GROWTH IN *VITRO* OF THE PROEMBRYO AND PROTHALLIUM OF *PINUS*

A STUDY OF THE GROWTH *IN VITRO* OF THE PROEMBRYO AND PROTHALLIUM OF *PINUS NIGRA* ARN. VAR. *AUSTRIACA* A. & GR.

By

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A Thesis

Submitted to the Faculty of Arts and Science

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

October 1955

MASTER OF SCIENCE(1955) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario.

TITLE: A Study of the Growth *in Vitro* of the Proembryo and Prothallium of *Pinus nigra* Arn. var. *austriaca* A. & Gr.

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NUMBER OF PAGES: iv, 97

SCOPE AND CONTENTS: Little is known about fundamental mechanisms of growth control on the growth of single or small groups of cells of the higher plants. Study of these effects *in vitro* requires the knowledge of the primary conditions for growth *in vitro*. In this work a procedure was outlined and used in an effort to establish a basis for the standard procedure. Also the associated effects of the physical, and to some extent, the chemical environment on the growth of the proembryo and prothallium of *Pinus* were studied.

PREFACE

In recent times much emphasis has been placed on growth and the factors causing and controlling it. In an effort to answer some of the many questions, research has been and is being conducted in many and often unrelated fields. This is not surprising, as the phenomenon of growth involves many and complex features; so many and so complex that they cannot all fall within the realm of one scientific discipline. Most fields can contribute something to further the understanding of this extensive problem. In this regard, this treatise deals with the alteration of growth expression, caused by the alteration of the chemical and physical environment of the growing cells, in particular, those of the prothallium and proembryo of Pinus nigra var. austriaca.

This work was undertaken as part of a programme which has been assisted through a grant from the Ontario Cancer Treatment and Research Foundation, for which Dr.N.W.Radforth is the grantee.

I would like to express my gratitude to Dr.Radforth for his guidance and understanding in conducting my research.

Also, I wish to express my appreciation to the Biology Department of this University for awarding me a Student Assistantship, without which I could not have undertaken this work.

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INTRODUCTION

It is increasingly evident that, although much has been accomplished regarding the interpretation and application of the factors affecting and controlling growth, there remains much more to be achieved, particularly in the realm of single and small groups of cells. This latter aspect has enjoyed greater interest in recent years because of the increased awareness of cancer, with associated growth and behaviour phenomena.

It is accepted that genetic inheritance provides the cell with its potentialities for growth. However, it is also accepted that chemical and physical stimuli affect the expression of that growth. This view is clearly stated by Thomson (14) as follows:"-that the morphological expression of any hereditary feature, whether it has an old constitutional basis or is due to recent mutation, cannot be used to determine the course of phylogeny until the extent of its variability under different conditions has been determined and taken into account." Ludford (8) has also shown that malignant cells react to physical and chemical stimuli in their growth and that this reaction varies with different strains.

Two facts are emphasized from this:firstly, that the chemical and physical environment affects the expression of growth and secondly, that this effect varies with the

materials used. This latter statement is not surprising as the different materials will vary in genetic constitution or when the materials are taken from different parts of the same organism, they differ in the type and degree of differentiation.

In spite of this, generalization regarding the effects of the factors could be made, if a sufficiently large range of material was studied, including that at different levels of morphological complexity.

As these are periods of minimum cellular differentiation, the zygote and early post-zygotic stages of embryonic development provide cells, or groups of cells, which have only the potentialities for extensive growth and differentiation. Then, if their chemical and physical environment is altered, with respect to their natural state, an alteration in the expression of their growth might also be expected. This was found to be true by Dr. Radforth (13) and Woods (15). Dr. Radforth, working with Ginkgo, found that in vitro, the formation of the suspensor, as well as the onset of asymmetrical development of the proembryo, can be deferred. Woods, working with Pinus, had similar results, noting that the presence or absence of the suspensor is a result of a physiological response. He also found that the radial or three dimensional growth in vitro can be initiated at fert-

ilization or imposed at subsequent stages, the extent of which depends on the degree of differentiation.

Except that the alteration of chemical and physical factors do impose changes on the growth of embryos, very little is known about their mode of action with respect to the regulation of growth. Their effects are known only by inference, as above and from the work of others such as Nutman, referred to by Skoog (11), who has correlated the degradation and reabsorption of tissues, enveloping the embryo, with the embryo's stages of development. From this, he has suggested that the enveloping tissues supply the nutrients or growth factors to the embryo as needed. Working with Datura, Van Overbeek et al (15) were able to develop mature embryos in vitro from relatively undifferentiated masses of cells, by adding coconut milk to the medium.

To investigate the mode of action of these factors, in this instance with respect to the proembryo of Pinus, the effect on growth of the basic conditions for in vitro culture must be known. By basic conditions is meant the simplest conditions in which optimum growth will occur, other than the natural state. Following this, variations can be made in the basic environment and the resulting effects can be compared, not only to those of the in vivo state but also to those of the basic conditions in vitro.

However, until this medium is obtained, relative comparisons can still be made among the effects of other conditions *in vitro* and to those of the *in vivo* state as well.

In order to obtain results, which could be used as a standard for comparison, the procedure involved in dissection, processing, staining and observation must also be standarized to yield consistent results.

It is in this regard that the work for this treatise was undertaken; namely, to attempt to establish a standardized procedure, to contribute information regarding the requirements of the basic medium for growth of the proembyo of *Pinus nigra* var. *austriaca* *in vitro*, and to evaluate the relative changes, if any, in the expression of growth in both the proembryo and prothallium of this species under several environments *in vitro*.

MATERIALS AND PROCEDURE

The standard procedure must be one in which each of the component steps is such that it can be performed repeatedly with accuracy and consistent results. The methods and materials of the following procedure were selected in an effort to fulfill these requirements.

The female gametophyte and the proembryo of Pinus nigra var. austriaca were selected because they were used by Woods (18) in his work which demonstrated the need for this investigation. Also their relatively large size affords reasonable ease of manipulation, thereby allowing rapid dissection which decreased the possibility of contamination.

Woods (18) had found that dissection, using the micromanipulator, produced material which was unable to survive in vitro, apparently due to shock, and he was only able to get satisfactory results when the whole gametophyte with the inclosed proembryo was used as the inoculum. However, since accurate and controlled dissection is necessary in the standardized procedure, it was decided that the micromanipulator would be used. It was thought that if care was exercised the incidence of lethal shock could be kept to a low level or avoided completely. In the effort to prevent this shock, the dissection was designed to leave a buffer zone of prothallial cells to protect the

inner proembryonic masses. This zone was several cells in thickness and was small enough to allow the proembryos to have, as much as possible, a uniform environment in the media.

The dissection procedure was as follows:

The female cone was cut along a median line from the distal to the basal end to allow ready removal of the scales. The prothallium was removed from its integument and nucellus, using a sterile probe and exercising care to prevent injury. It was then transferred to a sterile moist chamber, used for micromanipulation, which was provided with a floor of sterile 4% agar gel to prevent excessive breakage of the glass implements of the micromanipulator. The chamber containing the gametophyte, was placed on the stage of a dissecting microscope, where the dissection, according to the above stated requirements, was accomplished.

In order to insure that all stages, from fertilization to the establishment of the pr embryo, would be obtained, the dissections began on June 23rd, 1954, one week before the suspected time of fertilization (1), and continued on every second day until July 19th, 1954.

As the establishment and maintainance of sterility is essential for success in tissue culture, precautions were taken to insure as high a degree of sterility as pos-

sible. As well as autoclaving the media and agar, and dry sterilizing (heat for one hour at 170 degrees centigrade) the serological tubes and pipettes used in the transfer of media and penicillin, three solutions used to render sterile such things as equipment and working area. These were: 70% ethanol, 5% aqueous phenol, and saturated calcium hypchlorite solution, prepared by adding 20 g. of calcium hypochlorite to 280 c.c. of water and filtering the solution, after allowing it to stand for three hours.

Each has disadvantages which prevented its use throughout the whole procedure. The 70% ethanol was used to sterilize the glass instruments and the moist chamber, the phenol for the working area, the equipment and the surrounding air, the calcium hypochlorite for cooling the flamed metal instruments and for sterilizing the piece of tissue, used as the inoculum. Calcium hypochlorite does not affect the plant cells if used for a short period of time, but is effective against surface contamination (10).

After the dissection, the cell population was removed, using a small, sterile wire loop, transferred to the calcium hypochlorite solution, for one-half to one minute, and then placed in a tube containing the medium. Its mouth was flamed before and after the inoculation in the manner of microbiologists, to insure the maintenance of sterility.

The medium is one of the most critical factors in a standardized procedure for the culture of tissues. Since many types of media have been used for various materials, experimentation is necessary before the best one can be selected for the proembryo of Pinus nigra var. austriaca. In this connection, several media were designed and employed. All had, to provide mineral salts, the modified Crone's solution. This was used by Dr. Radforth (10), in his work with Ginkgo, and was selected by Woods (18) over Knudson's formula, in his work with Pinus.

It is prepared as follows: 10 g. potassium chloride; 2.5 g. calcium sulphate; 2.5 g. magnesium sulphate; 2.5.g magnesium phosphate; 2.5 g. iron phosphate are thoroughly mixed and one litre of distilled water added to 1.5 g. of this mixture. This is the '100 percent' modified Crone's solution. By adding distilled water to this solution in the correct proportions, solutions were obtained that were 50%, 25% and 12.5% concentrations of modified Crone's solution. The hydrogen ion concentration was adjusted to a pH of 5.5.

Following this, each solution was divided into eight portions and to each was added one of the following nutrient combinations: dextrose, 2 g. per 100 c.c.; yeast extract, 2 g. per 100 c.c.; yeast extract, 1 g. per 100 c.c. plus dextrose 1 g. per 100 c.c.; dextrose,1 g. per 100 c.c.; yeast extract, 1 g. per 100 c.c.; sucrose, 1 g. per 100 c.c.; yeast extract,

1 g. per 100 c.c. plus sucrose, 1 g. per 100 c.c.; sucrose, 2 g. per 100 c.c.

The resulting twenty-four solutions were dispensed, in 10 c.c. portions, in ordinary 20 c.c. test tubes, which were then fitted with cotton plugs and placed in an autoclave for twenty minutes at 15 pounds pressure and 100 degrees Centigrade.

To half of each medium, penicillin, dissolved in sterile, distilled water, was added such each ml. of the resulting solutions contained 10 units of penicillin. The media were then dispensed, under sterile conditions, into sterile serological tubes, each tube receiving 0.5.ml. of the medium. These tubes were also fitted with cotton plugs.

All glassware, associated with the preparation of the media, was made chemically clean using a saturated solution of chromic acid in concentrated (98%) sulphuric acid. This was rinsed out with tap water followed by sever l washings with distilled water.

The inoculated media were placed, for ten days, in an insulated cabinet where the temperature fluctuated between 25° C and 28° C. The length of the incubation period was chosen arbitrarily on the basis of Woods' (18) disclosures. The fluctuation was not considered injurious to the growth of the tissues, as it was found that there was a

variation in the internal temperature of the cone in its natural state and this variation exceeded that in the cabinet. To determine this internal temperature, a thermometer was inserted into the cone while on the tree and the temperatures were noted for several cones under a variety of conditions, including size, relative position on the tree, degree of sunlight and external temperature.

After the incubation period, the tubes were removed from the cabinet. The media were decanted and replaced with distilled water as a wash, which in turn was decanted to be replaced by Carnoy's solution (5), a killing and fixing reagent. This was allowed to remain for one-half hour. The pieces of tissue were then removed and placed in vials, containing 70% ethanol, which were corked and placed in a refrigerator to prevent excessive evaporation.

A condition of the investigation was that the fixative must not contain compounds with aldehyde groups, as their presence would interfere with the Feulgen reaction (3). Carnoy's solution was selected from the group of fixatives without such compounds, as it permitted immediate transfer to 70% ethanol for storage, made necessary by the fact that the plant material was only available for short periods of time. This made continuous processing impossible when large amounts of material were used.

Using 70% ethanol as the storage medium, permitted the direct transfer of the material from it to the first solution of the butyl alcohol series, used for the dehydration and infiltration procedure. This series was selected, rather than the ethanol-xylol method, because of the greater flexibility in procedure, while results are comparable.

The material was embedded in Tissue Mat and sectioned to a thickness of 12 micra.

Since the Feulgen reagent is considered to be highly specific for nuclei, its use in the standard procedure is desirable, as it would make observations more consistent and prevent misinterpretations. Since this is a chemical reaction, it yields quantitative as well as qualitative results; the degree of the former depending on the method of preparation and examination. In this instance, it was possible to make comparative estimations, with respect to size, chromosome number (whether the nucleus was diploid or haploid) and the condition of the nucleus.

The sections were mounted on slides using Meyer's adhesive (5) and were treated to remove the Tissue Mat, replacing it with water, using the method outlined by Johansen (5). Following this, the sections were submitted to a period of hydrolysis in 1 N. HCl, at 60° C for 6 minutes. The hydro-

lysis medium is as recommended by Hillary (3) and the time was determined experimentally for this particular material. After rinsing with distilled water the sections were treated with the Feulgen reagent, prepared according to Hillary (3), for not less than four hours. This period of reaction was also arrived at through experimentation.

Removed from the reagent, the sections were placed in three differentiated baths of sulphur dioxide water, each ten minutes in duration, after Hillary (3); washed in running water for five minutes; dehydrated, using the ethanol series; cleared in clove oil solution (5); treated with xylol and mounted in Canada balsam.

In vivo examples for controls were excised on each day of inoculation and were placed directly into Carnoy's solution. They then underwent the same processing as the *in vitro* material.

RESULTS

Effects of dissection and transfer to media:

In the dissection accomplished using the micromanipulator and procedure outlined in the preceding section, the percentage of failure, due to shock, was less than 40% and probably as low as 20%; the exact percentage was difficult to determine because failure to grow in the media could result from other causes. The early post-zygotic stages indicated a greater degree of sensitivity to this shock.

Therefore, the zone of prothallial cells that was left to protect the proembryo was adequate in most cases. Except for the cases showing later stages of embryonic development, this zone of cells was also narrow enough to encourage the view that the environment within the medium would be adequately uniform around the prothallium. In the later stages of development, the prothallial cells interfered with the growth of the lower situated embryonic masses.

Effects of sterility precautions:

Contamination occurred in only 7.8% of the 672 cultures. This was considerably lower than that experienced by Woods (18), as he found contamination a serious impediment which was not the case here.

Penicillin, in the concentrations used, was found to be ineffective in preventing contamination. In the penicillin-containing media, the contamination was slightly higher than in those without it. This difference is not significant, however as 8.0% contamination occurred in the penicillin treated media as compared with 7.7% occurring in the non-treated media.

There was a great increase in the incidence of contamination in the cultures inoculated on the 11th and 13th of July. This coincided with a significant increase in the size of the inocula and, it was considered that the time of decontamination in the calcium hypochlorite solution was not sufficient for these larger pieces of material. Accordingly, the time was increased to approximately one minute for the dissected material of the 15th, 17th, and 19th of July. The incidence of contamination in their cultures dropped to the level of the earlier group.

The evaluation of the Feulgen reagent:

In the majority of the slides, the Feulgen reagent gave the desired results, making the nuclei and mitotic figures clearly outstanding against the background of the unstained cytoplasm. Also the quality of the reaction was such that the haploid and diploid nuclei could be clearly distinguished and the condition of the nuclei,

as compared to those of the *in vivo* material, could be determined.

There were several slides which exhibited faint or no reaction with the Feulgen reagent. Those with no reaction may be classified into two groups: those with no nuclear material containing desoxyribose nucleic acid essential for the reaction and those which apparently had this material, but which failed to enter into the reaction for some other reason. There appears to be a connection between the second group and that material which showed only a faint reaction.

Assessment of the media:

The media were assessed on their ability to promote growth, which was determined in accordance with arbitrarily selected criteria. This ability was classified under three main headings; poor, fair, and good. Any medium with a poor ability was considered as not supporting growth.

On examination of the material, it was observed that there was not always agreement in the response of the gametophytic and embryonic cells to each medium. Hence, the media were assessed separately with respect to their effect on the growth of both the prothallial and embryonic cells. The data obtained are found in Tables I to VII.

In all of the tables the figures were obtained by determining the percentage of masses in which growth occurred,

in the total number of tissues in the particular group indicated.

Table I shows the data for growth in the separate media for the whole culture period. The overall percentage represents the percentage of masses indicating growth in a single nutrient combination regardless of the concentration of the modified Crone's solution.

Table II shows the percentage of growth with respect to the concentration of the modified Crone's solution used, disregarding the nutrient combination used.

Table III and IV show the percentage of meterial indicating growth with respect to the nutrient combination. The data here are for the gametophyte only, as fertilization had not occurred or its occurrance was not frequent enough to yield sufficiently significant figures.

Tables V,VI and VII are similar to Tables III and IV but include the data for the embryonic cells.

In the tables, code letters are used to represent the nutrient combinations in order to facilitate the compiling of the tables.

