was employed as an assistant in Zoology and Botany at Phillips University. From 1948 till 1950 he was employed by the Botany and Plant Pathology Department of Oklahoma A & M College as a teaching fellow in Botany. During the period from 1950 until 1953 he was associated with the Botany and Plant Pathology Department and was employed as a Research Associate on an Atomic Energy Project by the Research Foundation at Oklahoma A & M College.

THESIS TITLE: DEVELOPMENT OF AN AUTORADIOGRAPHIC TECHNIQUE FOR USE IN BOTANICAL INVESTIGATIONS

AUTHOR: Oran D. Steffey

THESIS ADVISER: Dr. Roy M. Chatters

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