The key to these code letters is as follows:

A-- 2% dextrose

B-- 2% yeast extract

C-- 1% yeast extract plus 1% dextrose

D-- 1% dextrose

Y-- 1% yeast extract

F-- 1% sucrose

G-- 1% yeast extract plus 1% sucrose

H-- 2% sucrose

The division of the total culture period, into the sub-periods used, was determined by the stage of embryonic development in the majority of the inocula at the time of inoculation. The sub-periods are:June 23rd to June 25th, the period of prefertilization; June 27th to July 1st, the period of fertilization; July 3rd to July 7th the period of suspensorial growth; July 9th to July 13th, the period of establishment of the cells at the tip of the suspensor; July 15th to July 19th, the period of established embryonic masses in the lower portions of the prothallia.

TABLE I

Incidence of Growth for the Total Period of Culture

Nutr't comb.	% prothallia showing growth	overall %	overall %	%embryos showing growth	overall %
	50%	44.4		41.7	
A	25%	55.5	52.5	20.0	34.1
	12.5%	56.5		41.2	
	50%	4.0		25.5	
B	25%	14.3	10.9	20.0	22.0
	12.5%	17.7		23.0	
	50%	61.9		60.0	
C	25%	71.5	61.6	59.1	57.1
	12.5%	50.0		53.8	
	50%	41.3		30.3	
D	25%	40.0	45.5	46.2	34.9
	12.5%	46.2		26.6	
	50%	8.7		36.7	
E	25%	15.4	12.7	22.2	28.6
	12.5%	13.3		25.0	
	50%	26.3		42.9	
F	25%	47.1	38.0	50.0	43.2
	12.5%	42.9		36.4	
	50%	40.0		60.0	
G	25%	61.5	5 .8	76.9	55.5
	12.5%	52.3		35.3	
	50%	45.0		15.4	
H	25%	47.8	47.8	36.8	28.6
	12.5%	52.2		29.4	

Percentage of total gametophytes showing growth is 41.2 %

Percentage of total embryos showing growth is 37.3 %

TABLE II

Incidence of Growth for the Total Period of Culture

Concentration of Crone's Sol'n	% of prothallia showing growth	% of embryos showing growth
50%	32.5	39.2
25%	42.2	38.8
12.5%	44.6	33.9

TABLE III

Incidence of Growth for the Sub-period of June 23rd to June 25th

Nutr't comb.	% of prothallia showing growth
A	25.3
B	0.0
C	70.0
D	0.0
E	33.3
F	37.5
G	71.4
H	44.4

Incidence of growth for all media is 39.0%

TABLE IV

Incidence of Growth for the Sub-period of June 27th to July 1st

Nutr't comb.	% of prothallia showing growth
A	45.5
B	20.0
C	71.4
D	50.0
E	0.0
F	36.4
G	60.0
H	27.3

Incidence of growth for all media is 37.2%

TABLE V

Incidence of Growth for the Sub-period of July 3rd to July 7th

Nutr't comb.	% of prothallia showing growth	% of embryos showing growth
A	27.3	18.2
B	23.0	9.1
C	61.6	37.5
D	28.6	20.0
E	7.7	18.2
F	0.0	22.2
G	62.5	64.3
H	35.7	15.4

Incidence of growth in prothallia for all media is 33.0%
Incidence of growth in embryos for all media is 26.4%

TABLE VI

Incidence of Growth for the Sub-period of July 9th to July 13th

Nutrient comb.	% of prothallia showing growth	% of embryos showing growth
A	81.3	37.5
B	0.0	31.3
C	56.3	68.8
D	68.8	37.5
E	6.7	40.6
F	46.2	38.5
G	33.3	58.3
H	58.8	35.3

Incidence of growth in prothallia for all media is 44.6%

Incidence of growth in embryos for all media is 43.0%

TABLE VII

Incidence of growth for the Sub-period of July 15th to July 19th

Nutr't comb.	% of prothallia showing growth	% of embryos showing growth
A	60.0	33.3
B	12.5	12.5
C	53.3	53.3
D	38.5	38.5
E	29.4	41.2
F	61.5	69.2
G	37.5	37.5
H	60.0	73.3

Incidence of growth in prothallia for all media is 43.3%

Incidence of growth in embryos for all media is 44.2%

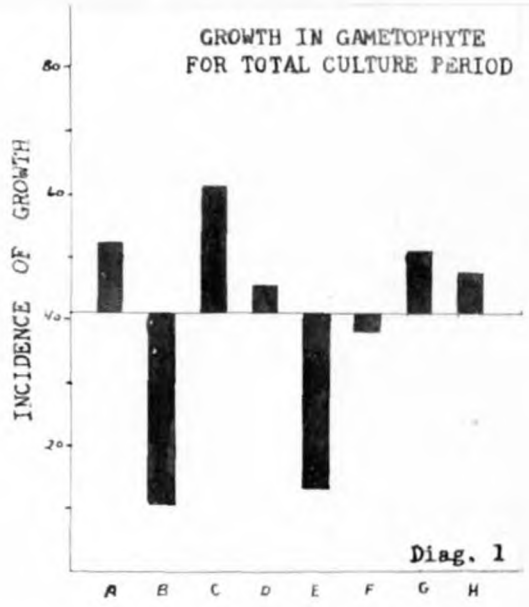
The data of the preceeding tables are graphically represented in the following diagrams.

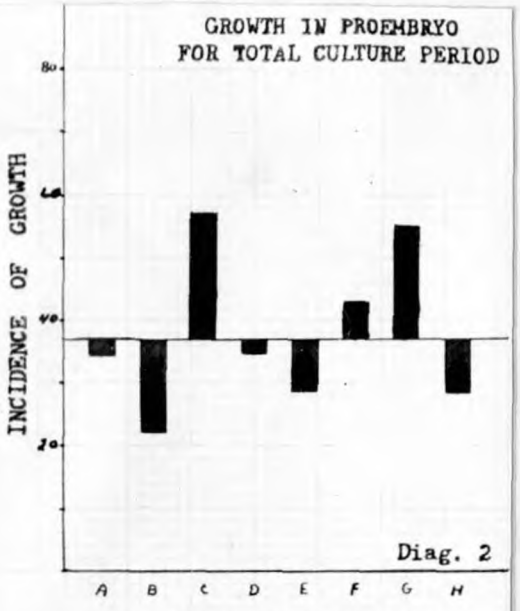
Graphs 1 and 2 represent table I; graphs 3 and 4 represent tables III and IV; graphs 5 and 6 represent table V; graphs 7 and 8 represent table VI; graphs 9 and 10 represent table VII.

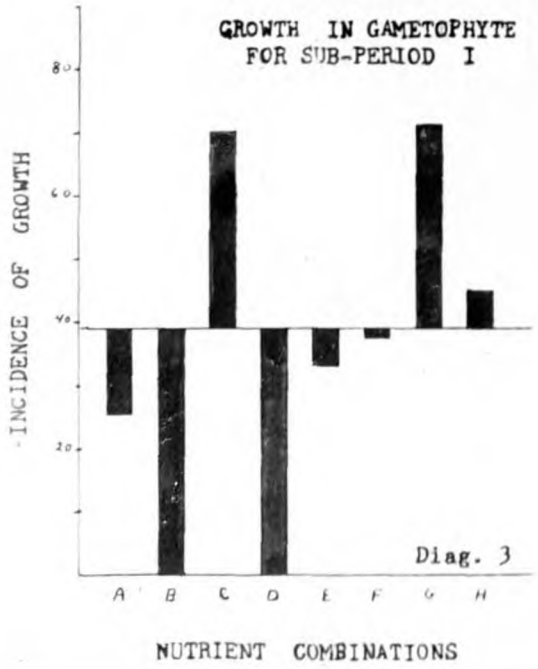
In text-figures IV to VII, diagrams 11 to 26 illustrate the data of the first ten diagrams. Here the data has been resorted to indicate the effect of individual nutrient combinations on the expression of growth in vitro.

In the first ten diagrams, the horizontal line represents the percentage of total masses, either prothallial or embryonic, which indicate growth in vitro. The vertical columns represent the difference in the percentage of masses indicating growth for each nutrient combination, relative to the percentage expressed by the horizontal line. In the rest of the diagrams, the horizontal line is placed at 0.0% and the vertical columns represent the increase or decrease in the percentage of masses showing growth relative to the percentages represented by the horizontal lines in diagrams 1 to 10 for each sub-period.

TEXT-FIGURE I







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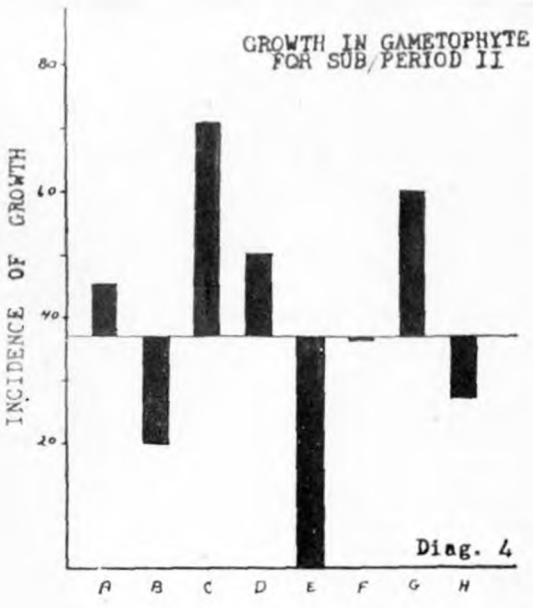

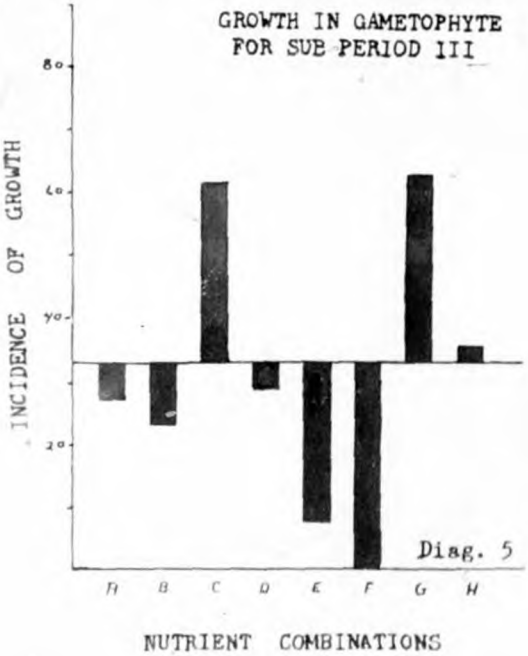

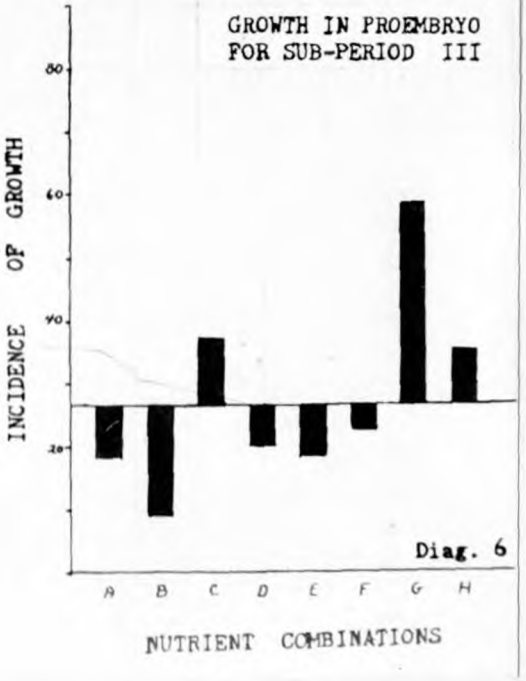

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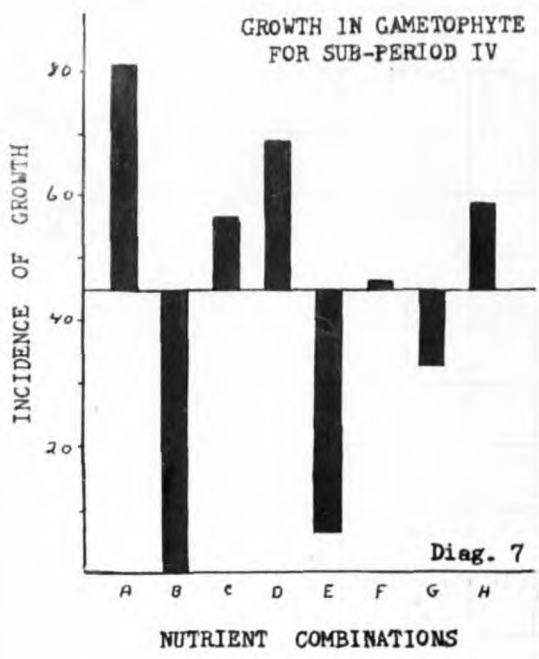

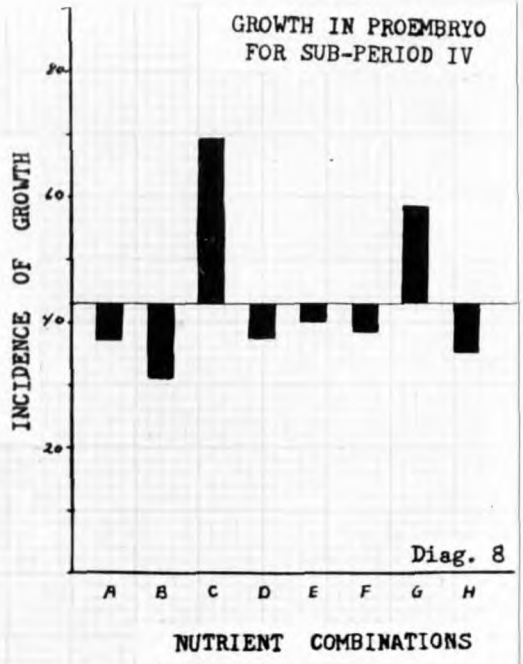

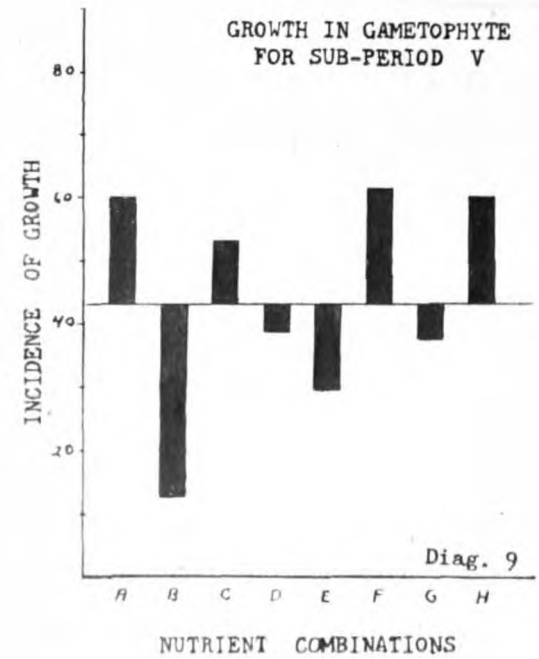

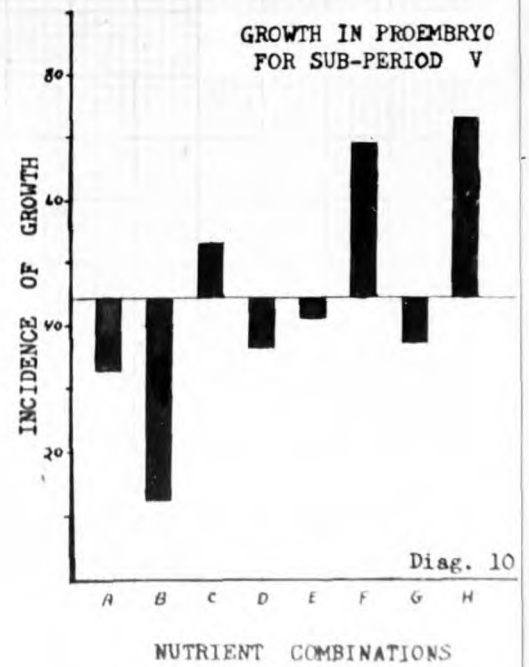

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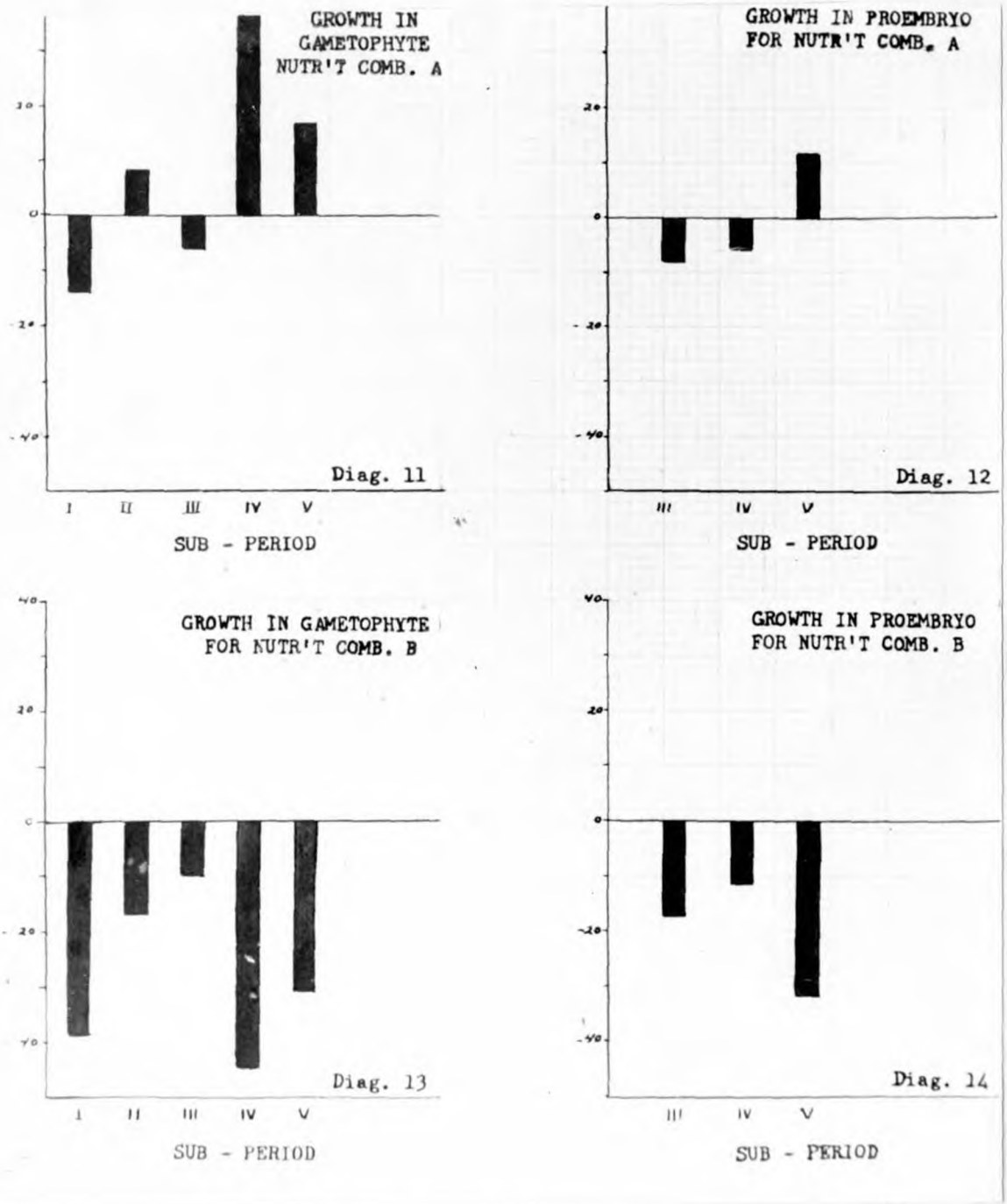
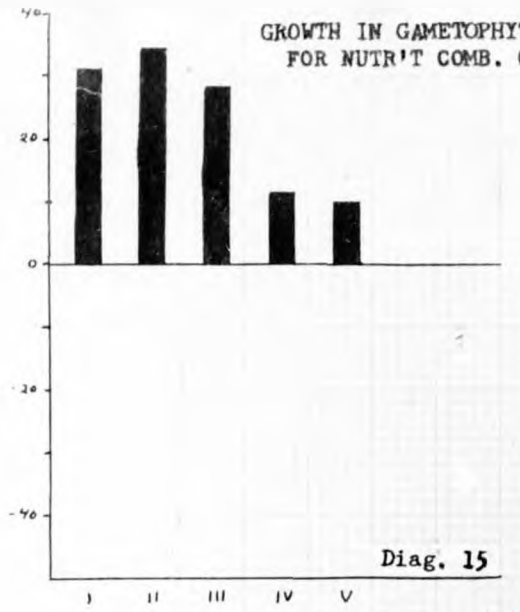
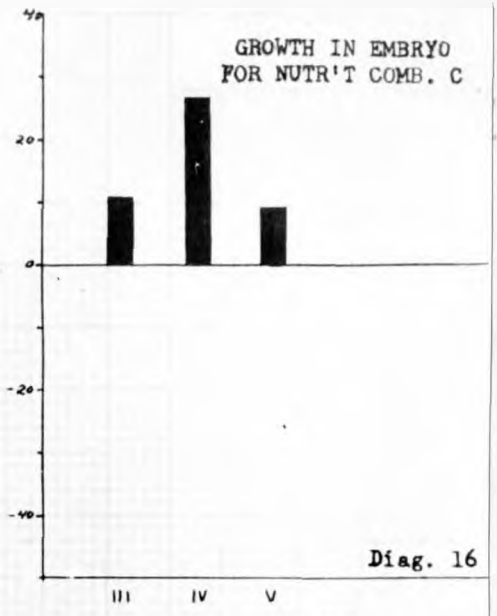
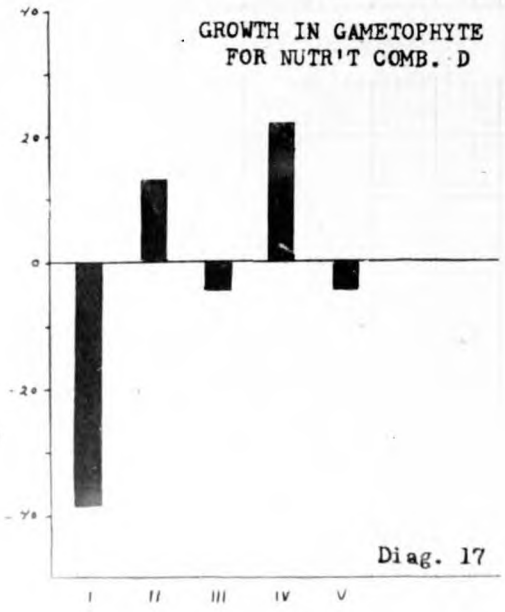
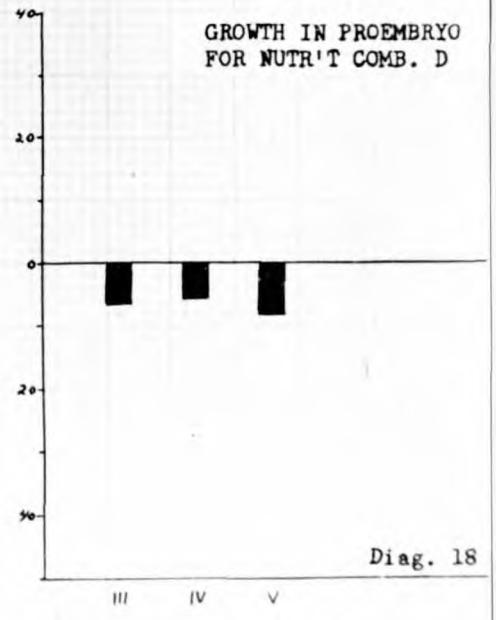


TEXT-FIGURE V

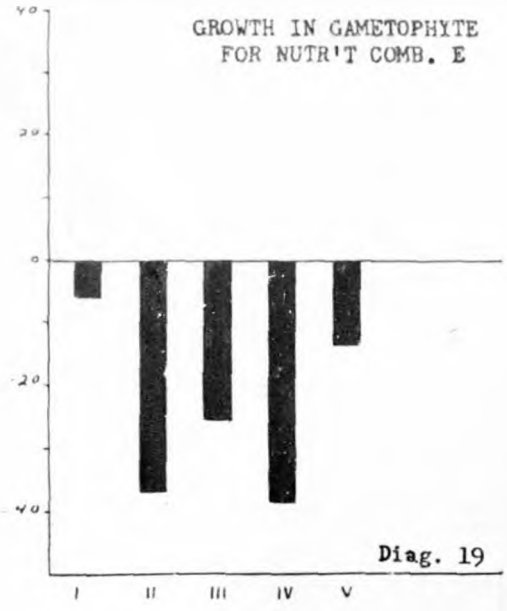


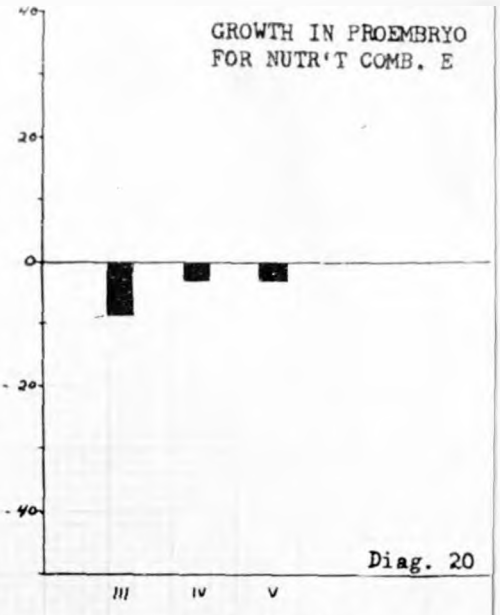


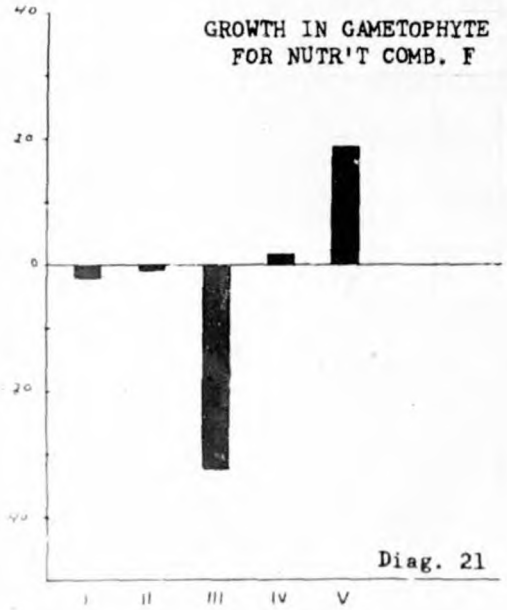


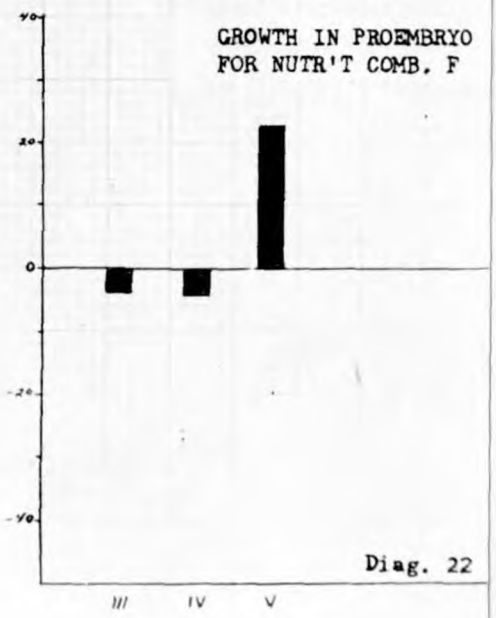


TEXT-FIGURE VI

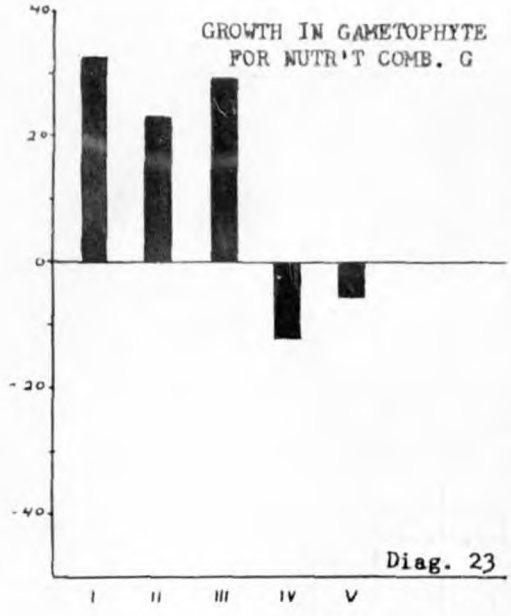


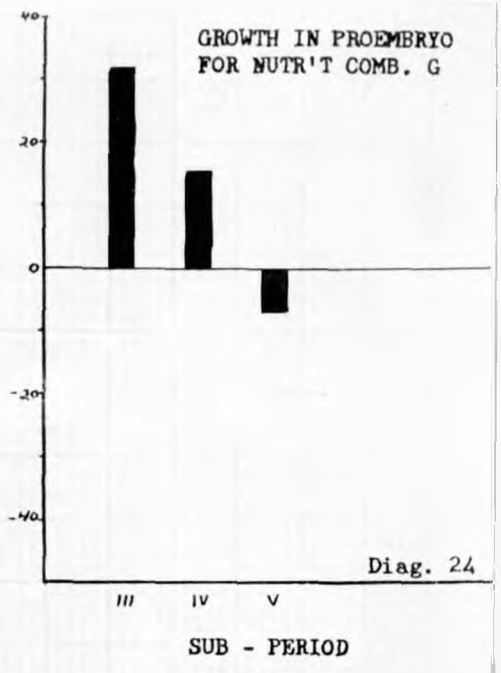


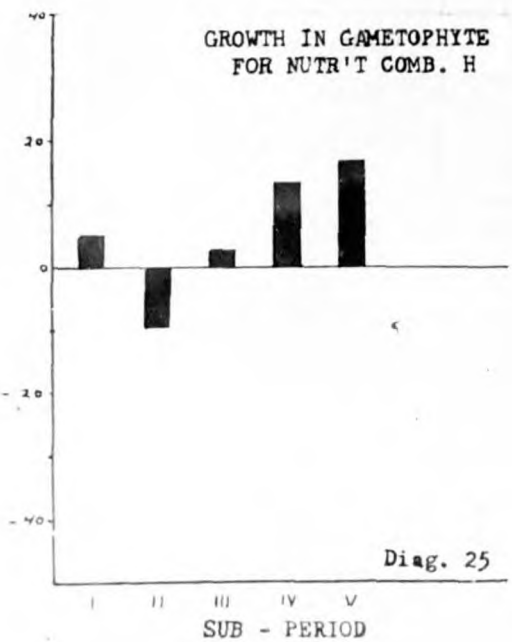


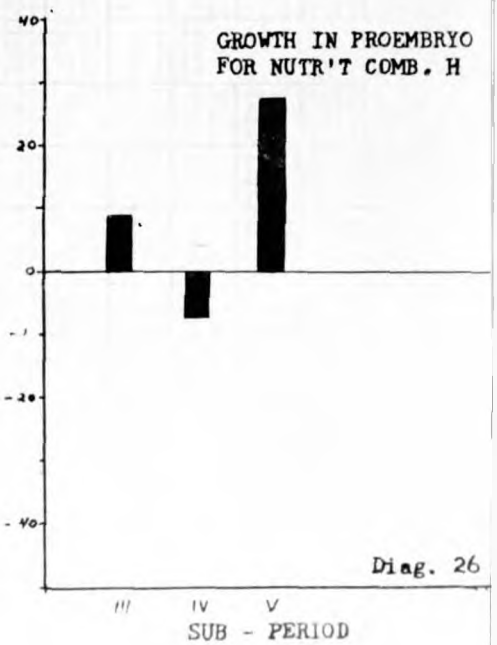


TEXT-FIGURE VII









Observation in growth and form:

Great irregularity, with respect to the date of fertilization in this species was noted. This is in accordance with the findings of Woods (18) but in disagreement with Coulter and Chamberlain (1), who set the fairly rigid date, July 1st, for the fertilization of Pinus. Corroborating the observations of Woods still further, it was noted that there was a variation, with respect to the time of fertilization, among the archegonia of a single prothallium and among the prothallia of a single cone. It was found that fertilization occurred most frequently between June 27th and July 1st. However, it was also observed to have occurred as early as the 25th of June and as late as the 5th of July.

Whereas Woods reported a high percentage of non-fertilization in his material, it was found that, except for those ovules dissected in the pre-fertilization period, a very high percentage of fertilization had occurred in the material under study. No ovule was found without at least one case of fertilization and frequently more than one archegonium was fertilized.

Two instances of what may be termed 'twin gameto-

phytes' were observed; that is a single ovule contained two gametophytes, complete with the usual complement of archegonia. The members of these pairs were smaller than usual and were somewhat misshapen, due possibly to their existence in the confined space normally occupied by only one gametophyte. Except for this, they appeared normal and showed no difference in behaviour in vitro as compared to the normal material. One of the two pairs was dissected in the prescribed manner and placed in culture.

On examination of the material in vitro, it was found that growth of some form had occurred in more than half of the cultures. This figure is slightly higher than those appearing in Table I; the reason being that, in some cases, growth had occurred in the proembryo without having occurred in the prothallium. The reverse was also noted.

The following criteria were used to determine whether growth had occurred in the tissues while in the culture media;

1. The appearance of mitotic figures in the in vitro material.

2. The increase in mean cell size, as compared to the cells of the in vivo material.

3. The increase of tissue mass relative to in vivo condition.

44. Change in growth direction.

5. Change in other morphological features.

6. The appearance of developmental forms not encountered in the in vivo situation,

7. The presence or absence of nuclei and the evaluation of their condition, relative to in vivo material.

Illustrations of the first criterion are found in Pl. VIII, figs. 2,4 & 6, and in Pl.VII, fig. 11; of the second in Pl.VII, figs. 1 & 3; as compared to Pl. VII, fig 8; of the fourth in Pl.VI, fig. 9; of the fifth in Pl. VI, fig. 14; as compared to Pl. VII, fig.8, showing thickening of the cell walls in vitro which is not observed in vivo; of the sixth in Pl. I, fig. 3; and of the seventh criterion in Pl.VII, figs. 3 & 5, as compared to Pl. VII, fig. 8.

Observations regarding the nuclei would seem to indicate that nuclear decline could occur in more than one manner. A normal nucleus as seen in vivo, has a discontinuous appearance when treated with the Feulgen reagent. Nuclei of this type are seen in Pl.VII, fig.11. Of the in vitro nuclei observed, some appeared to be continuous. By 'continuous' it is meant that there is, on treatment with Feulgen reagent, a uniform distribution and intensity of the resulting colour throughout the whole nucleus, masking any structural features if they are present. Some of the discontinuous, normal appearing nuclei gave the same response photographically as the continuous type, especially those of embryonic origin. An example

of this is shown in Pl.VII, fig.3. Of the continuous appearing group, of nuclei, several shapes were observed, ranging from the normal, near spherical, to the narrow bar-like shape. A crescent shaped nucleus was not uncommon.

The other possible mode of decline was represented by a decrease in the intensity of reaction with the Feulgen reagent, while the discontinuous nature was still observed. It is worthy of note that the second abnormal state did not occur in embryonic cells.

A difference was observed in the size and shape of the prothallial cells *in vitro* as compared to those *in vivo*. Although there was an increase in the relative mean cell size of the prothallia *in vitro*, the degree of this difference varied. This difference can be seen by comparing Pl.VII, figs. 3 & 4, to Pl. VII, fig. 8. Only relative and approximate estimations of the size of these cells could be made because of the nature of the preparation of the material and the state of the cells themselves. The absence of stain in the cell walls made it impossible to distinguish them clearly enough for accurate measurements. Also, the shape of the cells *in vitro* was very irregular and inconsistent.

There was only a slight increase in the size of the prothallial cells grown in the media which contained yeast extract and carbohydrate as well as mineral salts, while those

cells grown in a medium with only carbohydrate added were significantly larger than the cells *in vivo*. There was little difference, if any, between the size of the cells of material grown in the media which contained one percent carbohydrate as compared to that of the cells in media with two percent carbohydrate added. The cells which were seen on the outer edges of the growth area were larger than those in the inner portions. The degree of difference varied with respect to the amount of dead cellular remains in the outer regions; the greater the amount of this material the less the difference in size. There was little difference in size among the *in vivo* cells with respect to their position within the prothallium.

As mentioned previously, there was a significant alteration in the shape of the cells grown *in vitro* as compared to those of the *in vivo* material. In the latter instance, a high degree of regularity, with respect to shape, was observed among the cells of the prothallium, and their shape could be described as spherical, modified by the close association with adjacent cells. *In vitro* a variety of shapes were noted as seen in Pl.VIII, fig. 9, the cells of the outer regions of the growth area were more spherical than those of the inner regions, which exhibited a great variety of shapes. These shapes could be described as oblong, almost triangular and distorted rectangular. These are illustrated in Pl. VIII, fig. 6 & 7. The

cells along the channel generally resembled elongated rectangles. Those cells grown in media which contained yeast extract as well as carbohydrate tended to be more regular in shape than those grown in media without the yeast extract. There was little difference in the effects of different concentrations of sugar on the shape of the cells.

In vitro the walls of the prothallial cells were thicker than those *in vivo*, as shown in Pl. VI, fig. 14, and Pl. VII, fig. 8. Usually the degree of thickening was greater in the larger cells, except when they were located in the outer regions of the growth area.

Granular inclusions, prominent in the *in vivo* material, are found in great density in those cells below the lower regions of the channel, as seen in Pl. VII, fig. 8. These were suspected to be starch granules but iodine tests were inconclusive in establishing this fact. *In vitro* the density of these inclusions varied inversely with the degree of growth in those regions and in several instances the inclusions were not seen. Also, they were not seen in those cells which were in poor condition.

In most instances, the cells growing *in vitro* had very little cytoplasm, as compared to their size, while the cells *in vivo* had very abundant and dense cytoplasm and few vacuoles. The vacuoles in the cells *in vitro* were so large that only a

thin layer of cytoplasm was seen next to the cell wall and occasionally a thin strand was seen transversing the cell. As a result, the nuclei of these cells were located near the walls rather than being centrally located as in the normal cells. Generally the nuclei were somewhat smaller than normal and this was emphasized by the increase in cellular size. This was not true in all cases, as Pl. VII, fig. 4, shows cells with unusually large haploid nuclei. These conditions were less extreme in the smaller in vitro cells.

Binucleate cells were seen in several instances, and this was in accordance with the observations by Woods of this phenomenon. These are shown in Pl. VIII, fig. 10, but were difficult to represent photographically as the cell walls were not distinct. However, inspection of the slides left no doubt that these cells contained at least two nuclei, and in some instances even more. This phenomenon occurred only in the material cultured in media with only yeast extract added to the salt solution. It occurred most frequently in the medium containing two percent yeast extract than in that with only one percent. In some cases one of the nuclei appeared to be normal and the other was continuous in appearance after treatment with the Feulgen reagent. These latter types were smaller in size. In the same material, nuclei were seen which resembled budding yeast cells. These bud-like pro-

trusions were much smaller than the nucleus itself and were seen in various numbers on all surfaces of the nucleus.

Another observation worthy of note was that concerning the disorganization and degeneration of the jacket surrounding the archegonia. *In vivo*, following fertilization, the jacket is distorted by the beginning of suspensorial elongation. Continuation of this elongation results in the rupture of this jacket and the cells break apart and die. Their nuclei become small and irregular in shape and give a continuous appearance after the Feulgen reaction. This process begins at the end of the archegonium where the suspensor is formed and gradually progresses to the opposite end. There the cells remain intact for some time but eventually they too degenerate.

In vitro the course of events was found to be much the same but, instead of the apparently organized degeneration, seen *in vivo*, a disorganized break up and decline of the cells was observed.

This occurrence was most evident in the early post-fertilization stages of embryonic development. In both situations, dead cells were seen in the cavity where remnants of the egg cytoplasm were also seen. As a result of the thickness of the sections these appeared to be part of this cytoplasm, and in some instances, only the nuclei were observed. This

phenomenon was difficult to depict photographically as it usually involved several sections, but Pl. VII, fig. 6,7,9, & 10, shows some of the features involved.

Growth did not occur at random in the prothallium, nor did it occur uniformly throughout the tissue. To facilitate further description, two terms of reference should be described. The term 'channel' has been used previously and refers to that cavity created in the prothallium at the time of suspensorial elongation. This channel increases in size as embryonic development progresses, and is conical in shape with its base opening into the chamber. The chamber is that space previously occupied by the archegonial cluster. Several layers of dead cells line the channel in both the in vitro and in vivo condition. Pl. VII, figs. 4 & 8, illustrate this feature.

In vitro growth, when it occurred, was most frequently and extensively located about the channel and extended outwards to a greater or lesser degree. Generally, it could be described as occurring in a band which encircled the channel and this band was in turn encircled by a band composed of dead cells. The extent of these two regions usually varied inversely with respect to each other. The region enclosing the chamber was not as consistent with respect to the presence or absence of growth. Frequently, when growth was observed in

the channel region, it was conspicuously absent from the chamber region. This situation was more prominent at the time of suspensorial growth. Tissues excised at later stages of embryonic growth showed the occurrence of growing cells in this region, but its appearance was such as to indicate that the origin of growth had been in the lower channel region.

At the time of this apparent inhibition of growth in the region described, growth had occurred in the region above the chamber. The cells involved here were generally smaller and so occurred in greater density. Later, when growing cells were observed in the region about the chamber, no growth was seen in that area above the chamber. This apparent inhibition of growth seems to occur just after the degradation of the jacket cells in the particular centres.

In the pre-fertilization stages of the prothallia, growth was observed involving the cells between the jackets of the archegonia and those of the region about the archegonial cluster. Pl. VII, figs. 1& 2, illustrate this situation. The jacket cells themselves appeared to have been involved and in some instances, even appeared to have been the centre of growth. The growth pattern, as illustrated in Pl. VII, figs. 1 & 2, and Pl. VIII, fig. 9, indicate a degree of radiality.

Examination of the embryonic masses in the <u>in vitro</u>

material, indicated that there were several variations in growth as compared with the *in vivo* material.

The conditions of healthy embryonic cells were much similar to those occurring *in vivo* except for those of the smaller *in vitro* masses. Here the nuclei were usually smaller then the others. In some of the masses the conditions were quite poor and desoxyribose nucleic acid appeared to have diffused into the cytoplasm. Pl. II, fig.1, and Pl.IV, fig.12, show this condition.

There was a significant increase in the number of these groups of embryonic cells *in vitro*, especially in the later stages of embryonic development. For example, in one piece of tissue seven groups were seen, six of which are shown in Pl. III, figs. 10,11 & 14, and Pl.IV, figs.1,2, &4. *In vivo* material of comparable embryonic development would have about three proembryos. This increase in number of groups, as compared to *in vivo* conditions, was found in all material examined in which growth had taken place.

In vitro the frequent occurence of small or medium sized groups was observed, while *in vivo* they were seldom encountered, except in the stages of suspensorial elongation and proembryo establishment. Illustrations of this type of groups are found in Pl. I,figs. 1,2,&4;Pl.III, figs.1,9,11 & 13, and Pl.VI, fig. 7.

The number of the larger groups of embryonic cells that were seen in vitro was closely related to the number of proembryos seen in the in vivo material, of comparable embryonic development. Examples of this group are seen in Pl.I fig.7; Pl.II, fig.1;Pl.III,figs.5 & 6,;Pl.IV, figs. 3 & 5; Pl.V,figs. 1,2,& 14;and Pl.VI, fig. 2. Also Pl.III, figs.10, 11, 12, & 14, and Pl.IV, fig.4, illustrate the renge in size of embryonic masses found in one piece of tissue.

The size of the group of embryonic cells was determined by the number of nuclei that were seen in the largest section of the group, rather than by the physical extent of the mass. The groups with fewer than eight nuclei were classified as small, those with eight to twenty as medium, and those with more than twenty nuclei were classified as large.

Embryonic groups were seen throughout the channel and chamber, and generally there was a correlation between the size of the groups and their position. The largest groups of cells were most frequently found in the lower third of the channel and the size decreased as the distance from the chamber decreased. However, there were exceptions to this, as in several instances relatively large to medium sized groups were seen in the chamber, or only a short distance from it, in the channel. Pl.I, figs. 4 & 9;Pl.II, fig.8; Pl.III,

figs. 2 & 5, and Pl.V, fig.7, are illustrations of this exception.

In the channel there were several small and medium sized masses observed, but usually these were closely associated with the edges of the channel as seen in Pl.II, fig.14; Pl.III, figs. 1 & 3; Pl.IV, fig. 13, and Pl.VI, figs. 3 & 7, or they were remnants of declining proembryos as in Pl. I, fig.12, and Pl.II, fig.2.

There did not seem to be a preference of position in the chamber, as groups were observed in both the central portion and near the edges, including the top. Examples of these placements are seen in Pl. II, figs. 3,9,10 & 11;Pl.III, fig. 2; Pl.IV, figs. 6,7, & 9, and Pl.V, fig. 6 & 10, showing those associated with the edges and Pl.I, figs. 3, 13 & 14;Pl.III, figs. 11, 12 & 13; Pl.IV, fig.8, and Pl.VI, fig.4, showing those that were centrally located.

According to the literature (1,4,9 & 18) and the writer's experience, the proembryos *in vivo* are oblong in shape with parallel sides and rounded tips. However, *in vitro* the proembryos exhibit radial or three dimensional growth to a greater or lesser extent. The degree of this radiality was generally found to be least in the larger groups, and increased as the size of the mass decreased. A comparison of Pl.I,figs. 7,8 & 9; Pl.III, fig.14, and Pl.IV, fig.9, to Pl.I, fig.1,12

& 13, and Pl.V, fig.5, illustrate this statement. Exceptions to the above are illustrated by Pl.III, figs.2,5,6,7 & 8; Pl.IV, fig. 4, and Pl.V, figs. 1,12 & 14, which show large proembryos with pronounced radiality. Size appeared to be related to position, and therefore, radiality had an apparent relation to position. Generally, radiality increased as the distance of the group of embryonic cells decreased with respect to the chamber. Here the masses with the highest degree of radiality were seen. Just as there were exceptions to the generalization regarding size and position and size and radiality, so there were exceptions to that regarding radiality and position. The greatest expression of radiality was noted in the medium sized groups of embryonic cells, as seen in Pl.I, fig.1; Pl.II, fig.4; Pl.III, fig.11; Pl.V, figs.5,8,9 & 10 and Pl.VI, figs.5 & 8.

In a few instances, there were indications that radiality existed in the arrangement of groups of cells as well as in the arrangement of cells within the group. Pl.I, fig.3, and Pl.IV, fig.10, are illustrations of this, but it is difficult to illustrate adequately in a photograph, as inspection of several sections is necessary to observe it completely.

From the literature (1,4,9 & 18) and from inspection of the *in vivo* material it was found that the axes of the proembryos coincided with those of the archegonia. *In vitro*

this was not observed in every instance, and in some proembryos the axis of development was seen at various angles to the axis of the archegonia. The extreme expression of this tendency is revealed in those groups where the direction of growth was completely opposite to that *in vivo*. This phenomenon was seen in its various degrees of expression in the following figures: Pl.I, figs.8,9, & 10; Pl.II, fig.4; Pl.III,fig.4; Pl. IV, figs. 3,4, & 14; Pl.V, figs. 11,12,13 & 14, and Pl.VI, figs. 1 & 9. The ultimate expression of alteration of growth direction is that of radiality where growth tends to occur in all directions, rather than in one only.

The phenomena of increase in numbers, variation in size of the groups, and the variation in growth direction and radiality seem to have correlation with the media in which the tissues were grown. Material cultured in media with both sugar and yeast extract added showed the greatest number of embryonic groups which were larger and exhibited greater degree of radiality as compared with those cultured in other media. Also there was an increase in the appearence of groups with abnormal directions of growth and the number of exceptions to the generalities drawn with respect to size, position and radiality in this type of medium.

There was a decrease in the incidence and extent of the alterations in the expression of embryonic growth, when only carbohydrate was added to the modified Crone's solution. This

decrease was greatest when one percent rather than two percent carbohydrate was added.

There was little difference in the results observed with respect to the sugar used, at the concentration of one percent. However, there was a significant difference at the two percent level; sucrose affected the growth expression in a manner resembling the effect caused by media with sugar and yeast extract added, more closely than that caused by the medium with two percent dextrose added.

There were a few abnormal phenomena which occurred in only one or two instances but are worthy of note at this time. One, as shown in Pl.V, fig.3, was the appearance of a group of embryonic cells located within the archegonium, completely enclosed by jacket cells. These showed little, if any distortion of shape, as compared with those of the prefertilization period. These jacket cells also did not show any degree of degeneration. The arrangement of the cells within this group was definitely spherical, while those in the outer regions were significantly larger than those inwardly situated. There was no evidence of a suspensor having been formed.

A similar arrangement of embryonic cells located in the chamber was encountered and is shown in Pl.V, fig.4.Although both masses were similar in general appearance there were significant differences. There was no enclosing jacket seen in the second instance, nor was there any evidence of jacket cell

remains. However, cellular remains were seen surrounding the second mass. Its nuclei were smaller than those of the first mass, and there was a decrease in the amount of cytoplasm. The first group described was found in tissue excised on July 5th, 1954, and the second in tissue excised on July 11th, 1954.

Pl.VI, fig.1, shows a proembryo which exhibited curvature in its shape resulting from growth *in vitro*. It was not spherical but its appearance indicated that the direction of its growth had gradually changed. It was located in the lower portion of the channel. More or less similar tendencies were noted in material shown in Pl.II, fig.6, but in these instances, the tendency was not as definitely expressed.

The group of cells, shown in Pl.IV, fig.5, had an indefinite shape and did not indicate a definite direction of growth, or radiality. The component cells resembled meristematic cells *in vivo*, and there was a high percentage of mitotic figures. Pl. VIII, fig.5, shows the same material as photographed under high power. The mass of cells was longer than it was wide and was situated about midway down the channel, which was quite narrow especially at that point. A few other instances of the phenomenon were noted, but they were much weaker in expression, although they could be classified as material showing this tendency.

Pl.VIII, Fig.1, a high power photograph of the material shown in Pl.VI, Fig.4, shows a mass of substance of very unusual appearance, located in the chamber. This has never been recorded as occurring in this material for either *in vitro* or *in vivo* conditions, and appears to consist of an irregular mass of cytoplasm with nuclei and nuclear material dispersed throughout. The nuclear material occurred as irregular masses or in groups of structures which resembled chromosomes. Two other instances of this unexpected phenomenon were noted in the material studied but they were not as large or distinct.

DISCUSSION

The procedure developed seems to provide a satisfactory basis for the establishment of a standard procedure for the culture *in vitro* of the proembryo of *Pinus*.

The method of dissection is adequate but requires some modification. In this instance there was a low incidence of shock reaction resulting from dissection with the micromanipulator as compared with that experienced by Woods (18). Because of this, the use of the micromanipulator was justified. Also, its use is desirable in the standard procedure as it permits more accurate and controlled dissections.

In regard to the pattern of dissection of the prothallia, it would be advisable to increase the extent of the zone of residual, prothallial cells surrounding the proembryo at the early stages of its development, as it was found that these stages are more sensitive to shock. This zone could be decreased in the later stages of proembryo development as these are less sensitive to shock. Thus a more uniform environment for the proembryo would be insured, for with fewer cells there would be less obstruction to the uniform disposition of artificial nutrients with respect to the proembryos.

The precautions taken against contamination were suc-

cessful in reducing its occurrence as compared with that experienced by Woods (18). However, refinements could be made to decrease it still further. One might be in connection with the use of the moist chamber of the micromanipulator. During this work, only two chambers were available and this required that many dissections had to be made in the same chamber. This was not critical for the first group of excisions but became so in later stages of the work, as indicated by the increase in contamination which occurred at this stage. Contamination occurs mainly at the steps where the excised prothallia are placed in and removed from the chamber. The first of these steps introduces contaminants into the chamber where they can be spread to other prothallia. The large gametophytes are more susceptible to contamination from the air, possibly by virtue of their size, and so for this stage of development the chamber receives more contaminants than at earlier stages. The use of several chambers, especially at these later stages of development of the gametophyte, would diminish the amount and extent of contamination.

The ineffectiveness of penicillin is not considered a loss, because contamination can be reduced by other means. It can be omitted in the standard procedure. This is advantageous as it is desirable, according to the preliminary terms

of the experiment, to preserve simplicity in constitution of the medium.

The acceptance of the Feulgen reagent into the standard procedure warrants some consideration. In a survey of the literature, it was found that several workers questioned the specificity of the reagent for desoxyribose nucleic acid. The basis for this doubt was the fact that there were instances recorded where the Feulgen reagent imparted colour to material other than the nucleic acid, and other instances where it did not give a positive reaction with desoxyribose nucleic acid. The latter instance was experienced in this work.

The occurrence of the former was discussed by Hillary (3), who reported the occurrence of colour in structures containing starch, suberin and other similar substances. Stowell (12) reported on the application of the Feulgen reagent, and described it as a reaction occurring between aldehyde groups, produced on hydrolysis of desoxyribose nucleic acid and fuchsin sulphurous acid which results in a product having the characteristic magenta colour. Thus, the reaction is not specific for the aldehyde group of the hydrolysis product of the nucleic acid, but will occur with any aldehyde group of compareble reactivity. This would account for the occurrence of a positive reaction with starch, suberin and lignin which contain carbohydrates, if these carbohydrates could produce the aldehyde

groups of the required activity. Stowell (12), however, accounted for the presence of the apparent reaction with these compounds by indicating that their colouration is not due to a reaction with fuchsin sulphurous acid, but to their absorption of impurities which may occur in the reagent. He reported that this reagent, prepared using the Coleman modification of filtering the reagent through activated charcoal before use, did not give this apparent positive response to the Feulgen reagent.

Any factor which would inhibit the availability of the aldehyde group would result in a negative response to the reagent. Hillary (3) reported that the presence of tannins impeded the reaction, and Lessler (7) showed that aldehyde coupling reagents, such as bisulphite, semicarbazides, phenylhydrazine and hydroxyamines, when applied to the material before the Feulgen reagent, caused a negative response. It would follow that similar groups, such as the amino groups of certain amino acids, could have the necessary reactivity to combine with the aldehyde group, thereby removing it from the reaction with fuchsin sulphurous acid and so cause an apparent negative reaction.

This could account for the failure in the appearance of the expected results with the Feulgen reagent in the second group of material, described in the preceding section. As

there appeared to be a correlation between this second group and the tissues which gave a faint reaction with the Feulgen reagent, this explanation should also account for this latter type of response. Being a chemical reaction, the Feulgen reaction obeys the laws of such reactions. Thus, if the concentration of the aldehyde-binding groups is less than that of the aldehyde groups produced on hydrolysis, there will be an excess of the latter groups and they would still be free to react with the fuchsin sulphurous acid. In this case the concentration of the aldehyde groups would be the limiting factor and so decrease the amount of product. Thus, the intensity of the colour would be decreased.

Nuclei of the proembryonic cells were not observed to show the second type of failure in response to the Feulgen reagent, but they were observed to give a weak reaction. This would be expected on the basis of the above hypothesis, as they contain twice the amount of nuclear material as the nuclei of the prothallial cells. Therefore, it seems reasonable to assume that they would have a greater concentration of aldehyde groups available for reaction with the Feulgen reagent. It would take a very large concentration of aldehyde-binding groups to inactivate all of these aldehyde groups.

Non-response occurred in those tissues with a high degree of cellular degeneration. Cellular degeneration in-

volves the destruction of structural material into its simpler component parts, such as proteins into smaller peptides and amino acids. Freed from their structural location, such compounds, under the influence of the solvents and solutions, used to remove the paraffin and replace it with water in the cells, could diffuse over the cut surface of the section and so enter the nucleus. There, upon the production of the aldehyde groups, these compounds could react with them, making them unavailable for the Feulgen reaction.

If this was the case, these undesirable compounds might be removed with prolonged washing before the Feulgen reagent is applied. A possible solvent would be 95% ethanol which Stowell (12) recommended for the removal of carbohydrates.

Of the media utilized, one would appear to have outstanding attributes to afford the development of a good primary medium. The figures appearing in the tables and graphs do not indicate the actual efficiency, but only the relative efficiency of the media with respect to their ability to support growth in vitro, because they are not adjusted to account for the failure in growth resulting from shock. This adjustment could not be made under the conditions of this experiment.

Analysis of the tables and graphs indicate that the media containing yeast extract and sugar in addition to the mineral salts of the modified Crone's solution, gave the high-

est and most consistent incidence of growth. Media with only sugars added supported a lower incidence of growth and were not consistent in their ability to do so. This was more prominent in the media with one percent of the sugar than in those with two percent of the sugar added. Media composed of yeast extract and mineral salts were unsatisfactory in the promotion of growth and there was little difference in this ability between the media with one percent and that with two percent yeast extract.

The results are not conclusive enough to warrant a selection of sucrose or dextrose as the most suitable carbohydrate for the basic medium for the growth of Pinus tissue in vitro. They were also unsatisfactory for the selection of the optimum concentration of the modified Crone's solution. They effectively indicate however, that the proembryonic tissue is less sensitive to the concentration of Crone's solution than is the prothallial tissue. The proembryo revealed a slightly higher incidence of growth in the higher concentrations, while the results for the prothallium indicated that the lower concentrations were more suitable for its growth. In the latter, the concentration of Crone's solution was a limiting factor only in the poor media. It is possible that osmotic effects are involved.

Reports by La Rue,(6), White (17), and van Overbeek (16) substantiate the findings. White stated that, for growth in

vitro beyond ' residual growth', the medium should contain salts, to provide the major and minor ions, carbohydrates, vitamins, and some organic supplement. The term 'residual growth',(White acknowledges Carrel as the author) is used to designate that increment in growth which occurs at the expense of internal nutrition.

White indicated that sucrose and dextrose are the best carbohydrates and that sucrose is probably the better of the two. However, he thoughtthat the efficiency of sucrose depends upon the concentration of phosphate present in the medium and that the percentage to be used in the medium is largely determined by osmotic effects. Van Overbeek *et al* (16) reported that sucrose was a better carbohydrate source than dextrose and that two percent concentration was optimal for growth *in vitro* of *Datura* embryos. In view of this, sucrose rather than dextrose seems to be the better source of carbohydrate for the *in vitro* culture of some plant tissues, and it is recommended that it be used in the development of the basic medium, which should also include yeast extract or a substitute giving comparable results.

Yeast extract, used in this instance, supplies vitamins and other organic compounds. Also, it probably supplies the organic factor or equivalent mentioned by White. La Rue (16) used media similar to the one outlined in the procedure, for the

growth in vitro of maize endosperm. He reported that yeast extract was essential for growth, supplying amino acids and other substances essential for growth. Also he found that sucrose, lactose, and dextrose were effective in promoting growth and their efficiency was in the order named. This was determined on the basis of wet weight, which he used as the criterion of growth.

The irregularity in the time of fertilization, observed in this instance and reported by Woods (18) would indicate that the development of the ovule determines the time of fertilization and that its development is in turn controlled by the environment.

With reference to the discussion of the Feulgen reagent, it would seem that the second method of nuclear decline might have been on artifact. The appearance of the nuclei of this group suggested that there was a possible decrease in the amount of desoxyribose nucleic acid present as compared with normal nuclei. Possibly some of it had combined with other groups. The fact that this type of nuclear decline was not very frequent in the prothallial cells and did not occur in the embryonic cells would substantiate this explanation.

Comment on the response of growth in vitro of gametophytic tissue to the chemical and physical factors of the environment may lead to theoretical conclusions. The size and shape of the cells were affected by the result of the inter-

relation of both types of factors in some cases, and in others one type was the critical and determining one. In vitro, the cells tend to become enlarged, and this enlargement tends to be spherical in its expression. However, the presence of adjacent cells decreased the degree of the expression of this tendency, with the greatest expression being observed in the outer edges of the group. In some instances, where there was a great amount of cellular remains surrounding these outer edges, the tendency was not as great and varied indirectly with the extent of this material. Also, when the material was very actively growing and indicated cellular reproduction, the cells tended to be smaller than those in growth areas with a low rate of cell reproduction. The evaluation of the above would indicate that the presence of physical barriers is instrumental in the determination of the size of cells in tissues. Also, the great irregularity in shape might be interpreted as the result of the competition for space among the individual cells. Frey-Wyssling (2) attributes cell growth, with respect to shape, to the difference in growth potential within a cell wall. If this is true, then the physical presence of adjacent cells or other obstacles influences the location and quality of these potentials.

The chemical factors of the media affects the shape and size of cells by regulating growth in its developmental

aspects. Media with sugar and yeast extract added increased the tendency for cellular reproduction, and as a result, the cells involved were smaller in size and more regular in shape, and had relatively dense cytoplasm and thin walls. Media with only sugars added supported the growth of individual cells rather than their reproduction. As a result, the cells were larger, more irregular in shape, and had thicker walls. Cells on the outer regions of the growth area still had thin walls as these cells were probably still increasing their volume. Secondary wall formation, according to Frey-Wyssling (2) occurs after the cell has attained its capacity for expansion. In the case of the internally situated cell with the thick walls, their capacity for expansion could be determined by the amount of space available to it for expansion.

There has been much controversy regarding the fate of the jacket cells after fertilization and the elongation of the suspensor. This has been reviewed by Coulter and Chamberlain (1), and the following account is derived from that source.

The jacket cells differ from the rest of the prothallial cells in that they have thick walls which have pits. At first, it was supposed that these cells emptied their contents through these pits into the enlarging central cell and egg. Strasburger did not agree, and showed that these so-called migratory nuclei were in reality protein vacuoles.

Later, Arnoldi described the jacket cells as becoming ameboid and passing through the walls.

Some clarification of this problem is offered through the use of the Feulgen reagent. Bodies which might be similar to those seen by Strasburger and Arnoldi were noted in the present material and these were definitely nuclear in origin. However, they did not occur in the egg cytoplasm by movement through the walls of the jacket cells, but as previously described, they only appeared to be so situated because of the thickness of the sections. They were the remains of the degenerating jacket cells.

The degeneration of the jacket *in vivo* appeared to be organized while that *in vitro* was disorganized. This would suggest that the degeneration is controlled by the development of the proembryo, as Skoog (11) suggested with reference to the findings of Nutman. *In vitro*, the nutritional requirements of the proembryo were met by the medium. Thus there was no control for the decline of the jacket, and so it proceeded in a random fashion.

It is possible that the jacket cells control the growth of the cells surrounding the chamber, for it was observed that growth did not occur in the chamber region immediately following the degeneration of the jacket cells. If this apparent inhibition of growth was associated with the jacket cells, it was only effective after their degeneration, as growth was seen in

this area before this occurrence. Also, there was growth in the region above the chamber when there were some cells left intact in the top of the chamber.

The appearance of binucleated cells in the material was substantiated by the findings of Woods (18), who attributed this phenomenon to rapid growth. However, in this instance it occurred in tissues grown in media which are extremely poor in their ability to support growth, and did not occur in any other media. An explanation for this phenomenon, in accordance with these results, is that yeast extract contains factors which promote mitosis. When mitosis occurred in a medium without a carbohydrate and so lacked the material for further growth, even of a primary lamella, growth could not proceed and in the majority of cases, degeneration of the nuclei occurred. In this regard, it is notable that the two nuclei of one cell did not degenerate concurrently, but there was ample evidence of the decline of one before the other.

The phenomenon of 'budding nuclei' appears to be inexplicable at this stage. It was observed to occur in the same tissues as the binucleated cells, but there was no evidence besides the fact of common occurrence, to suggest that the two phenomena were related. No references were found in the literature with respect to this abnormality. However, Straus (13) reported the occurrence of several nuclear ab-

normalities in the endosperm of maize grown *in vitro*, but none of the types that he has described resemble that which occurred in these investigations.

The occurrence of the 'twin gametophytes' has two possible explanations; either two cells, rather than only one, of the sporogenous tissue gave rise to linear tetrads, and the surviving spores of each independently gave rise to a gametophyte or two spores rather than one, of a single tetrad survived to give rise to independent gametophytes. No record of the previous occurrence of this phenomenon was found and it is difficult to assess its implications on the basis of only two examples. The set of twins which were placed in culture reacted in a manner similar to that of the average normal gametophytes of similar development. They were in the pre-fertilization stage of development.

To discuss the alteration in growth of the proembryo of *Pinus* caused by the environment *in vitro*, it is advisable to review briefly the condition *in vivo*. The following account is derived from Coulter & Chamberlain (1), Johansen (4), McLean & Ivimey-Cook (9) and from observation of the *in vivo* material.

Upon fertilization, two diploid nuclei are formed which undergo mitosis, forming four nuclei which become aligned in a plane perpendicular to the axis of the archegonium at the non-micropylar end. There, partial cell walls are formed and one

end remains exposed to the egg cytoplasm. Mitosis follows to produce two tiers of four cells each; those of the lower tier have incomplete ones. Two separate series of mitosis occur to produce three and then four tiers of four cells each. All those except the ones of the upper tier have complete cell walls.

The cells of the second tier (from the bottom) begin to elongate and become the suspensors. This forces the cells of the bottom tier into the channel created in the prothallium. The cells of the upper two tiers remain in the chamber; those of the third tier are the rosette cells which can, under special conditions, produce proembryos. The continuation of the elongation of the suspensors force the tip cells further into the channel, where they enter into the competition for the available food. There can be a total of twenty-four proembryos in the same channel if all of six possible archegonia are fertilized. However, some of these are never formed and others never grow beyond a few cells. Approximately two weeks after fertilization only three or four proembryos can be seen and these are usually quite large.

From the above account it can be seen that there are several embryonic cells or groups of cells present in the channel in the early stages of embryonic development, but in normal

circumstances only one proembyro develops into the immature plant of the seed. Thus many proembryos abort. This may be due to lack of nutriment, but on the other hand may be an effect of phylogenetic control. However, transfer of the prothallium to artificial medium seems to prolong the lives of these ill-fated masses. Therefore more embryonic masses are seen *in vitro* than *in vivo*, and the phylogenetic factor is not apparent.

There are several possible sources of these groups of cells. The cells of the upper tier, which are apparently nonfunctional, could be capable of reproducing under *in vitro* conditions. Also the rosette cells, which have been known to produce proembryos *in vivo*, should show an increase in this tendency when placed in culture. These two groups of cells would be responsible for the many embryonic groups seen in the chamber, especially the small or medium sized ones, as their source was probably unicellular in size.

Another potential source would be those embryos which were losing in the competition for survival. These would account for the masses seen in the channel, especially those located near the mouth of the chamber. The size of the resulting mass would depend on the size of the initial group. It was found *in vitro* that the larger masses were most frequently

located well down in the channel, and the size of the proembryo decreased as the distance from the chamber decreased. Those masses associated with the edge of the channel were probably derived from the remnants of large masses which had declined *in vivo* before inoculation. Thus these groups, seen *in vitro* were of the small or medium sized groups as their initial source had probably been reduced to a few cells.

Media containing yeast extract as well as sugars, were generally found to be the best media to revive and initiate proembryonic growth. This is substantiated by the fact that more groups were seen in individual pieces of material grown in such media, than in those grown in other media. Also, these groups were larger in this type of medium than those in the others.

The degree of radiality expressed by a group of embryonic cells was determined by its size at the time of inoculation by the position in the channel and the medium in which it was cultured (*cf.* Pl.III,figs.5,6,7, & 8; Pl.V, fig.2; Pl.VI, fig.2.)

The larger groups, by virtue of their differentiation, although slight, were less able to express three dimensional growth. Small or medium sized groups, which probably originated from single or small groups of cells, were relatively undifferentiated at the time of their inoculation and were apparently free to express this three dimensional growth. Also

the presence of physical barriers, such as the edges of the channel, would hamper the expression of this tendency. Thus, the groups situated lower showed a slight tendency towards radiality probably because of the presence of both of these factors; the physical barrier of the channel edges and their relatively high degree of differentiation. Small groups, located in the chamber or high in the channel, had neither of these apparent limitations. Possibly because of this, they indicated a high degree of radiality. Groups, associated with the edges of the channel, generally showed a lower degree of radiality, in spite of their small size. This could be attributed to their position next to the channel edge, and to the presence of cellular remains observed about the masses.

When the medium is one which supports a high degree of growth, these previously mentioned factors are no longer critical for the expression of radiality. This is corroborated by the appearance of large masses in the lower portion of the channel possessing a high degree of radiality.

The large masses found in the upper regions of the channel and in the chamber, were probably placed there by the mechanical treatment of the tissues in the course of processing. Proembryos are not fixed in their positions in the channel, but rather, they are suspended in it by their suspensors. Thus, such loose masses could be shaken free and re-

located in another position. This is especially true of the masses located in the channel in other than the lower region. Here the channel is narrow and would tend to inhibit this relocation.

If this occurred before inoculation, these groups would have a greater opportunity for radial growth, as the physical barrier of the channel edges would have been removed. Those groups which showed a degree of radiality, comparable to that of the masses located in the lower regions of the channel, were probably relocated after their removal from the media.

This postulated relocation of embryonic groups would account for those masses whose growth directions were observed to be at various angles to the normal direction. For in those instances, the relocation was not completed. That is, the groups were only re-orientated in their position, not removed from it. Growth in vitro occurred in these groups in abnormal directions as if no disturbance had occurred. Thus it would seem that the direction of growth was determined by the differention and not by the orientation of these groups. This is significant, as it would indicate that the direction of growth in vivo, which is parallel to the axis of the archegonia and directed away from them, is determined not by genetic factors alone, but by the chemical and physical factors of the environment within the prothallium. Included in these are the bar-

riers of the channel edges and the directional source of nutrients.

Of special significance is the single case of pro-embryonic cells developing within the undistorted and unbroken archegonial jacket without the formation of a suspensor. This group of embryonic cells, seen in Pl.V, fig.5, is probably the first recorded occurrence of this phenomenon, as no reference to it or similar masses was found in the literature. Its origin was probably the first cells formed after fertilization. Since this material was excised within the period of fertilization, recorded in this instance, therefore it is probable that fertilization had occurred just previous to, or shortly after, inoculation. The nuclei resulting from fertilization probably did not align themselves in the customary plane, for, if they had, the expression of growth would not have been so definitely spherical. The presence of the intact, undistorted jacket gives further evidence that the origin of this group of cells was the nuclei resulting from fertilization. The complete absence of any indication of suspensor development indicates that this feature is determined in its occurrence by physical and chemical factors, although genetic factors may give the material the potentiality for its development.

The occurrence of a second group, similar in appearance substantiates the statement that the expression of growth in an

environment uniform with respect to chemical and physical factors, is spherical when the origin of that growth was a single or small group of cells.

The origin of the second group of cells could not have been the same as that for the first, as there was a distinct difference in the condition of the cells and the size of the nuclei between the two groups. Also there was no indication of the presence of a jacket. The fact that this group was excised six days after the end of the fertilization period was influential in deciding against the possiblity that the origin of this group was from the nuclei formed after fertilization. It could have originated from the rosette cells.

Although there was only one incidence of curvature in form, there is no doubt that it resulted from a gradual alteration in the direction of growth in some of the cells. A possible explanation for its occurrence is that the rate of growth in the embryonic cells exceeds the rate of channel formation, and this created a physical barrier which affected the growth direction. The channel at that position was wide enough to permit the masses to curve away from the channel tip. However, there is not sufficient evidence to warrant an hypothesis, but only to present the above suggestion.

The reason for a group of cells to abandon all semblance of shape and form, as in the mass shown in Pl.IV, fig.5, in its expression of growth, can not be set forth at this time,

as there is not sufficient evidence. They were definitely proliferating, however, as there was a high degree of mitosis observed, and the appearance of the cells indicated that they were undergoing reproduction. As well, these cells appeared to behave as individuals, and showed no evidence of normal differentiation. The general outline of the mass seemed to be determined by the edges of the channel which were close together.

The mass of material in Pl.VIII, fig.1, seems to suggest that nuclei can lose all normal features and become a disorganized (not disintegrating) mass of material. Similar occurrences were noted by Straus (13) in maize endosperm grown in vitro, although the extent of disorganization was not as great in the maize endosperm as it was in the material of Pinus. Straus referred to a disorganization which occurs after treatment with colchicine, which was similar to abnormal mitosis in untreated human cancer cells. The reason for the occurrence of this mass in this material is not apparent, and none can be suggested on the basis of this experiment. It is however, very important in assessing effects of growth controlling agents which, in future, will be added to the basic medium substantiated in this work.

CONCLUSIONS AND SUMMARY

The results indicate that excission of early embryonic masses of *Pinus sp.* for growth control experiments can now be effected by a standardized procedure. The attainment of growth in a statistically reasonable number of cases supports this claim. Moreover, where growth was achieved *in vitro*, modification of growth form and cellular developmental adjustment, found to vary with manipulation of the medium, provided further encouragement and justification for a proposal that the standardized procedure, developed here, is not over-simplified. If a fundamental or primary medium is to be selected from those tested it would be one which would include salts to provide the required ions, a carbohydrate, preferably sucrose, and yeast extract. The concentration of these components would depend on the results expected. Augmenting this with additional nutriments (organic) and growth control agencies might be expected to provide adequate experimental possibilities in future culture work.

With the application of the Feulgen technique it is now known that by examination of nuclei with respect to their size, condition and distribution, the extent and the nature of growth as well as the distinction between haploid and diploid

tissues can be ascertained.

Although only relative estimations of the effects of alterations in the chemical and physical environment on the expression growth were possible in these investigations, it was shown that these factors were influential in the expression of the growth potentials of cells. These results, in particular, substantiated the results of Woods (18), that the presence or absence of a suspensor is the result of a physiological response. Also they showed that radial growth occurs *in vitro* when its *in vivo* barriers, of limited disposition of nutrients and presence of adjacent gametophytic cells, are removed by dissection and by transfer to an artificial medium. From the evidence of the post-fertilization embryonic mass found inclosed by the archegonial jacket, it is seen that nuclear dispersal without polarity can be achieved before the advent of cell walls and this is likely to be the true primitive growth pattern.

The degree of variation was greater in the proembryonic material than in the prothallial tissue, and this is probably due to the difference in their degree of differentiation; the prothallium being more differentiated, even though its component cells are very similar in many respects.

The occurrence of such phenomena as random proliferation, 'budding nuclei', disorganized nuclear material, and twin gam-

etophytes are significant, as they indicate that there is much more to know than there is known about cellular behaviour in growth, as influenced by chemical and physical factors in the environment.

The significance of this work lies in its contribution to the understanding of the requirements of the basic conditions for the growth of *Pinus nigra* var. *austriaca* *in vitro* and in the establishment of standard procedure. Furthermore, it has contributed some understanding of the influences of the chemical and physical stimuli upon the expression of inherited potentialities.

DESCRIPTION OF PLATES

PLATE I

Fig.1. - Spherical embryonic mass located near periphery of chamber, Mag. 85X. In culture July 11-21. Medium: 50% mod.Cone's sol'n plus 2% dextrose.

Fig.2. - Embryonic mass in mouth of channel. Mag.85X. In culture July 11-21. Medium: 25% mod. Crnne's sol'n plus 2% dextrose.

Fig.3. - Three embryonic masses located in chamber showing radial arrangement. Mag.85X. In culture July 19-29. Medium: 12.5% mod. Crone's sol'n plus 2% dextrose.

Fig.4. - Embryonic masses located in lower portion of channel. Mag.85X. In culture July 11-21. Medium:50% mod. Crone's sol'n plus 2% dextrose.

Fig.5. - Embryonic mass associated with edge of channel. Mag.85X. In culture July 11-21. Medium:12.5% mod. Crone's sol'n plus 2% dextrose.

Fig.6. - Embryonic mass associated with channel edges. Mag.85X. In culture July 11-21. Medium:12.5% mod. Crone's sol'n plus 2" dextrose.

Fig.7. - Proembryo located in lowest region of channel. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 2% dextrose.

Fig.8. - Proembryo in lower third of channel showing irregular polarity. Mag.85X. In culture July 15-25. Medium: 12.5% mod. Crone's sol'n plus 2% dextrose.

Fig.9. - Large embryonic mass in chamber showing reverse polarity. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 2" dextrose.

Fig.10.- Radial mass with abnormal polarity located in upper region of channel. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 2" dextrose.

Fig.11.- Radial embryonic mass located near channel mouth. Mag.85X. In culture July 15-25. Medium:12.5% mod.Crone's sol'n plus 2% dextrose.

Fig.12.- Declining proembryos located at tip of channel. Mag.85X. In culture July 9-18. Medium:25% mod. Crone's sol'n plus 2% yeast extract.

Fig.13.- Small embryonic mass located in the chamber. Mag.85X. In culture July 11-21. Medium:50% mod. Crone's sol'n plus 2% yeast extract.

Fig.14.- Small group of embryonic cells located in chamber. Mag.85X. In culture July 9-19. Medium:25% mod.Crone's sol'n plus 2% yeast extract.

Pl. Ta. I

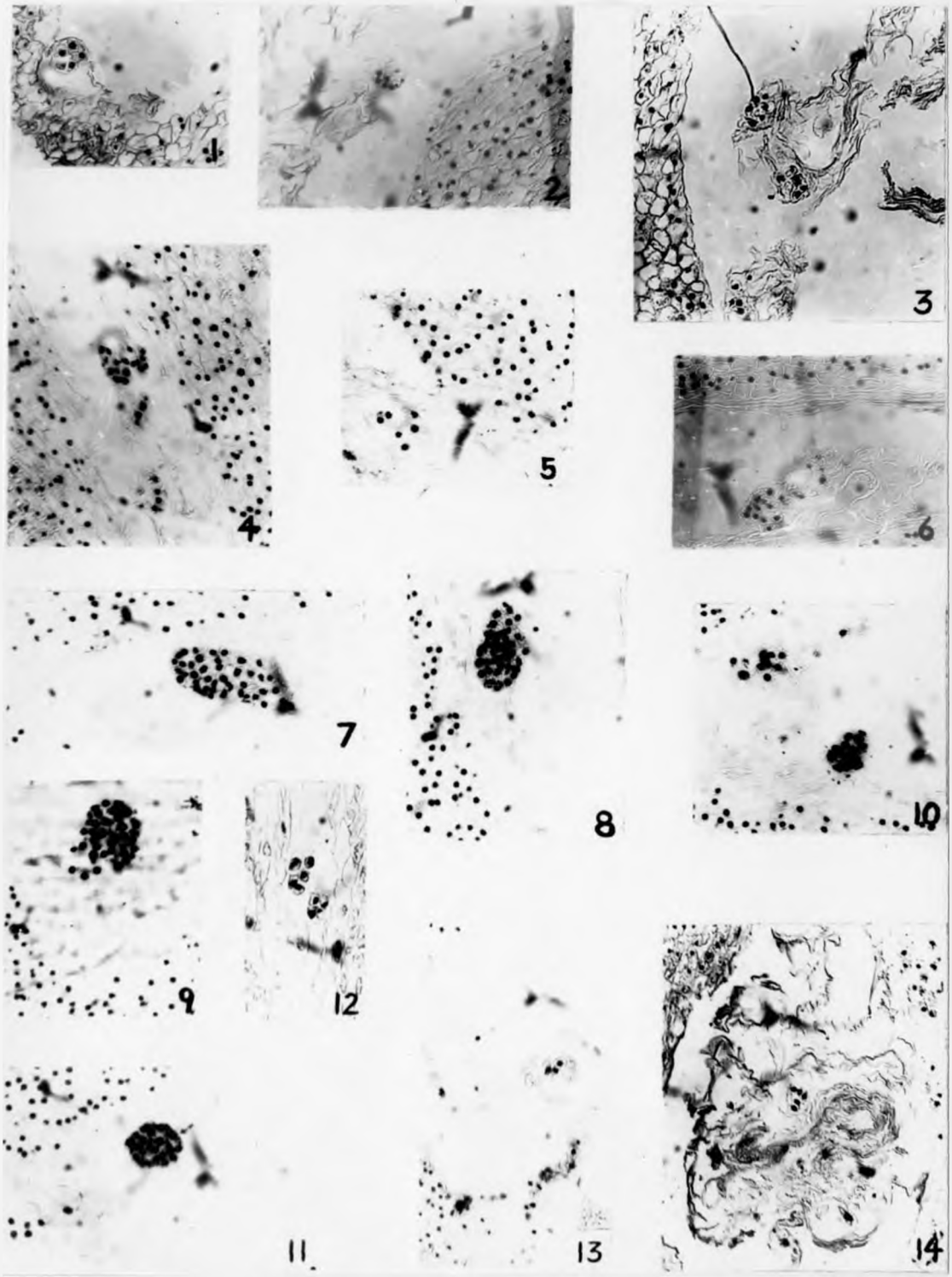


PLATE II

Fig. 1. - Several declining proembryos located mid-way down channel. Mag.85X. In culture July 11-21. Medium: 50% mod. Crone's sol'n plus 2% yeast extract.

Fig. 2. - Declining proembryo located in lower region of channel. Mag.85X. In culture July 13-23. Medium:50% mod. Crone's sol'n plus 2% yeast extract.

Fig. 3. - Small embryonic mass located in chamber. Mag.85X. In culture July 13-23. Medium:50% mod. Crone's sol'n plus 2% yeast extract.

Fig. 4. - Embryonic mass located in chamber with reversed polarity and some radiality. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 2% yeast extract.

Fig. 5. - Two small groups of embryonic cells located in chamber. Mag.85X. In culture July 17-29. Medium:25% mod. Crone's sol'n plus 2% yeast extract.

Fig. 6. - Degenerating embryonic mass located mid-way in channel. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 2% yeast extract.

Fig. 7. - Disorganizing mass of embryonic cells located in lower regions of channel.Mag.85X. In culture July 19-29. Medium:12.5% mod. Crone's sol'n plus 2% yeast extract.

Fig. 8. - Large embryonic mass located in chamber. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 2% yeast extract.

Fig. 9. - Embryonic mass in chamber showing poor condition. Mag.85X. In culture July 11-21. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.10. - Small mass located in chamber also shows growth pattern of cells above chamber. Mag.85X. In culture July 11-21. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.11. - Small group located in upper region of chamber. Mag.85X. In culture July 13-23. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.12. - Embryonic mass located in chamber.Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.13. - Small group of embryonic cells associated with channel edge. Mag.85X. In culture July 11-21. Medium: 50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.14. - Small declining proembryo associated with channel edges. Mag.85X. In culture July 9-19. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

PLATE II

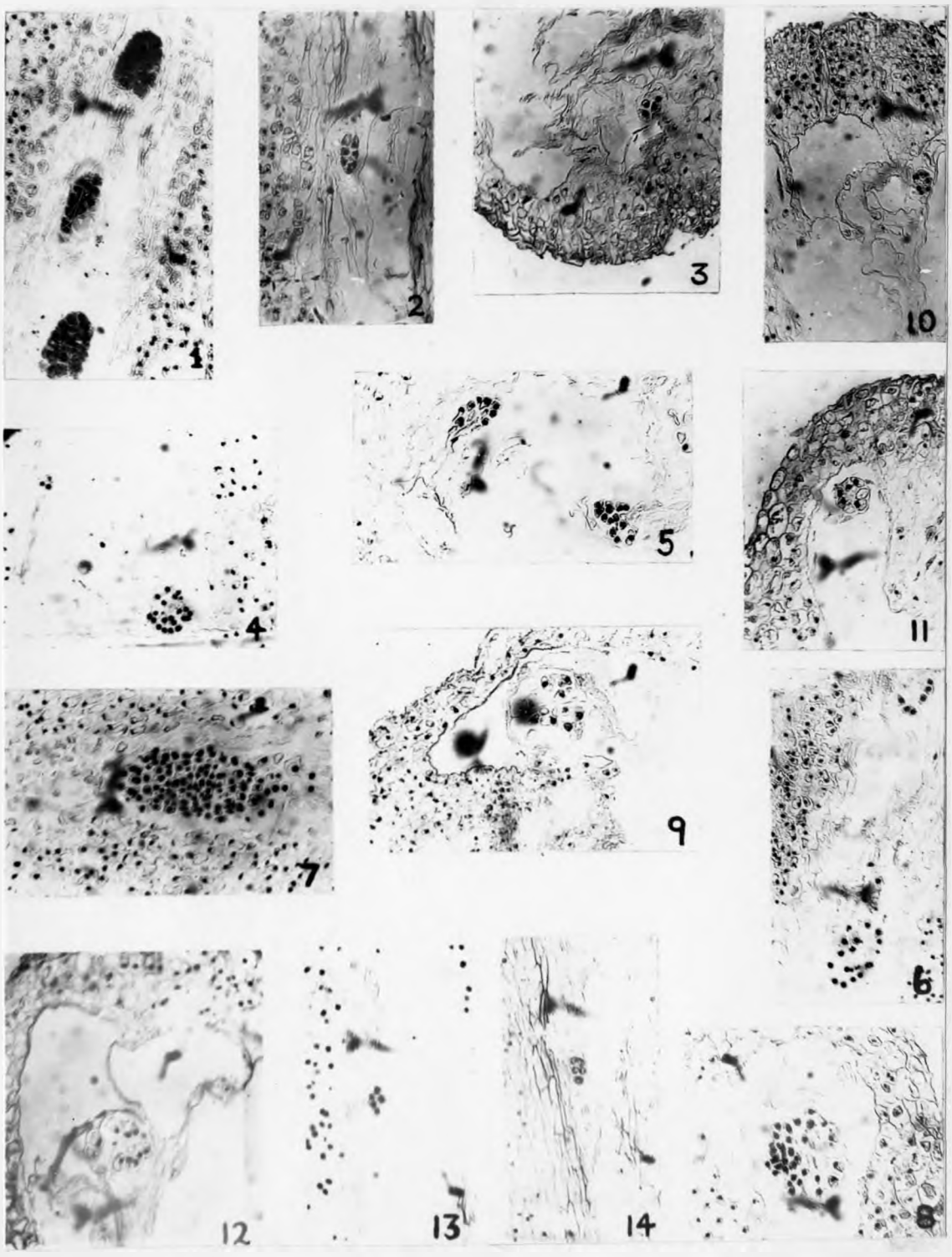


PLATE III

Fig. 1. - Small embryonic mass associated with edges of channel. Mag.85X. In culture July 9-19. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 2. - Medium-sized mass in chamber showing radiality. Mag.85X. In culture July 11-21. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 3. - Small embryonic cells associated with channel edge. Mag.85X. In culture July 19-29. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 4. - Group of embryonic cells in chamber showing abnormal polarity. Mag.85X. In culture July 19-29. Medium: 12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 5. - Large mass in lower regions of channel showing radiality. Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 6. - Two embryonic masses in lower regions of channel;lower one showing radiality. Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 7. - Large proembryo - lower regions of channel showing radiality. Mag.85X. In culture July 15-25. Medium: 50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 8. - Large embryonic mass at tip of channel showing high degree of radiality. Mag.85X. In culture July 19-29. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 9. - Small mass of embryonic cells located in chamber. Mag.85X. In culture July 13-23. Medium:12.5% mod. Crone's sol'n plus 1% dextrose.

Fig.10. - Embryonic mass associated with chamber. Mag.85X. In culture July 13-23. Medium:25% mod..Crone's sol'n plus 1% dextrose.

Fig.11. - Small embryonic mass located in chamber. Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% dextrose.

Fig.12. - Two embryonic masses located in chamber. Mag.85X. In culture July 13-23. Medium:50% mod. Crone's sol'n plus 1% dextrose.

Fig.13. - Embryonic mass located in chamber. Mag.85X In culture July 13-23. Medium:50% mod. Crone's sol'n plus 1% dextrose.

Fig.14. - Proembryo shown in lower portion of channel. Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% dextrose.

PLATE III

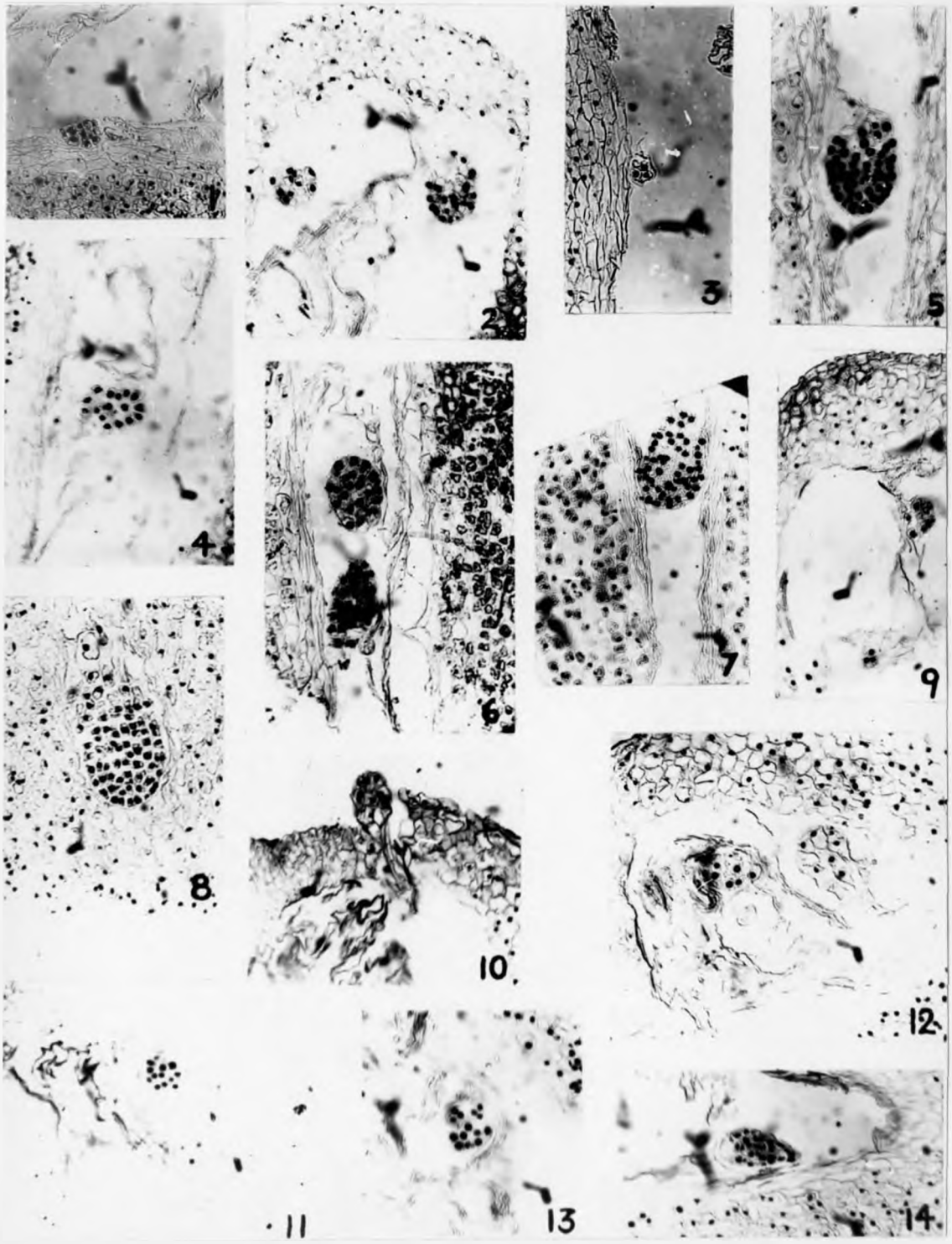


PLATE IV

Fig. 1. - Embryonic mass located in chamber. Mag.85X In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% dextrose.

Fig. 2. - Embryonic mass located in chamber. Mag.85X In culture July 13-23. Medium:25% mod. Crone's sol'n plus 1% dextrose.

Fig. 3. - Two proembryos in lower regions; one showing curvature tendency. Mag.85X. In culture July 15-25. Medium: 25% dextrose plus 1% yeast extract.

Fig. 4. - Embryonic mass mid-way down in channel showing radiality and abnormal polarity. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 1% dextrost.

Fig. 5. - Group of cells, halfway down channel showing high degree of proliferation. Mag.85X. In culture July 19-29. Medium:50% mod. Crone's sol'n plus 1% dextrose.

Fig. 6. - Embryonic mass located in chamber. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 7. - Embryonic mass located in chamber. Mag.85X. In culture July 17-27. Medium:25% mod. Crone's sol'n plus 1% yeast extract.

Fig. 8. - Embryonic mass located in chamber. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract.

Fig. 9. - Proembryo in channel mouth, showing reversed polarity. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 1% yeast extract.

Fig.10. - Proembryo located in chamber. Mag.85X. In culture July 19-29. Medium:50% mod. Crone's sol'n plus 1% yeast extract.

Fig.11. - Proembryo located at channel tip. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract.

Fig.12. - Declining proembryos located in lower portion of channel. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% sucrose.

Fig.13. - Embryonic mass associated with channel edge. Mag.85X. In culture July 15-25. Medium:25% mod. Crone's sol'n plus 1% sucrose.

Fig.14. - Proembryo in lower portion of channel showing abnormal polarity. Mag.85X. In culture July 15-23. Medium: 50% mod. Crone's sol'n plus 1% sucrose.

PLATE IV

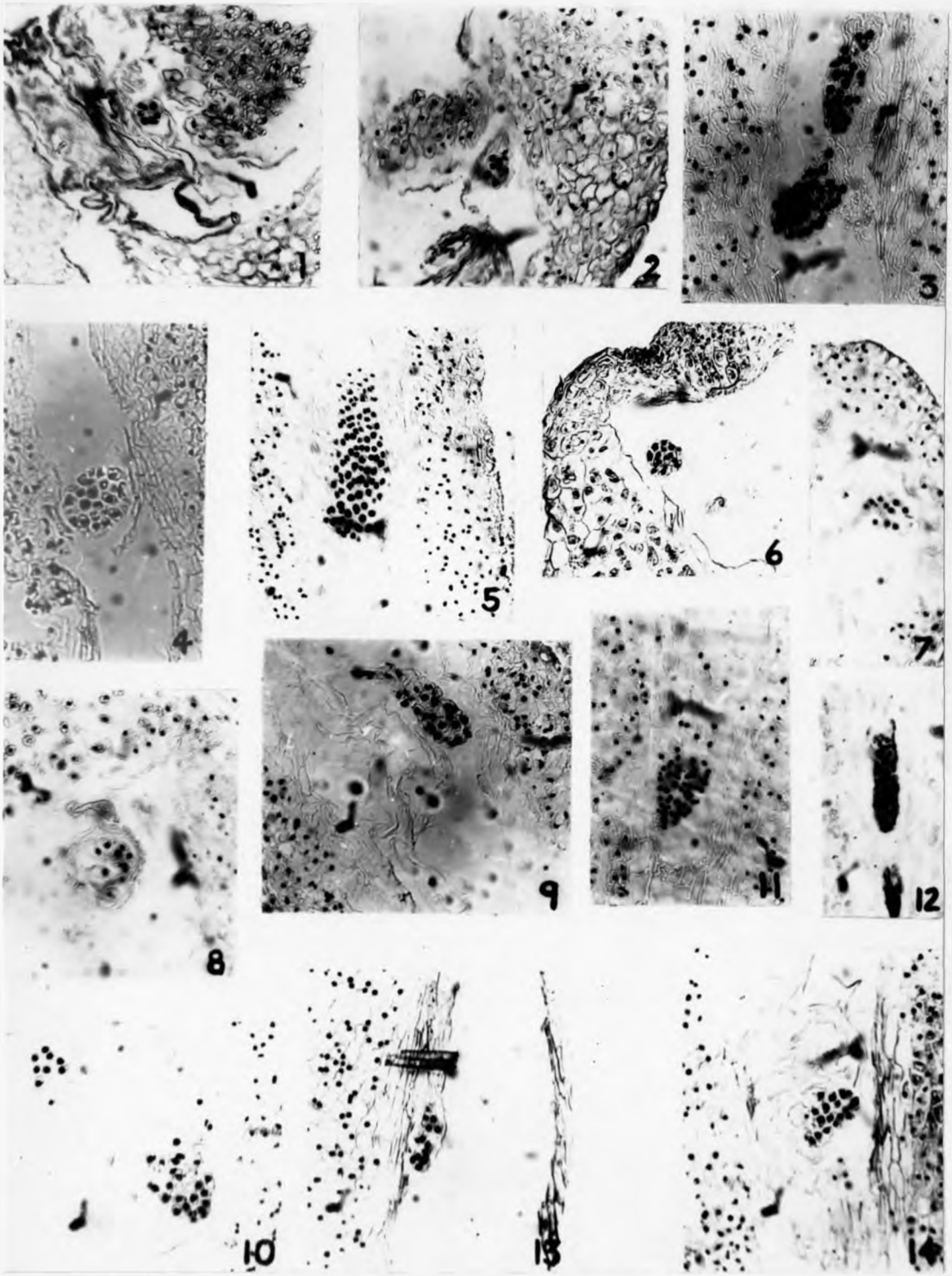


PLATE V

Fig. 1. - Two proembryos in tip of channel showing radiality. Mag.85X. In culture July 17-27. Medium:50% mod. Crone's sol'n plus 1% sucrose.

Fig. 2. - Proembryo located in tip of channel. Mag. 85X. In culture July 17-27. Medium:50% mod. Crone's sol'n plus 1% sucrose.

Fig. 3. - Mass of embryonic cells enclosed by jacket. Mag.330X. In culture July 5-15. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 4. - Embryonic cells in chamber. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 5. - Embryonic mass located in chamber showing radiality. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 6. - Proembryo located in chamber. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 7. - Proembryo located in chamber. Mag.85X. In culture July 15-25. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 8. - Mass embryonic cells located at top of chamber. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 9. - Mass of embryonic cells located at mouth of channel. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 10.- Mass of embryonic cells located at top of chamber. Mag.85X. In culture July 15-25. Medium:25% mod. Crone's sol'n plus 1% sucrose and 1% yeast extract.

Fig. 11.- Three groups of embryonic cells located in chamber. Mag.85X. In culture July 15-25. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 12. - Mass of embryonic cells located at mouth of channel. Mag.85X. In culture July11-21. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 13. - Proembryo located in channel showing abnormal polarity. Mag.85X. In culture July 15-25. Medium: 25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 14. - Two proembryos located in lower third of channel and showing radiality. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

PLATE V

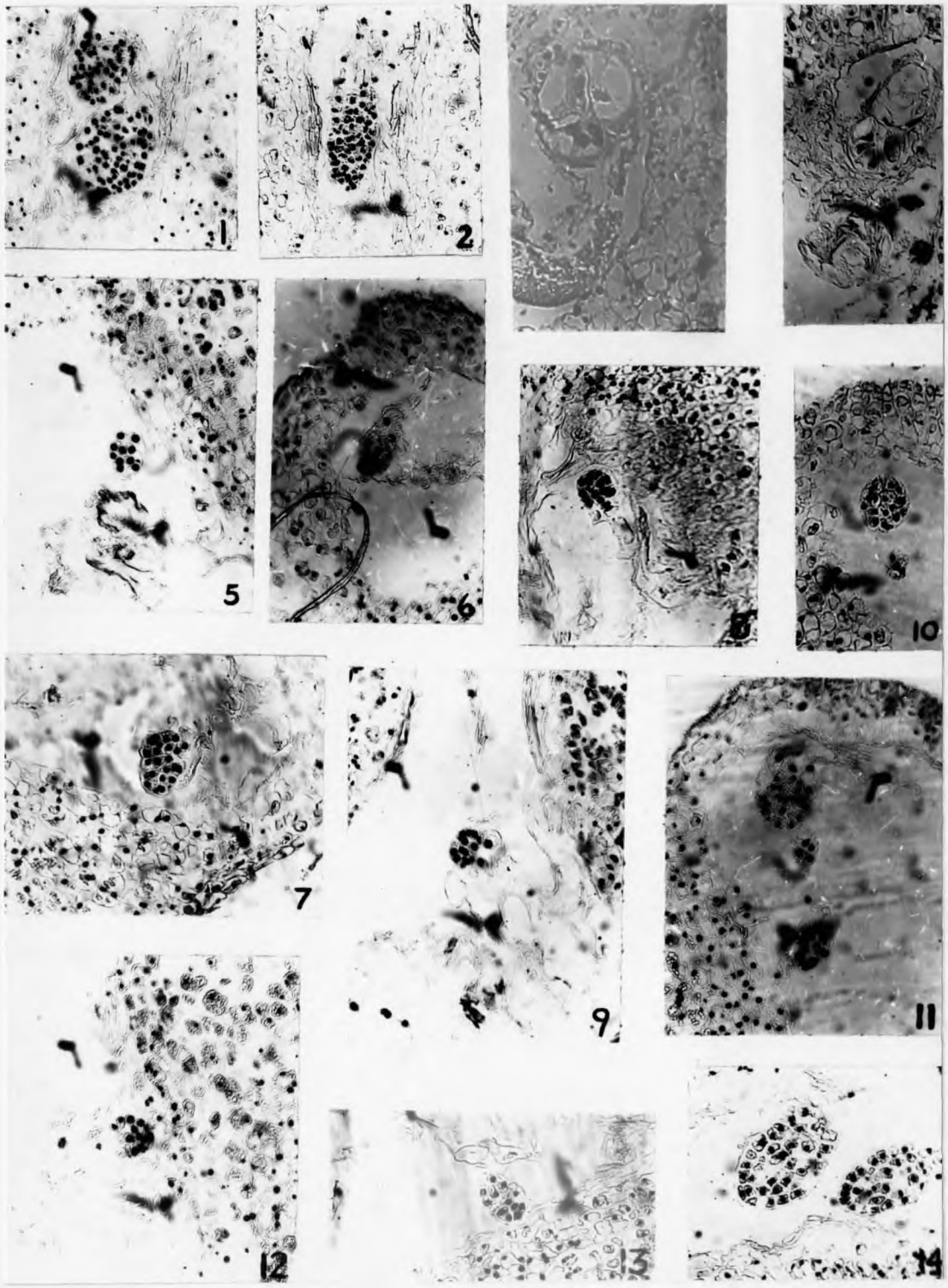


PLATE VI

Fig. 1. - Proembryo in tip of channel showing curvature in form. Mag.85X. In culture July 19-27. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 2. - Proembryo located in channel. Mag.85X. In culture July 15-25. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 3. - Masses of embryonic cells associated with channel. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig. 4. - Masses of embryonic cells and mass with disorganized nuclear material located in chamber. Mag.85X In culture July 13-23. Medium:25% mod. Crone's sol'n plus 2% sucrose.

Fig. 5. - Proembryo located in channel showing radiality. Mag.85X. In culture July 19-29. Medium:25% mod. Crone's sol'n plus 2% sucrose.

Fig. 6. - Mass of embryonic cells associated with channel edge and showing radiality. Mag.85X. In culture July 15-25. Medium:12.5% mod. Crone's sol'n plus 2% sucrose.

Fig. 7. - Mass of embryonic cells associated with channel edges. Mag.85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 2% sucrose.

Fig. 8. - Proembryo in tip of channel. Mag.85X. In culture July 11-21. Medium:12.5% mod. Crone's sol'n plus 2% sucrose.

Fig. 9. - Small proembryo in channel showing abnormal polarity. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 2% sucrose.

Fig.10. - Proembryo located at tip of channel. Mag. 85X. In culture July 13-23. Medium:25% mod. Crone's sol'n plus 2% sucrose.

Fig.11. - Large mass of embryonic cells located near edge of channel and showing slight degree of proliferation. Mag.85X. In culture July 19-29. Medium:25% mod.Crone's sol'n plus 2% sucrose.

Fig. 12. - Large mass of embryonic cells located at channel mouth. Mag.85X. In culture July 19-29. Medium:50% mod. Crone's sol'n plus 2% sucrose.

Fig. 13. - Prothallial cells. Mag.85X. In culture June 29-July 9. Medium:12.5% mod. Crone's sol'n plus 2% sucrose.

Fig. 14. - Prothallial cells with thick walls. Mag. 85X. In culture July 7-17. Medium:50% mod. Crone's sol'n plus 2% sucrose.

PLATE VI

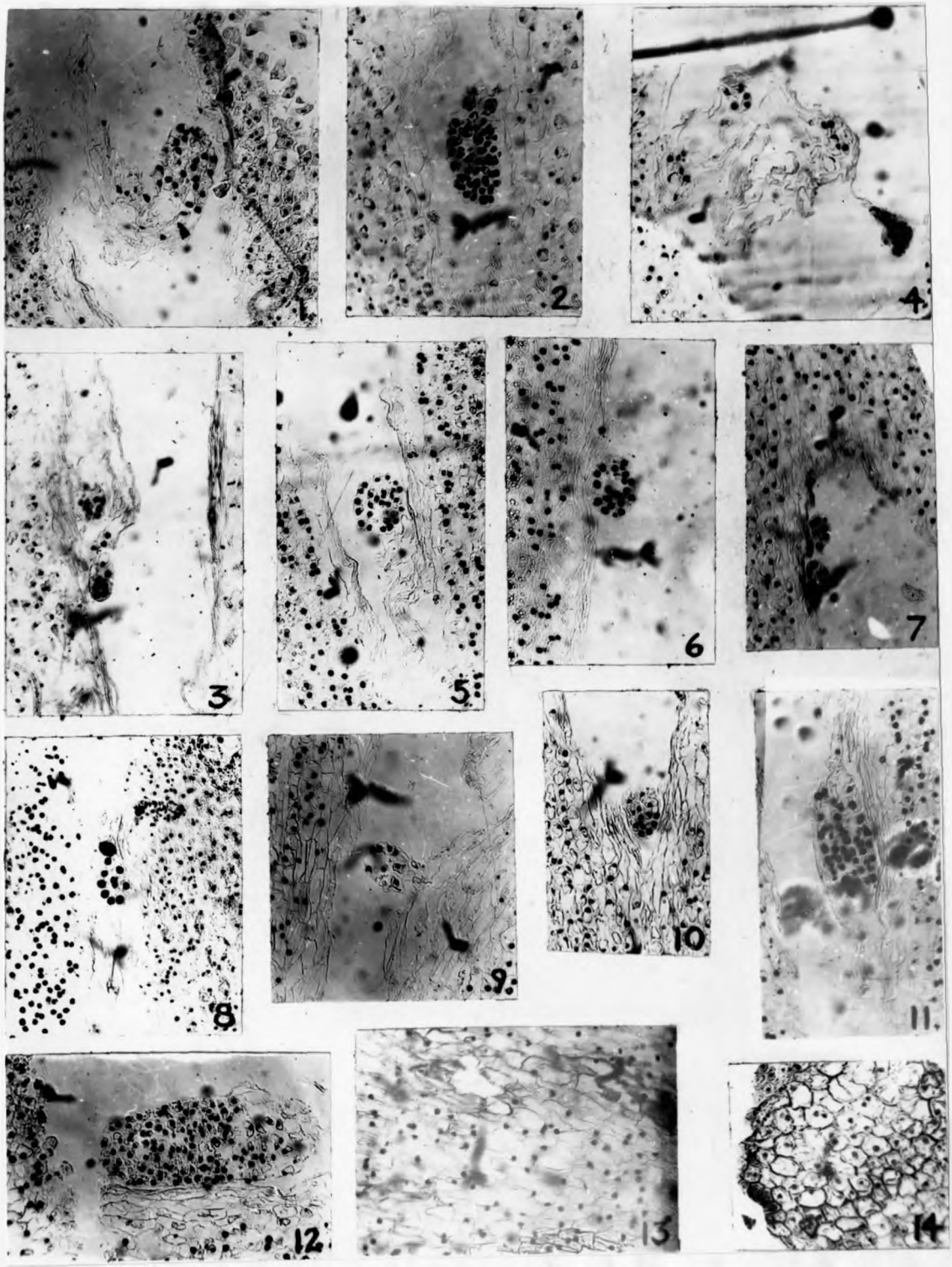


PLATE VII

Fig. 1. - Prothallial cells showing growth pattern. Mag.85X. In culture June 25-July 5. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 2. - Prothallial cells involved in growth between archegonia. Mag.85X. In culture June 25-July 5. Medium 25% mod. Crone's sol'n plus 1% dextrose.

Fig. 3. - Prothallial cells.Mag.85X. In culture July 9-19. Medium:50% mod. Crone's sol'n plus 2% sucrose.

Fig. 4. - Prothallial cells some with large nuclei. Mag.85X. In culture July 11-21. Medium:25% mod. Crone's sol'n plus 2% dextrose.

Fig. 5. - Dead prothallial cells. Mag.85X. In culture June 27-July 7. Medium:12.5% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 6. - Rosette cells and portion of suspensor. Mag.85X. In vivo material of July 3.

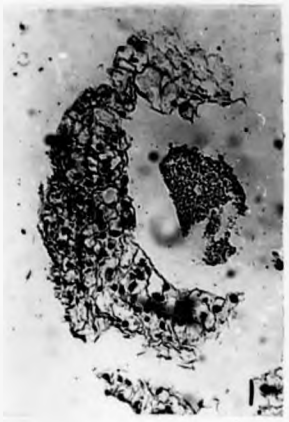
Fig. 8. - Prothallial cells showing granular inclusions. Mag.85X. In vivo material of July 15.

Fig. 9. - Jacket cells showing slight distortion. Mag.85X. In culture June 29-July 9. Medium:50% mod. Crone's sol'n plus 2% yeast extract.

Fig.10. - Jacket cells showing distortion. Mag.85X. In culture June 29-July 9. Medium:25% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig.11. - Small group of embryonic cells with mitotic figure in one. Mag.355X. In culture July 7-17. Medium:12.5% mod. Crone's sol'n plus 2% dextrose.

PLATE VII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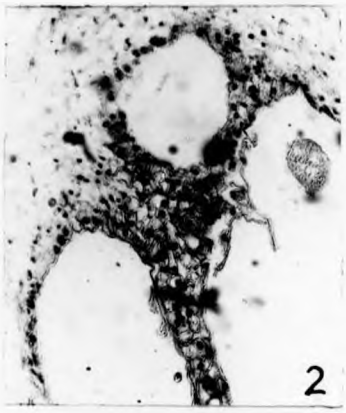
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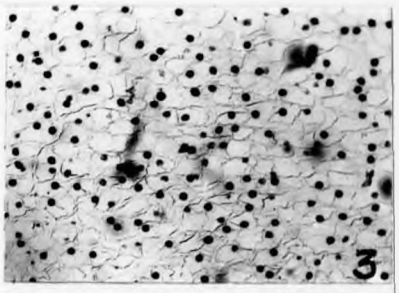
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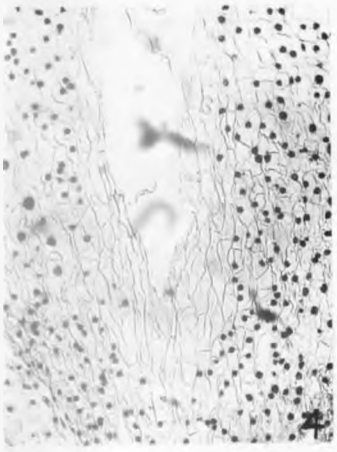
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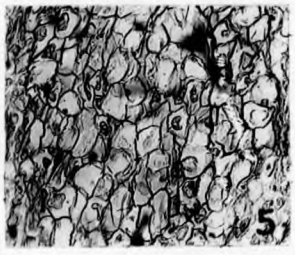
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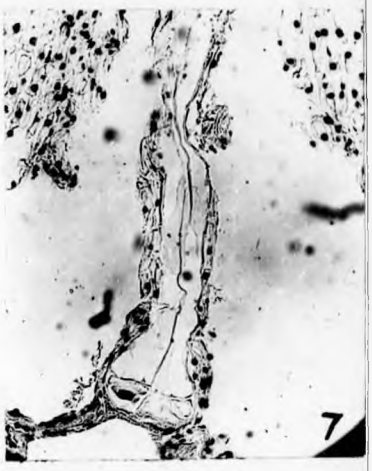
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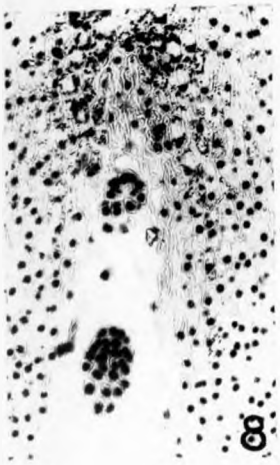
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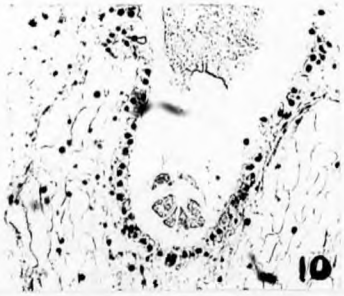
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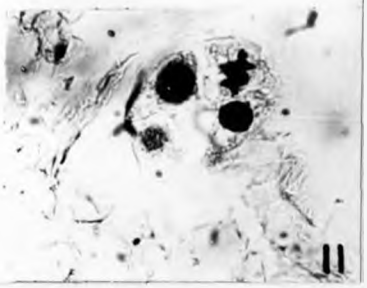

11

PLATE VIII

Fig. 1.- Mass of cytoplasm with disorganized nuclear material.Mag.355X. In culture July 13-23. Medium: 25% mod. Crone's sol'n plus 2% sucrose.

Fig. 2.- Group of embryonic cells one with mitotic figure.Mag.355X. In culture July 11-21. Medium:50% mod. Crone's sol'n plus 2% dextrose.

Fig. 3.- Few embryonic cells.Mag.355X. In culture July 11-21. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig. 4.- Embryonic cells, one with mitotic figure. Mag.355X. In culture July 17-27. Medium:50% mod. Crone's sol'n plus 1% dextrose.

Fig. 5.- Cells of mass of proliferating mass.Mag.355X. In culture July 19-29. Medium:50% mod. Crone's sol'n plus 1% dextrose.

Fig.6 and 7.- Prothallial cells showing mitosis. Mag. 355X. In culture July 1-11. Medium:12.5% mod. Crone's sol'n plus 2% dextrose.

Fig. 8.-Prothallial cells. Mag.355X. In culture July 7-17. Medium:50% mod.Crone's sol'n plus 2% sucrose.

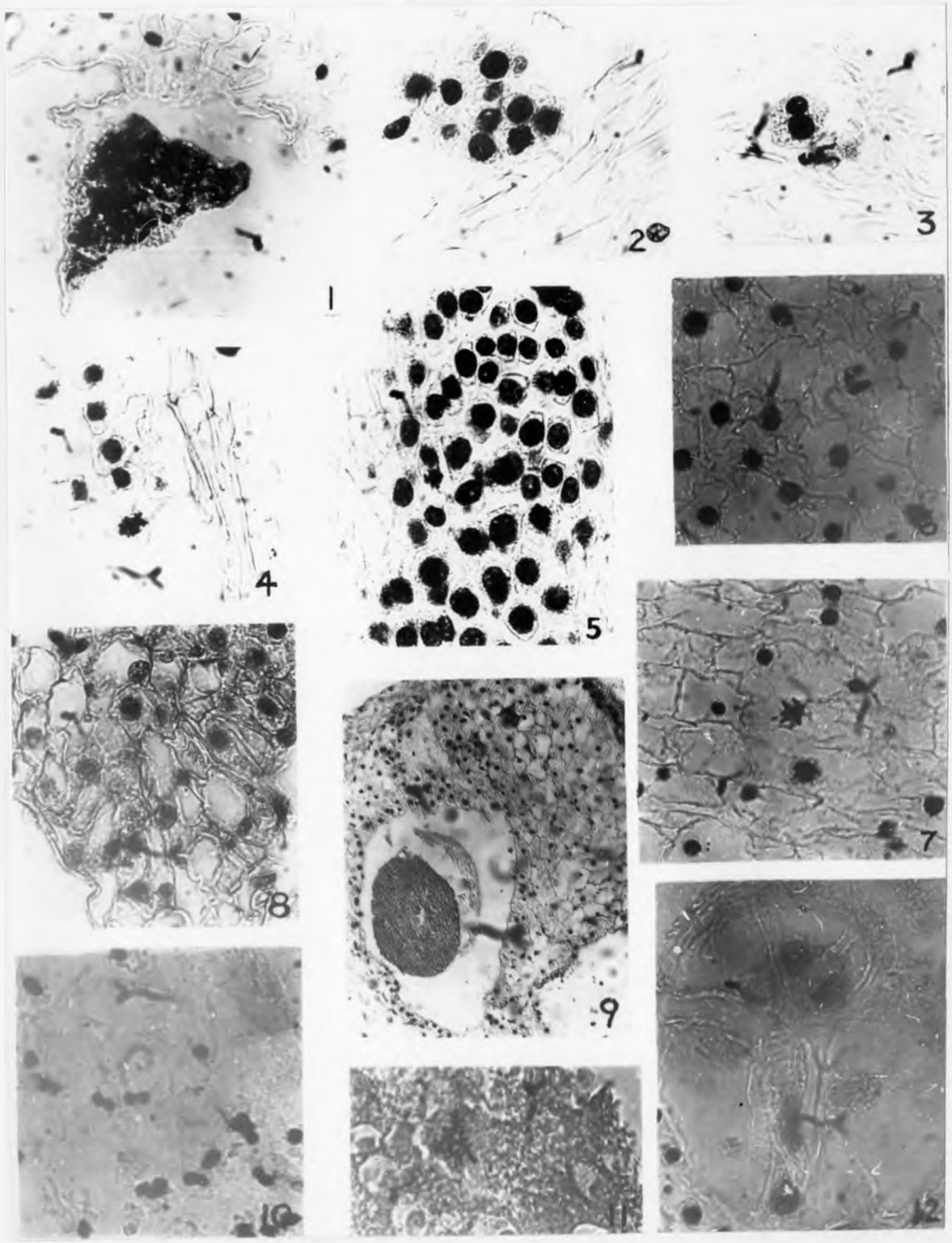
Fig. 9.- Prothallial cells showing growth pattern. Mag.85X. In culture June 23-July 3. Medium:50% mod, Crone's sol'n plus 1% yeast extract and 1% dextrose.

Fig.10.- Binucleated cells. Mag.355X. In culture June 29-July9. Medium:50% mod. Crone's sol'n plus 2% yeast extract.

Fig.11.- Mass of embryonic cells enclosed by jacket. Mag.355X. In culture July 5-July 15. Medium:50% mod. Crone's sol'n plus 1% yeast extract and 1% sucrose.

Fig.12.- Mitotic figure in egg sac. Mag.355X. In culture June 25-July 5. Medium:25% mod. Crone's sol'n plus 1% yeast extract.

PLATE VIII



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